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Aberrant epithelial differentiation by cigarette smoke dysregulates respiratory host defence

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Summary: Loss of highly expressed host defence proteins as a result of cigarette smoke-induced airway epithelial remodelling.

Abstract

Research question: It is currently unknown how cigarette smoke-induced airway remodelling affects highly expressed respiratory epithelial defence proteins and thereby mucosal host defence.

Methods: Localization of a selected set of highly expressed respiratory epithelial host defence proteins was assessed in well-differentiated primary bronchial epithelial cell (PBEC) cultures. Next, PBEC were cultured at the air-liquid interface and during differentiation for 2-3 weeks daily exposed to whole cigarette smoke. Gene expression, protein levels and epithelial cell markers were subsequently assessed. In addition, functional activities and persistence of the cigarette smoke-induced effects upon cessation were determined.

Results: Expression of pIgR, SLPI, long and short PLUNC was restricted to luminal cells and exposure of differentiating PBEC to cigarette smoke resulted in a selective reduction of the expression of these luminal cell-restricted respiratory host defence proteins compared to controls. This reduced expression was a consequence of cigarette smoke-impaired end-stage differentiation of epithelial cells, and accompanied by a significant decreased trans-epithelial transport of IgA and bacterial killing.

Conclusions: These findings shed new light on the importance of airway epithelial cell differentiation in respiratory host defence and could provide an additional explanation for the increased susceptibility of smokers and patients with COPD to respiratory infections.

Introduction

Respiratory infections and microbial colonization are a major health burden in smokers, and contribute to exacerbations and to the development and progression of chronic obstructive pulmonary disease (COPD) (reviewed by Sethi[1]). The mechanisms underlying this increased susceptibility of smokers with or without COPD are incompletely understood, but can be attributed in part to epithelial injury and remodelling resulting in a disrupted mucociliary clearance[2]. In addition to mucociliary clearance, the airway epithelium contributes to host defence with a wide variety of additional activities[3] that include secretion of antimicrobial peptides that act as endogenous antibiotics or modulate important antimicrobial immune responses via a variety of mechanisms[4]. Furthermore, the epithelium produces cytokines and chemokines that initiate an immune response to act against microbial invaders. Finally, transport of polymeric IgA and IgM to the lumen by the polymeric immunoglobulin receptor (pIgR) contributes to adaptive immunity in the lung by inhibiting adherence and facilitating clearance of pathogens, a process called immune exclusion[5]. Several of these respiratory host defence proteins (HDPs) in the airways are highly expressed during homeostasis by epithelial cells, suggesting their importance for airway epithelial barrier function. Highly expressed proteins and peptides include -but are not limited to- antimicrobial peptides such as human beta defensin-1 (hBD-1) and lipocalin 2 (LCN2), the secretory leukocyte protease inhibitor (SLPI), pIgR and the epithelial sodium channel regulators short and long palate, lung and nasal epithelium clone protein (s/lPLUNC or *BPIFA1/BPIFB1*)[6-8]. Expression of other peptides involved in airway host defence such as Ribonuclease 7 (RNase 7), LL-37 and human beta defensin-2 (hBD-2) is low during homeostasis but can be induced by e.g. inflammatory mediators, microbial products and upon injury of epithelial cells, and thus contribute to clearance of the pathogen and the resulting inflammatory process[4]. The pseudostratified airway epithelium is composed of several cell-

types, including goblet, club and ciliated cells that reach out toward the lumen of the airways, while basal cells do not reach this lumen in the intact epithelial layer[9]. Based on their distinct anatomical positioning, it is not surprising that these different cell-types also produce different types of mediators. For example, expression of pIgR is restricted to the luminal cells of the pseudostratified airway epithelium and is therefore largely regulated by airway epithelial cell differentiation[10], similar to e.g. mucin production by goblet cells. In contrast, expression of the antimicrobial protein RNase 7 is restricted to basal cells[11]. Cigarette smoke is known to induce airway epithelial remodelling in smokers and patients with COPD, characterized by an increase in goblet cells and a reduction in presence of ciliated cells[2]. As a result higher levels of mucus are produced by the epithelium, while mucus transport is impaired, thereby compromising mucociliary clearance activity of luminal airway epithelial cells. Currently it is unknown if the expression of proteins that are important for airway epithelial defence is polarized in the epithelium, and if so, how cigarette smoke-induced remodelling of the airway epithelium affects their expression. We hypothesized that cigarette smoke-induced alterations in epithelial cell differentiation result in a decreased expression of proteins that contribute to respiratory host defence (HDPs), which may render the host more susceptible to infection.

Methods

Cell culture

Primary bronchial epithelial cells (PBEC) were obtained from tumor-free resected lung tissue at the Leiden University Medical Center, Leiden, the Netherlands as described in the online data supplement and cultured as described[11]. PBEC were cultured at the air-liquid interface (ALI) for 13 to 19 days (Fig. 2A). Apical washes were performed daily; medium was refreshed every other day.

Fractionation of the airway epithelial cultures

Luminal and basal cell-enriched fractions were obtained from 3-4 weeks differentiated ALI-PBEC cultures as described previously[11]. The luminal cell fraction was spun down and either lysed in RNA lysis buffer or fixed with 1% paraformaldehyde (Millipore B.V., Amsterdam, the Netherlands) in PBS for 10 minutes on ice and washed afterwards in ice-cold PBS. The remaining basal epithelial cell fractions on the transwell inserts were also either lysed in RNA lysis buffer (Promega) or fixed with 1% paraformaldehyde (Millipore B.V.) in PBS for 10 minutes on ice and washed afterwards with ice-cold PBS. Next, cells were stained as described in the online data supplement with antibodies described in supplemental (s)Table 2.

Chronic cigarette smoke exposure

When confluent, PBECs were air-exposed (day 0) by removal of medium from the apical side of the transwell insert and 4 h later exposed to freshly generated whole cigarette smoke (CS) using 3R4F reference cigarettes (University of Kentucky, Lexington, KY). CS exposure was repeated daily as described in[11], in the figure legends of Fig. 2 and in sFig. 1 and illustrated in Fig. 2B and sFig. 1. Briefly, cells were exposed in modified hypoxic chambers for 4-5

minutes to either cigarette smoke from 1 cigarette or to room air, after which smoke was removed by ventilation with air during 10 minutes. and cells were subsequently placed back in the incubator overnight. Approximately 18-20 h later, ALI-PBEC were washed apically with PBS and 4 h hereafter exposed to cigarette smoke. This cycle was repeated every day until day 13-19. Cells were harvested for analysis 18-20 h after the last cigarette smoke exposure.

RNA isolation, cDNA synthesis and qPCR

Methods are described in the online data supplement with primers described in supplemental Table 1.

Confocal microscopy

Cells were fixed on transwell inserts in 1% paraformaldehyde (Millipore B.V) in PBS for 10 minutes on ice and washed afterwards with ice-cold PBS. Next, cells were stained as described in the online data supplement with antibodies described in sTable 2.

Transcytosis experiment

Methods are described in the online data supplement.

Antibacterial activity assay

Methods are described in the online data supplement

ELISA and Trans-epithelial electrical resistance

Methods are described in the online data supplement.

Inhibition of differentiation by DAPT

At day 0, PBEC were air exposed by removal of the medium in the insert and culture medium of ALI-PBEC was refreshed with medium supplemented with either 5 μM DAPT (Notch signalling inhibitor, Sigma Aldrich, Zwijndrecht, The Netherlands), or solvent control. Every other day, basal medium was changed in a similar fashion up to day 13 when the cells were harvested.

Statistics

Statistical analysis was conducted using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, U.S.A.). Data are shown as mean ± SEM and significance was tested with use of a paired t-test or two-way ANOVA with a Bonferroni corrected post-hoc test. Differences were considered significant at $p < 0.05$.

Results

Respiratory host defence proteins display a polarized distribution in airway epithelial cell cultures

In this study we have focussed on a set of proteins and peptides that are important for respiratory host defence. These host defence proteins (HDPs) were selected based on their constitutive and/or high expression by airway epithelial cells during homeostasis: i.e. SLPI, PLUNC (short/long), pIgR, hBD-1 and LCN2. We first investigated whether expression of these proteins was polarized in the airway epithelial cultures. To this end, we prepared luminal and basal epithelial cell-enriched fractions of well-differentiated primary bronchial epithelial cells (PBEC), cultured at the air-liquid interface (ALI, Fig. 1A). We confirmed the successful enrichment of fractions for luminal and basal cells by determining the gene expression of the typical basal cell markers *TP63* and *KRT5* and luminal epithelial cell markers *FOXJ1* (ciliated cells), *SCGB1A1* (club cells), *MUC5AC* and *MUC5B* (both goblet cells) (Fig. 1A), and by immunofluorescence staining for p63 (basal cells), CC16 (club cells) and acetylated α-tubulin (ciliated cells) (sFig. 2). Further analysis of these fractions showed that the luminal cell-enriched fraction expressed significantly higher levels of *BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *SLPI* (Fig. 1A). In contrast, *LCN2* and *DEFB1* expression did not differ between the luminal and basal cell-enriched fraction (Fig. 1A). The luminal cell-specific expression of SLPI and sPLUNC was further confirmed using confocal imaging in which the staining of both proteins did not co-localize with $p63^{+}$ basal cells, but was highly present at the apical side of the PBEC culture and in the luminal cell-enriched fraction (Fig. 1B and sFig. 2).

Chronic cigarette smoke exposure of airway epithelial cell cultures reduces expression of respiratory host defence proteins

We next investigated if cigarette smoke-exposure affected expression of this set of respiratory HDPs. To this end, ALI-PBEC cultures were exposed on a daily basis during 2-3 weeks of differentiation to whole cigarette smoke (CS) (Fig. 2 and supplemental (s)Fig. 1). Gene expression analysis showed that *DEFB1* (hBD-1) mRNA levels decreased during differentiation, but were not affected by CS exposure (Fig. 3A). On the other hand, expression of *SLPI, BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *PIGR* strongly increased during differentiation, and this increase was significantly prevented by CS (Fig. 3A). In contrast, gene expression of *LCN2* (lipocalin 2) was increased by CS exposure during differentiation (Fig. 3A). These findings were further confirmed by assessment of hBD-1 and SLPI protein levels in the apical wash and in basal medium from the ALI-PBEC cultures (Fig. 3B). Indeed, hBD-1 levels reduced over the time of differentiation in the apical wash, but were not significantly affected by chronic CS-exposure, whereas SLPI levels were significantly lower in chronic CS-exposed cell cultures (Fig. 3B). We next performed immunofluorescence staining of the airway epithelial cell cultures and found strongly reduced presence of SLPI-, sPLUNC- and pIgR-positive cells in chronic CS-exposed epithelium compared to air controls (Fig. 3C). These results confirmed selective impairment of specific respiratory HDPs by chronic CS exposure during airway epithelial differentiation.

Chronic cigarette smoke exposure reduces host defence of the airway epithelial cell cultures by decreasing apical release of secretory IgA and bacterial killing of *Moraxella catarrhalis* and *Klebsiella pneumoniae*

Next, we assessed if the strong reduction in *SLPI, BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *PIGR* expression levels in the CS-exposed airway epithelial cultures had functional

consequences for host defence. We selected pIgR-mediated transfer of dimeric (d)IgA across the epithelium as a proof-of-principle for the consequences on immunomodulatory host defence functions and found this to be significantly reduced in chronic CS-exposed cultures (Fig. 4A). We furthermore analysed bacterial killing by chronic CS-exposed cell cultures of the Gram-negative bacteria *Moraxella catarrhalis* and *Klebsiella pneumoniae*, pathogens that are found to be increased in the lungs of patients with stable or acute exacerbations of COPD [12]. We observed significantly higher bacterial counts (indicating lower antibacterial activity) in chronic CS-exposed PBEC cultures when compared to air-exposed cultures for both pathogens (Fig. 4B). These data indicate that various host defence mechanisms are functionally impaired in CS-exposed epithelial cell cultures, which corresponds with impaired expression of respiratory defence proteins.

Cigarette smoke affects end-stage differentiation of airway epithelial cells

Next we assessed if chronic CS exposure affected differentiation of ALI-PBEC by measuring gene expression of epithelial cell-specific markers. Gene expression of the basal cell markers cytokeratin-5 (*KRT5*) and *TP63*, and of cytokeratin-8 (*KRT8*) that is expressed by intermediate/committed progenitor epithelial cells[13], was not affected by CS (Fig. 5A). In contrast, expression of the specialized luminal epithelial cell-specific genes *FOXJ1* (ciliated cells), *SCGB1A1* (club cells) and *MUC5B* (goblet cells) increased during differentiation, and this increase was significantly prevented by CS (Fig. 5A). Confocal imaging confirmed the aberrant epithelial differentiation in CS-exposed cultures as cells positive for cilia marker acetylated α-tubulin, the club cell marker CC16, and the goblet cell marker MUC5AC were reduced in chronic CS-exposed cultures, while cytokeratin-8 $(CK\text{-}8)^+$ and $p63^+$ cells remained unchanged between air and CS-exposed cultures (Fig. 5B).

Reversibility of cigarette smoke-induced effects on host defence protein expression

To assess the persistence of the CS-induced reduction in *SLPI, BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *PIGR* expression levels and of its effect on cellular composition, we allowed the cultures to recover from 13 days of CS exposure by culturing the cells for an additional 6 days without CS exposure. Chronic CS-exposed cultures were able to (partly) recover from the lack of end-stage differentiation, since all specialized luminal cell markers, except for *SCGB1A1* (club cells), significantly increased in expression (Fig. 6A). Furthermore, also *SLPI, BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *PIGR* showed enhanced expression compared to day 13 (Fig. 6A). In addition, *KRT5* (basal cell marker), and *DEFB1* and *LCN2* increased upon CS cessation, whereas *TP63* and *KRT8* were unaffected (sFig. 3A). This indicates that the inhibitory effects of cigarette smoke exposure on epithelial differentiation and expression of specific respiratory defence proteins are at least in part reversible. Furthermore, in an attempt to better mimic the *in vivo* situation and establish if the effects observed after chronic CS exposure also can be obtained when exposing an already partly differentiated epithelium to chronic CS, we performed a separate experiment. In this experiment, we first allowed the cultures to differentiate for 1 week, after which we started chronic CS exposure for an additional 12 days. Here we found similar effects of CS exposure on ALI-PBEC cultures regarding cell-type specific markers and HDP expression compared to CS exposure starting from day 0 (Fig. 6B and sFig. 3B).

Notch signalling inhibition impairs host defence protein expression during differentiation

Our results so far showed an impaired end-stage differentiation into specialized luminal epithelial cells in CS-exposed cultures resulting in reduced levels of SLPI, sPLUNC, lPLUNC and pIgR. Previous studies have shown that Notch signalling is involved in airway

epithelial differentiation and that the airway epithelium of smokers displays reduced Notch signalling[14]. Therefore, we next examined if Notch signalling was impaired in CS exposed cultures, and if Notch signalling inhibition modulates HDP expression during differentiation. First, we assessed gene expression of components of the Notch signalling cascade and found that chronic CS exposure did not influence gene expression of Notch ligands, receptors or transcriptional co-activators in these cultures (Fig. 7A). However, the Notch signalling target genes, *HEY1* and *HEY2*, were significantly reduced by chronic CS exposure, while *HES1* was not (Fig. 7B), indicating that chronic CS exposure selectively affects target genes of the Notch signalling pathway.

To further investigate the importance of Notch signalling in the expression of host defence proteins, we examined the effect of the γ–secretase inhibitor DAPT, which acts as an inhibitor of Notch signalling (Fig. 8A). After 15 days of PBEC differentiation in the presence of DAPT, we measured expression of HDPs. *DEFB1* (hBD-1) and *LCN2* were not affected by DAPT, while gene expression of *SLPI*, *BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *PIGR* were strongly reduced by DAPT incubation (Fig. 8B). Furthermore, DAPT-exposed cultures showed a skewing of cell differentiation away from a secretory phenotype towards an increase in ciliated cells (Fig. 8C) that was also confirmed by confocal imaging (Fig. 8D).

Discussion

Here we demonstrate that cigarette smoke negatively affects expression of the respiratory HDPs: pIgR, SLPI, lPLUNC and sPLUNC. Their expression was significantly reduced in epithelial cells daily exposed to CS during differentiation as a result of an impaired end-stage differentiation of specialized luminal cells. As a consequence, remodelling of the airway epithelium by cigarette smoke has a significant impact on respiratory host defence, underscored by the severely diminished IgA transport across the CS-exposed epithelium and impaired antibacterial defences against *M. catarrhalis* and *K. pneumoniae*. Our data suggest that increasing expression of specific respiratory HDPs (or preventing their decrease) could be of therapeutic interest to improve host defences in the lungs of COPD patients. Furthermore, this (selective) loss may also contribute to changes in lung microbiome composition, which is increasingly recognized as an important contributor to chronic inflammatory lung diseases[15, 16].

The cellular composition of the ALI-PBEC cultures changed drastically upon chronic CS-exposure. While presence of intermediate CK-8^{+} cells (or also called early, intermediate or committed progenitor epithelial cells[13, 17]) was not affected by chronic CS exposure, the specialized luminal cell markers were reduced in chronic CS-exposed cultures. These results indicate that specifically end-stage differentiation seems impaired by CS exposure. The effects of chronic CS exposure were also observed when the cells were first allowed to differentiate for one week in absence of CS. Furthermore, upon cessation of CS exposure, gene expression of most luminal cell markers showed a strong recovery. In contrast, *SCGB1A1* mRNA expression remained absent after almost 1 week of recovery, suggesting an exceptional detrimental effect of CS on club cell differentiation or the regulation of *SCGB1A1* gene expression. This is underscored by a recent study showing that expression of the club cell-protein CC16 (*SCGB1A1*) is reduced in COPD patients and in CS-exposed mice.

Loss of CC16 was correlated with increased severity of the disease and CS-induced pulmonary inflammation was lower in mice overexpressing CC16[18].

Cytotoxicity is unlikely to have a major contribution to the CS-induced effects on the ALI-PBEC cultures since we detected no difference in trans-epithelial electrical resistance (TEER) as a measure of barrier function during the course of differentiation between CS and air-exposed controls. We previously observed transient decreases in TEER after acute single CS exposures, normalizing after 24 h [11]. In our chronic CS set-up we measured TEER 18-20h after the previous CS exposure, which may explain why we did not observe significant differences in TEER between air and CS-exposed cultures. Previous studies have shown decreases in TEER by CS, but often use cigarette smoke extract (CSE) and not whole CS [19, 20]. CSE has a different composition and concentration than whole CS used in our study. In addition, in some studies CSE was added to the basal medium, resulting in stimulation from the basal side of the transwells [19]. This may also contribute to differences found in effects on TEER. Lastly, LDH release was not increased in our CS-exposed cultures, while cellular size appeared larger in the CS-exposed cultures for some donors. Finally, the CS-exposed cultures produced higher amounts of IL-8 and displayed increased mRNA expression of the inducible antimicrobial peptides RNase7 and LL-37 (but not hBD-2). These results are described in the online data supplement and in sFig. 4

Unexpectedly, we did not observe goblet cell hyperplasia in cultures that were exposed to CS, shown previously in smokers [21], guinea pigs [22], rats [23] and in cell lines [23, 24] or PBEC exposed to cigarette smoke extract (CSE)[19]. However, Brekman et al. [25] also observed a decline in goblet cells markers in PBEC continuous exposed to CSE. Data are therefore conflicting and dependent on the type of cell culture used. Obviously, in our primary differentiated cultures, whole CS exposure alone is insufficient to induce goblet cell hyperplasia within 19 days. We strongly consider that the findings in patients are more likely

explained by a secondary effect of the CS-induced inflammation (which is obviously incompletely represented in our *in vitro* model). For example, neutrophil recruitment as a consequence of the CS-induced inflammation and subsequent release of proteases and other molecules, may help to explain goblet cell formation in patients. In addition, also the presence of other cell types besides neutrophils such as macrophages seem important for goblet cell hyperplasia. This has also been suggested in literature [26-29]. Furthermore, several studies suggest that various other factors might have an important role in promoting goblet cell hyperplasia that are involved in COPD pathogenesis, including bacterial and viral infections [30, 31].

Whereas previous studies have shown that CS reduces the presence of ciliated cells [19, 25, 32], so far CS-induced airway epithelial remodelling was not yet linked to changes in levels of the highly expressed respiratory HDPs, despite the fact that changes in expression in these proteins have been reported in smokers or patients with COPD. Aarbiou *et al.* showed that expression of SLPI was significantly reduced in damaged bronchial epithelium of COPD patients compared to non-COPD individuals[33], and Gohy *et al.* showed reduced levels of pIgR in the lungs of patients with COPD, however not in smokers with a normal lung function compared to healthy controls[10]. Finally, reduced levels of PLUNC were detected in bronchial brushes performed in current smokers compared to never smokers[34].

We observed impaired anti-bacterial activity of the CS-exposed airway epithelial cultures against *Moraxella catarrhalis* and *Klebsiella pneumoniae*. We also evaluated direct anti-bacterial activity of the chronic CS-exposed cultures using a grid assay with live/dead staining of bacteria [35, 36] and via conventional plating methods against *Pseudomonas aeruginosa* and non-typeable *Haemophilus influenzae,,* but could not detect any differences (data not shown). These data suggest that the observed CS-induced impairment of antimicrobial activity may be pathogen-specific, since it is not observed with all pathogens

studied. In addition to impaired antibacterial host defence activities, the loss of HDP expression by the airway epithelium can have further negative effects for the host. For example, loss of SLPI expression (highly expressed in normal lung tissue) can promote inflammation in the lungs of patients with COPD. SLPI acts as an inhibitor of detrimental proteases such as neutrophil elastase, acts as an inhibitor of NFκB activation and it modulates macrophage functions[37-39]. sPLUNC is involved in regulation of the epithelial sodium channel (ENaC), thereby regulating airway surface liquid (ASL), and reduced levels could result in lowered ASL volume and impaired mucociliary clearance[40].

Since Notch signalling was previously reported to be impaired in COPD[14], we first analysed the effect of chronic CS exposure on components of the Notch signalling pathway and Notch target genes, and found that CS decreased the expression of selected Notch target genes. When we next used the Notch signalling inhibitor DAPT to inhibit airway epithelial cell differentiation, we found similar effects compared to CS exposure on expression of the selected set of respiratory HDPs. To our knowledge, this is the first study linking Notch signalling to expression of a range of host defence proteins that are increased upon differentiation. Whereas chronic CS exposure resulted in a reduction of all luminal-cell markers, DAPT-exposed cultures showed higher levels of ciliated cells when compared to control-treated cells, in line with previous studies[41, 42]. These results suggest that expression of the luminal cell-restricted HDPs is confined to mature secretory epithelial cells, rather that the ciliated epithelium. Further studies using single cell RNA sequencing, may reveal whether the expression is restricted to a certain secretory cell phenotype. The partial similarity between the DAPT-incubated cultures and the CS-exposed cultures suggests involvement of altered Notch signalling in the CS-induced effects. However, alterations in other signalling pathways might also be involved in the observed effects of CS, such as those involving EGFR[32], TGF-β[25] Wnt[43] and BMP[44]. Most likely a more systems/-omics

approach has the potential to elucidate in detail all effects of chronic CS exposure on Notch and other signalling pathways[45].

In summary, these findings shed new light on the role of dysregulated host defence in smokers and patients with COPD by highlighting the importance of airway epithelial cell differentiation in the expression of respiratory HDPs. Further investigations into how suppression of epithelial cell differentiation by cigarette smoke contributes to microbial colonization and infections of the airways are warranted in order to develop new therapeutics that restore airway epithelial host defence in patients with COPD.

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Figure legends

Figure 1. Respiratory host defence proteins display a polarized distribution in air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC).

(A) PBEC were seeded on coated transwells and cultured in submerged conditions until confluent. At day 0, cultures were air-exposed and cultured at the air-liquid interface. After 3-4 weeks of differentiation luminal and basal cell-enriched fractions were separated followed by RNA isolation, cDNA synthesis and qPCR analysis. Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin *(B2M)* and Ribosomal Protein L13a *(RPL13A)*, n=5-7 different donors. Open bars are basal cell-enriched fractions; grey bars are luminal cell-enriched fractions. Statistical significance was tested using a paired t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (B) Confocal images to visualize polarized distribution of secretory leukocyte protease inhibitor (SLPI) and short palate, lung and nasal epithelium clone protein (sPLUNC) in differentiated ALI-PBEC cultures cells. After 3 weeks of differentiation, cells were fixed in 1% paraformaldehyde and stained using immunofluorescence with primary antibodies against p63 (basal cell marker, red) in combination with primary antibodies against SLPI and/or sPLUNC (both green) and DAPI for nuclear staining (blue). Z-stacks and images of the apical and basal side of stained cells were made by confocal imaging, scale bars equals 50 μm. Images shown are representative for results obtained with cells from 4 different donors.

Figure 2. Cell culture set-up and cigarette smoke exposure of primary bronchial epithelial cells differentiated at the air-liquid interface (ALI-PBEC).

(A) Primary bronchial epithelial cells (PBEC) were seeded on coated transwells and cultured in submerged conditions until confluent. At day 0, cultures were air-exposed and cultured for additional 13-19 days to allow mucociliary differentiation. (B) Each day, starting at day 0, cultures were exposed to cigarette smoke (CS) by placing them in an exposure chamber that was infused with either cigarette smoke or with air for 4-5 min. Next, residual smoke in the chamber was removed for a period of 10 min. by infusing the chambers with air derived from the incubator. Approximately 4 h before each CS exposure the apical surface of the cultures was washed to remove mucus. Basal medium was changed every other day.

Figure 3. Chronic cigarette smoke exposure of air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) lowers the expression of luminal cell-restricted host defence proteins.

(A) ALI-PBEC were daily exposed to whole cigarette smoke (CS) or air as a control (AIR) during differentiation for 13-19 consecutive days. Cells were lysed at several points during this course of time and RNA was isolated followed by cDNA synthesis to assess gene expression of *DEFB1* (human beta defensin-1), *SLPI* (secretory leukocyte protease inhibitor), *BPIF1A* (short palate, lung and nasal epithelium clone protein), *BPIF1B* (long palate, lung and nasal epithelium clone protein), *PIGR* (polymeric immunoglobulin receptor) and *LCN2* (lipocalin 2). Open circles: air-exposed controls, black circles: CS-exposed cell cultures; data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin *(B2M)* and Ribosomal Protein L13a *(RPL13A)*; day 0, 7, 13 n=8 different donors and day 19 n=4 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ between AIR and CS. # $p<0.05$, ## $p<0.01$, ### $p<0.001$, #### $p<0.0001$ between AIR at day 7, 13 and 19 and unexposed cultures at day 0. (B) ELISA for hBD-1 and SLPI was performed on the apical wash (Apical) and basal medium (Basal) of these cultures. Open bars: air controls (AIR), black bars: CS-exposed cell cultures (CS); day 7 and 13, n=8 different donors and day 19: n=4 different donors. Statistical differences on T=7 and T=13 (not T=19) was tested using a paired two-way ANOVA to compare AIR and CS. * $p<0.05$, ** $p<0.01$. (C) ALI-PBEC were differentiated for 2-3 weeks in which they were daily exposed to CS or air as a control (AIR). Subsequently the cells were fixed in 1% paraformaldehyde and stained using primary antibodies against SLPI, sPLUNC and pIgR (all green staining) in combination with DAPI to stain the nuclei (blue staining). Scale bars are

equal to 50 μm. Images shown are representative for results obtained with cell cultures from 4 different donors.

Figure 4. Chronic cigarette smoke exposure of air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) impairs host defence activities.

A) ALI-PBEC were daily exposed to whole cigarette smoke (CS) or air as a control (AIR) during differentiation for 13 consecutive days. After 13 days of chronic CS exposure, dimeric (d)IgA transcytosis capacity of the epithelial cultures was assessed by determining secretory (S)-IgA levels in apical washes by ELISA (no S-IgA could be detected in the basal medium, as a control of the assay that does only recognize S-IgA and not d-IgA), n=10 different donors. Open bars: air-exposed cell cultures, black bars: CS-exposed cell cultures. B) After 13 days of chronic CS exposure, ALI-PBEC were cultured for 48 h in antibiotics-free cell culture medium after which they were exposed for 2 h to *Moraxella catarrhalis* or *Klebsiella pneumoniae* at the apical surface of the ALI-PBEC. The surviving bacteria are depicted as colony forming units (CFU)/ml, n=8 different donors. Significance was determined using a paired t-test. *$p<0.05$, **** $p<0.0001$.

Figure 5. Chronic cigarette smoke exposure of air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) changes cellular composition.

(A) ALI-PBEC were exposed during differentiation for 13-19 consecutive days to whole CS. Cells were lysed at several time-points and RNA was isolated followed by cDNA synthesis, to assess gene expression of basal cell markers cytokeratin-5 (*KRT5*) and *TP63*, of early progenitor cell marker cytokeratin-8 (*KRT8*) and of specialized cell markers *FOXJ1* (ciliated cells), *SCGB1A1* (club cells) and *MUC5B* (goblet cells). Open circles: air-exposed controls (AIR), black circles: CS-exposed cell cultures (CS); data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin *(B2M)* and Ribosomal Protein L13a *(RPL13A)*; day 0, 7, 13 n=8 donors and day 19 n=4 donors. Significance was determined using a two-way ANOVA and Bonferroni post-hoc test. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ between AIR and CS. # $p<0.05$, ## $p<0.01$, ### $p<0.001$, #### $p<0.0001$ between AIR at day 7, 13 and 19 and unexposed cultures at time 0. (B) ALI-PBEC were differentiated for 2-3 weeks and daily exposed to CS. Subsequently the cells were fixed in 1% paraformaldehyde and stained using primary antibodies against basal cells (p63), presented as a red staining, in combination with primary antibodies against cytokeratin-8 (CK-8), acetylated α-tubulin (ciliated cells), CC16 (club cells) and MUC5AC (goblet cells), which are presented as a green staining; DAPI was used to stain the nuclei (blue staining). Z-stacks and images of the apical and basal side of stained cells were obtained by confocal imaging. Scale bars equals 50 μm. Images shown are representative for results obtained with cells from 4 different donors (CK-8; n=3 different donors).

Figure 6. Cigarette smoke-induced impairment of host defence proteins and differentiation markers are partly persistent upon cigarette smoke cessation.

(A) Air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) were exposed during differentiation for 13 consecutive days to whole cigarette smoke (CS) after which cultures were continued for another 6 days without CS exposure. Cells were lysed at several points during this course of time and RNA was isolated followed by cDNA synthesis, to assess gene expression of the cell specific markers: *FOXJ1* (ciliated cells), *MUC5B* (goblet cells) and *SCGB1A1* (club cells) and of respiratory defence proteins: *SLPI*, *BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *PIGR*. Open bars: air-exposed controls (AIR), black bars: CS-exposed cell cultures (CS), grey bars: CS-exposed cultures that were cultured for an additional week without CS exposure (CS cessation). Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin *(B2M)* and Ribosomal Protein L13a *(RPL13A)*. n=8 different donors. Statistical differences were evaluated only for the difference between cessation and previous CS expression using a two-way ANOVA and Bonferroni post-hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. (B) ALI-PBEC were air exposed at day 0 and cultured for 7 days under standard conditions. At day 7 cultures were exposed to CS for 12 consecutive days after which the cells were lysed and similar analysed as in (A). Grey bars (start point of culture at day 7): unexposed, open bars: air-exposed controls, black bars: CS-exposed cell cultures. Data are shown as target gene expression normalized for the geometric mean expression of the reference genes *ATP5B, B2M* and *RPL13A;* n=6 different donors. Statistical differences were evaluated using a two-way ANOVA and Bonferroni post-hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure 7. Chronic cigarette smoke exposure of air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) results in selective impairment of Notch signalling.

(A) After 2 weeks of differentiation and daily cigarette smoke exposure, ALI-PBEC were lysed, RNA was isolated and cDNA synthesized. Subsequent qPCR analysis was performed on notch signalling ligands *DLL1*, *JAG1* and *JAG2*, on Notch receptors 1-3 and on the transcriptional co-activators *MAML1* and *MAML3*; data are shown as target gene expression normalized for the geometric mean expression of ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin *(B2M)* and Ribosomal Protein L13a *(RPL13A)*. Open circles: air-exposed controls (AIR), black circles: CS-exposed cell cultures (CS); n=8 different donors. (B) Subsequent qPCR analysis was performed on Notch signalling target genes *HEY1*, *HEY2* and *HES1*. Data are shown as target gene expression normalized for the geometric mean expression of the reference genes *ATP5B*, *B2M* and *RPL13A;* n=8 different donors. Statistical significance was tested using a two-way ANOVA and Bonferroni post-hoc test . ** $p<0.01$, *** $p<0.001$ between AIR and CS.

Figure 8. DAPT inhibits host defence protein expression in air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC)

(A) Mechanism of action of the Notch inhibitor DAPT, a γ-secretase inhibitor that prevents proteolytic cleavage of the Notch intracellular domain (NCID). (B) PBEC were seeded on coated transwells and cultured in submerged conditions until confluent. At day 0, cells were differentiated for an additional 15 days in the presence of 5 μM of the Notch signal transduction inhibitor DAPT in the basal medium or solvent as control. At day 0, 7, and 15 cells were lysed, RNA was isolated and cDNA synthesized. Subsequent qPCR analysis was performed to assess expression of respiratory defence proteins and epithelial cell-specific genes such as: *DEFB1* (human beta defensin-1), *SLPI* (secretory leukocyte protease inhibitor), *BPIFA1* (short palate, lung and nasal epithelium clone protein), *BPIFB1* (long palate, lung and nasal epithelium clone protein), polymeric immunoglobulin receptor (*PIGR*) and *LCN2* (lipocalin 2). Data are shown as target gene expression normalized for the geometric mean expression of the reference genes ATP synthase, H^+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), β2-microglobulin *(B2M)* and Ribosomal Protein L13a *(RPL13A)*; n=7 different donors. Statistical significance was tested using a two-way ANOVA and Bonferroni post-hoc test . ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ between CTRL and DAPT. (C) qPCR analysis was performed to assess mRNA expression of the epithelial cell markers *SCGB1A1* (club cells), *MUC5B* (goblet cells), *FOXJ1* (ciliated cells), *TP63* (basal cells), and cytokeratin-8 (*KRT8)* (intermediate cells) after 15 Days of differentiation with DAPT or solvent control, n=6 different donors. Statistical significance was tested using a paired t-test * $p<0.05$, ** $p<0.01$ between CTRL and DAPT (D) ALI-PBEC were differentiated for 15 Days with DAPT or solvent control. Subsequently the cells were fixed in 1% paraformaldehyde and stained using primary antibodies against CC16 (club cells), MUC5AC (goblet cells), and acetylated α-tubulin

(ciliated cells), which are presented as a green staining; DAPI was used to stain the nuclei (blue staining). Scale bars equals 50 μm. Images shown are representative for results obtained with cells from 3 different donors.

Figure 1

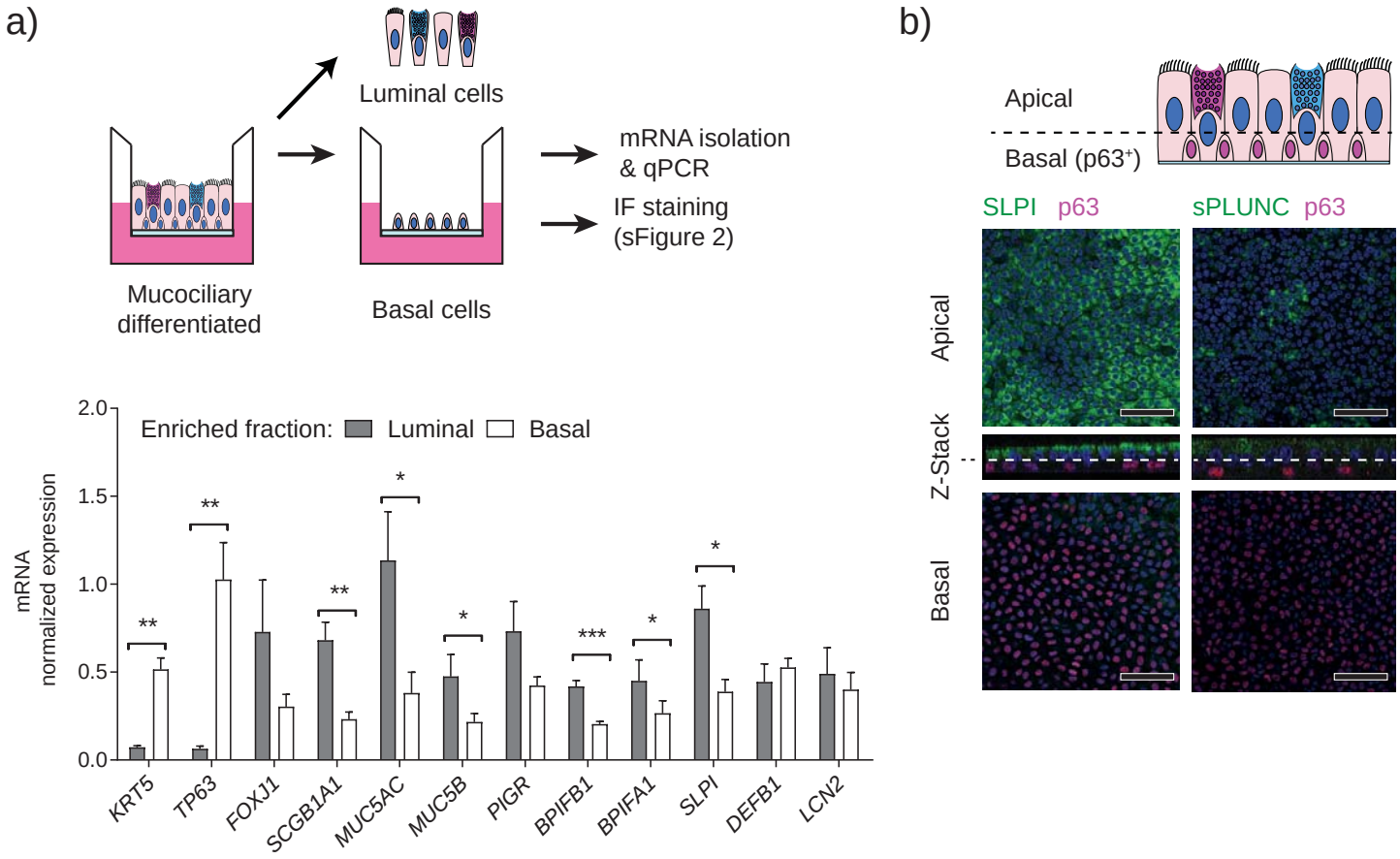

a)
Luminal cells
mRNA isolation
& qPCR
IF staining
(sFigure 2)
Mucociliary
differentiated
Basal cells
Enriched fraction: Luminal Basal
mRNA
normalized expression
2.0
1.5
1.0
0.5
0.0
KRT5
TP63
FOXJ1
SCGB1A1
MUC5AC
MUC5B
PIGR
BPIFB1
BPIFA1
SLPI
DEFB1
LCN2
b)
Apical
Basal (p63+)
SLPI p63
sPLUNC p63
Apical
Z-Stack
Basal

Figure 2

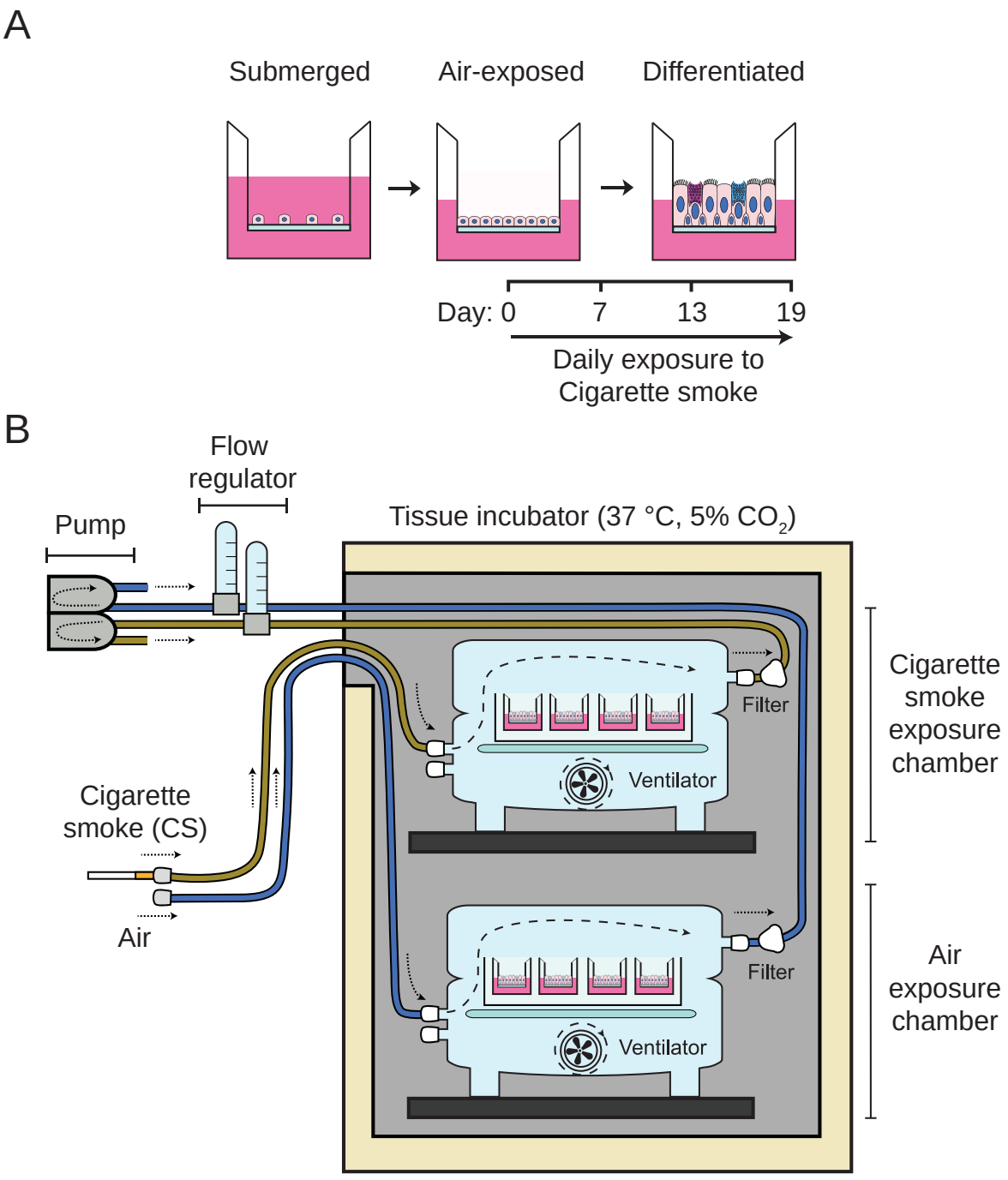
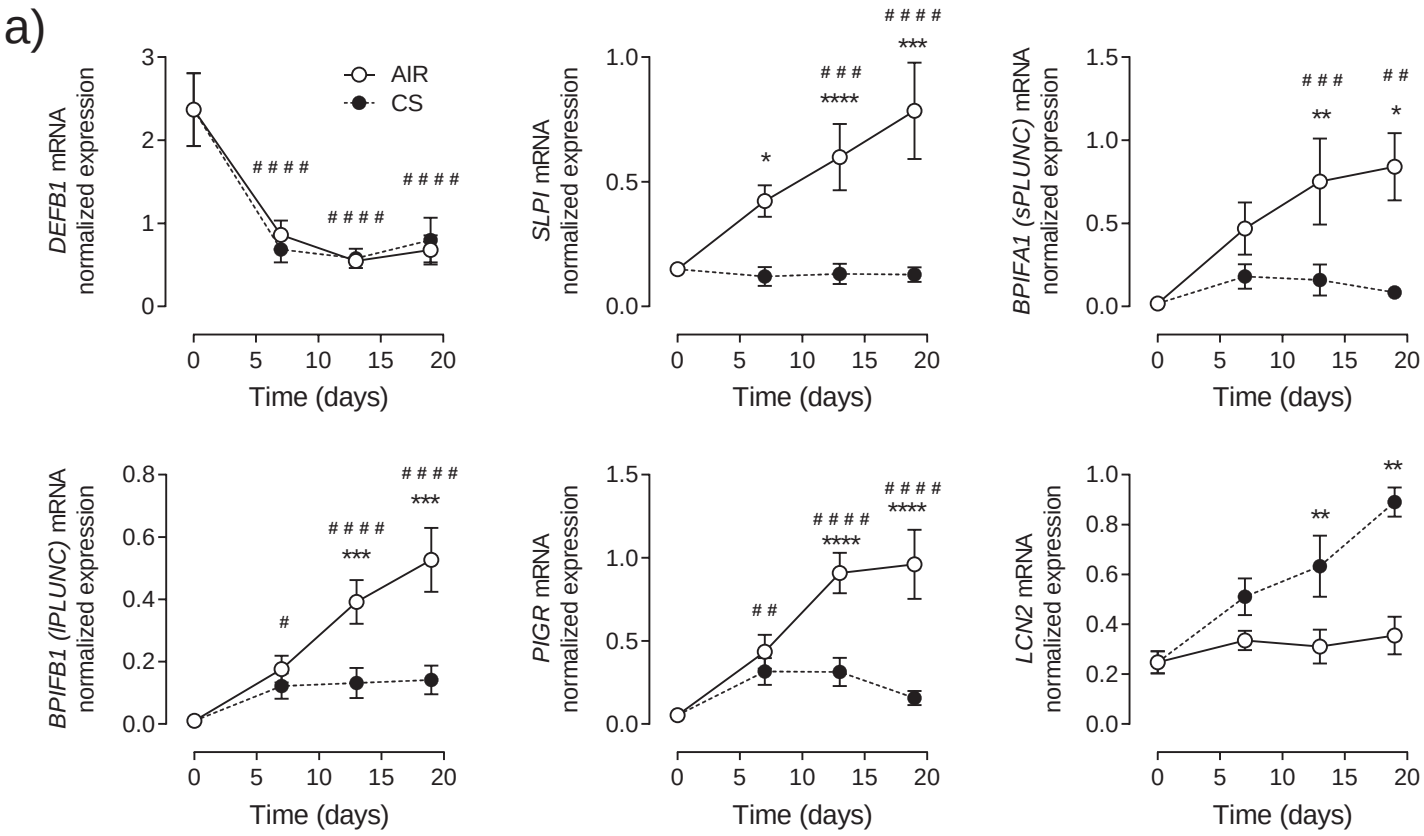
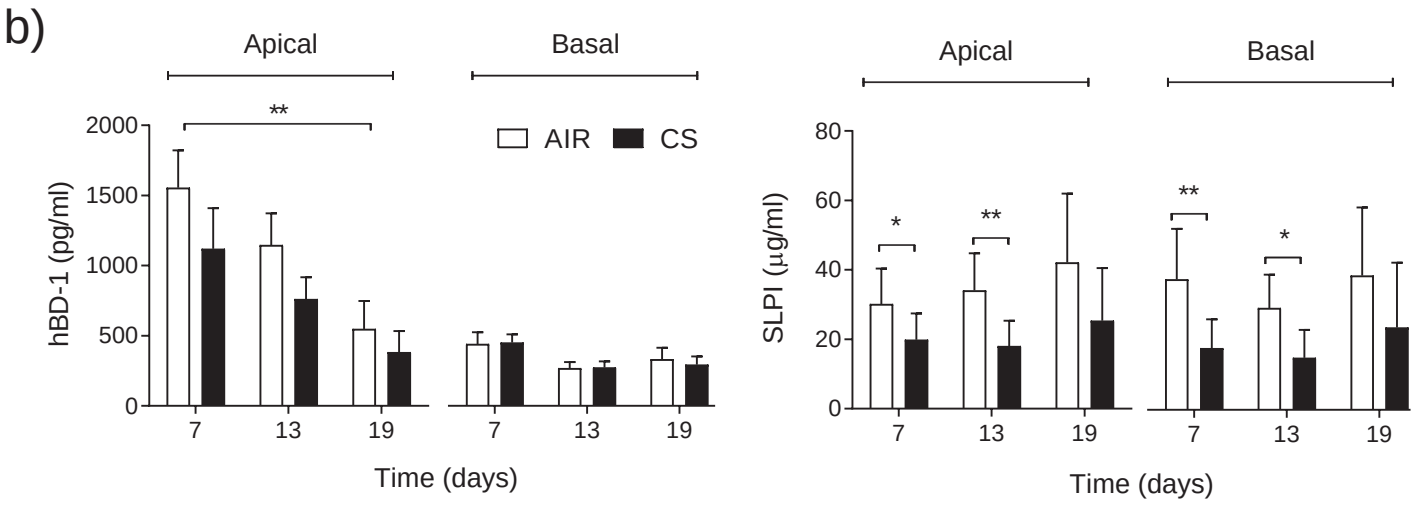

A
Submerged
Air-exposed
Differentiated
Day: 0
7
13
19
Daily exposure to
Cigarette smoke
B
Flow
regulator
Pump
Tissue incubator (37 °C, 5% CO_2)
Filter
Ventilator
Cigarette
smoke (CS)
Air
Filter
Ventilator
Cigarette
smoke
exposure
chamber
Air
exposure
chamber

Figure 3


a)
DEFB1 mRNA normalized expression
AIR
CS
Time (days)
SLPI mRNA normalized expression
BPIFA1 (sPLUNC) mRNA normalized expression
BPIFB1 (IPLUNC) mRNA normalized expression
PIGR mRNA normalized expression
LCN2 mRNA normalized expression


b)
Apical
Basal
AIR
CS
hBD-1 (pg/ml)
SLPI (µg/ml)
Time (days)

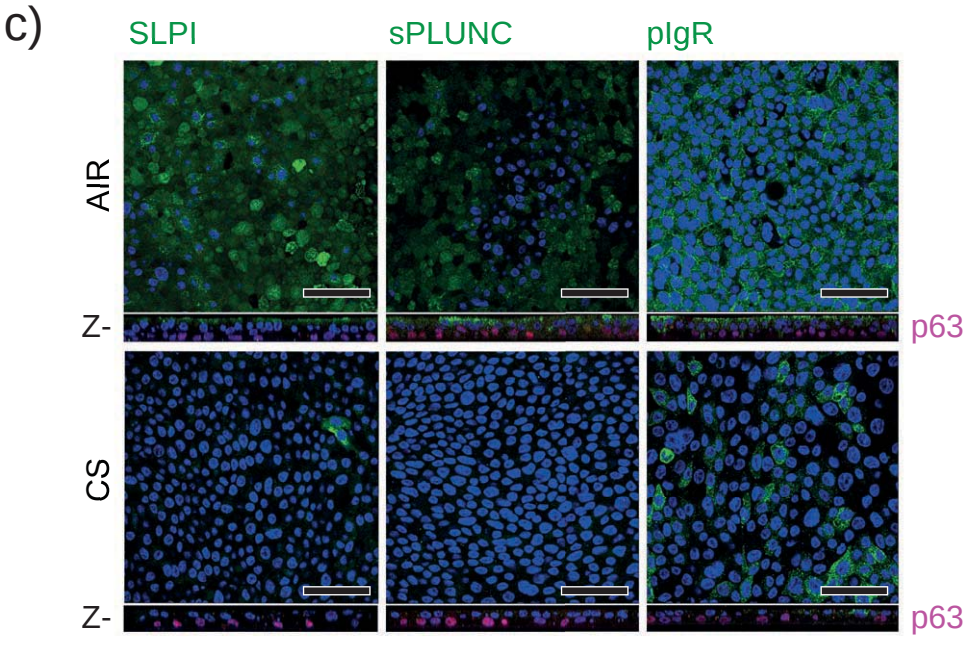

c)
SLPI
sPLUNC
pIgR
AIR
CS
Z-
p63

Figure 4

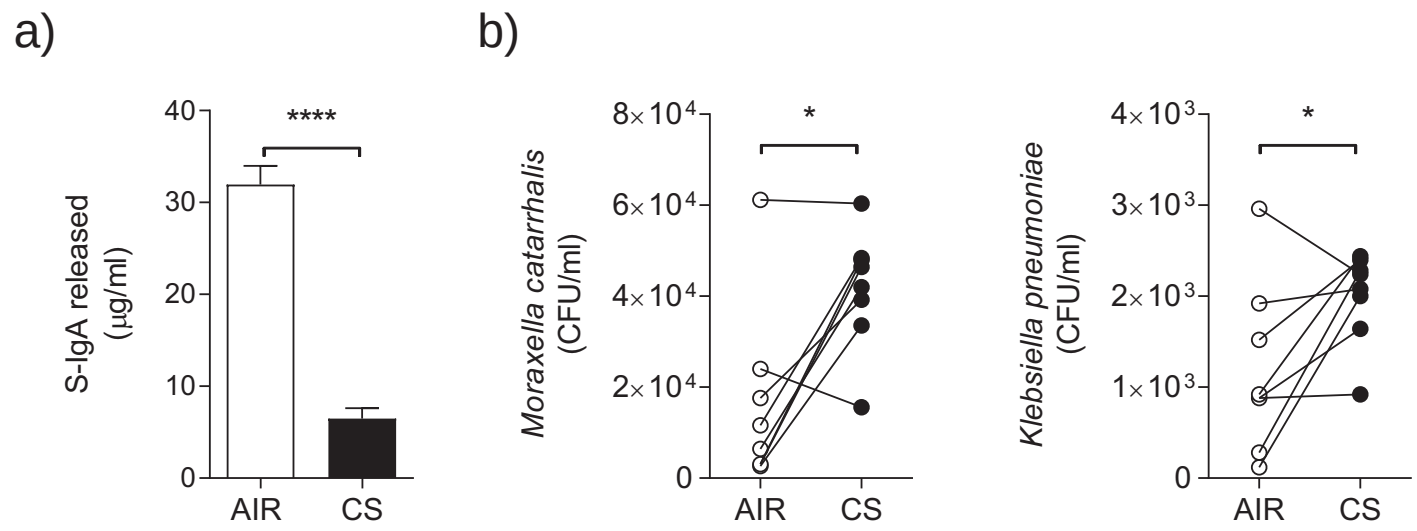
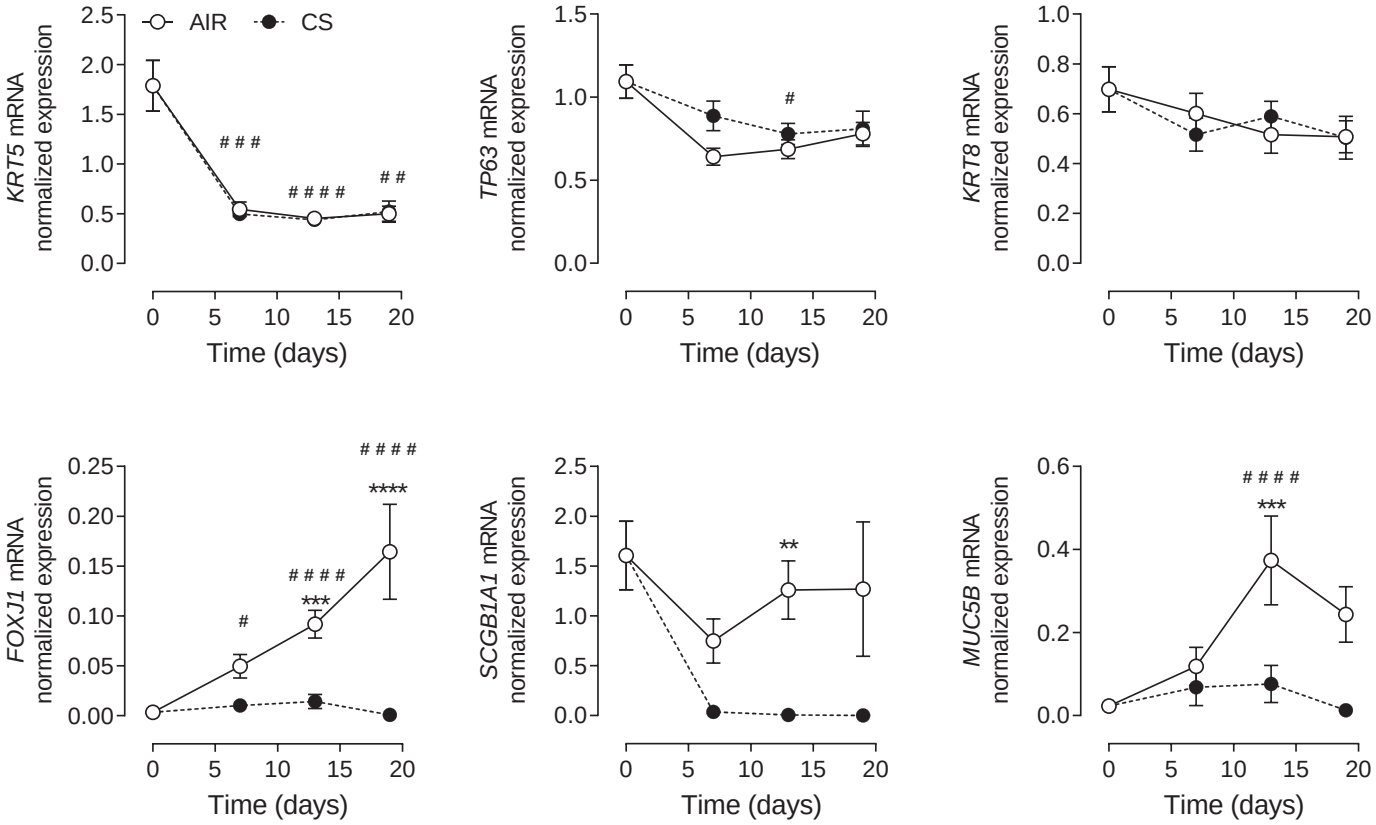

a)
b)
S-IgA released (µg/ml)
40
30
20
10
0
****
AIR
CS
Moraxella catarrhalis (CFU/ml)
8×10^4
6×10^4
4×10^4
2×10^4
0
*
AIR
CS
Klebsiella pneumoniae (CFU/ml)
4×10^3
3×10^3
2×10^3
1×10^3
0
*
AIR
CS

Figure 5

a)


AIR
CS
KRT5 mRNA normalized expression
TP63 mRNA normalized expression
KRT8 mRNA normalized expression
FOXJ1 mRNA normalized expression
SCGB1A1 mRNA normalized expression
MUC5B mRNA normalized expression
Time (days)
####
*** ****

b)

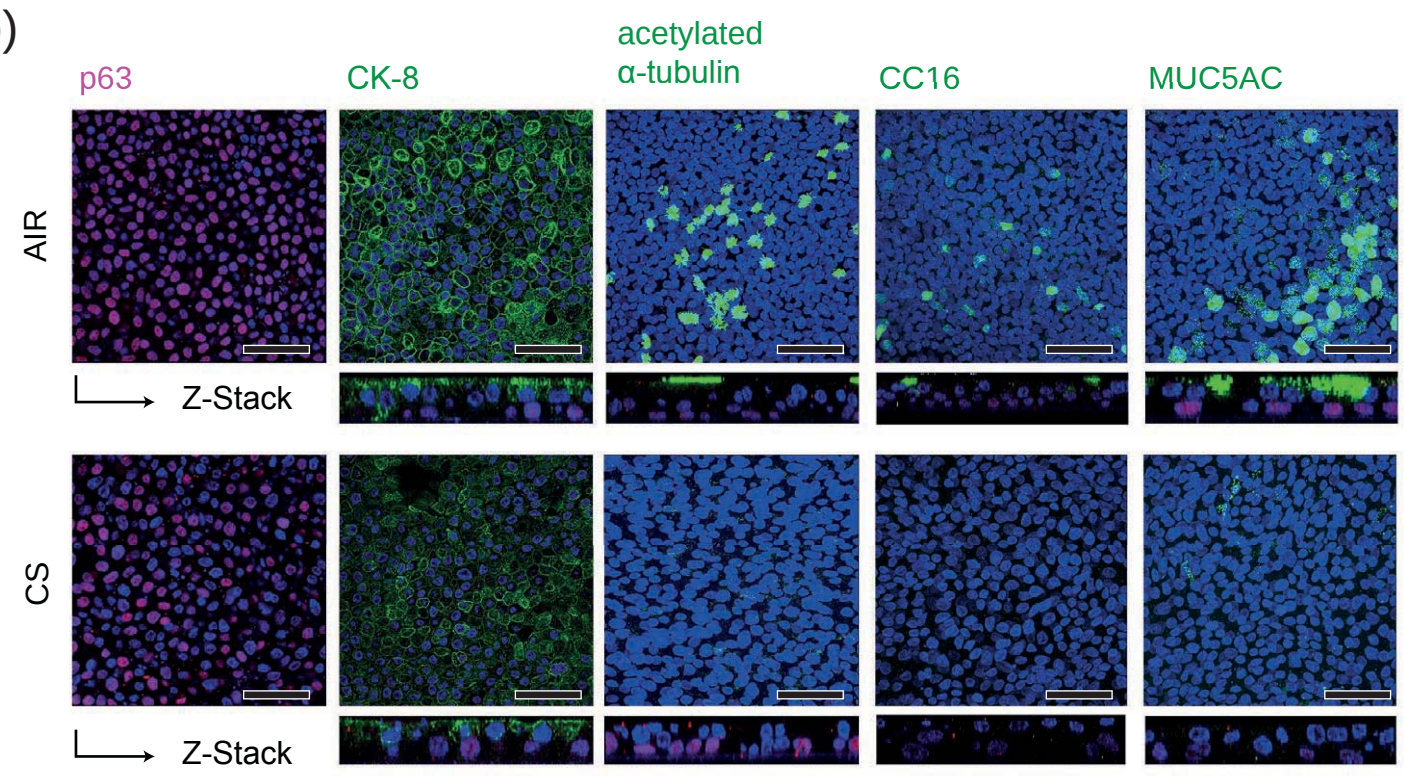

p63
CK-8
acetylated α-tubulin
CC16
MUC5AC
AIR
CS
Z-Stack
Z-Stack

Figure 6

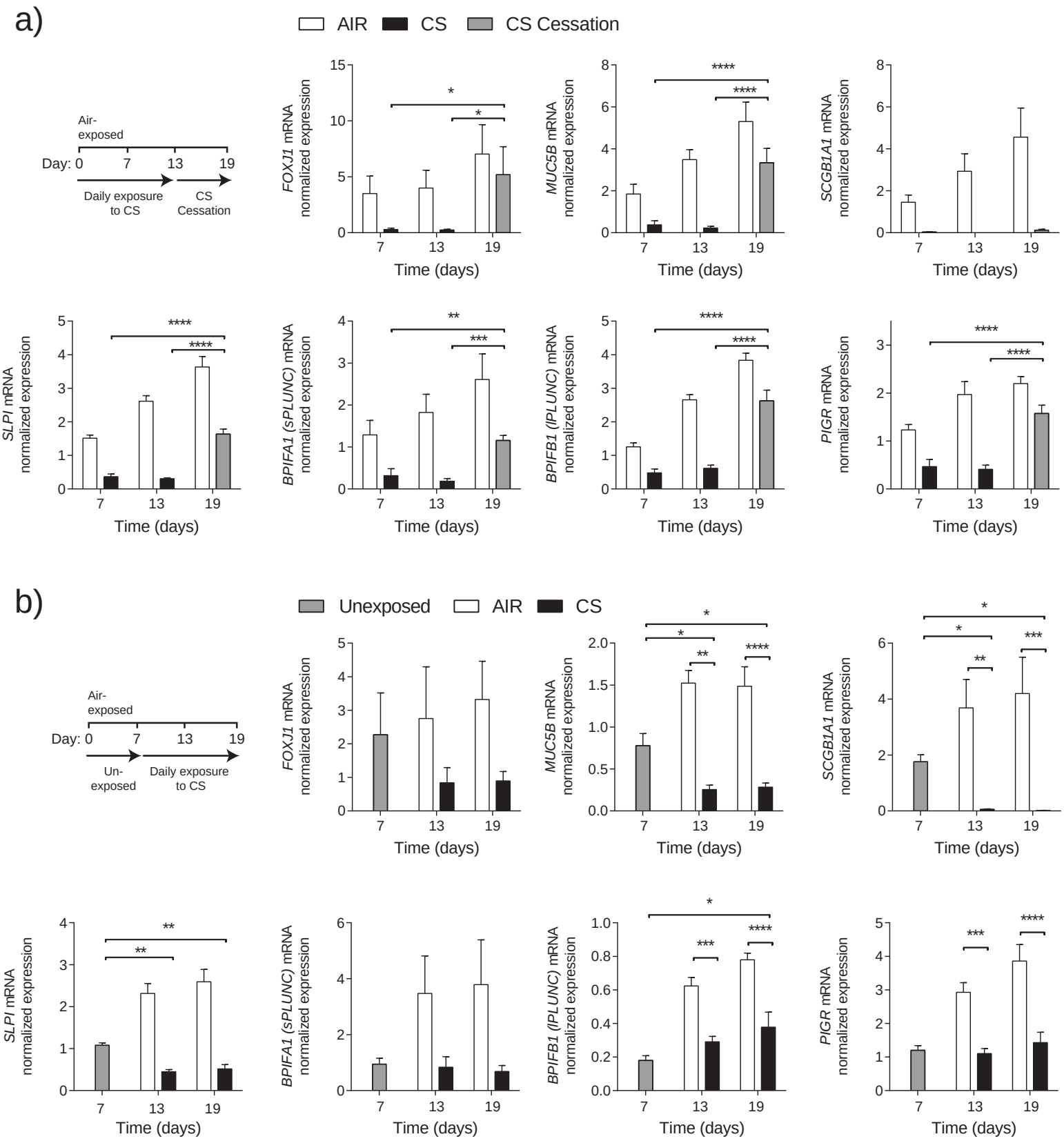

a)
AIR
CS
CS Cessation
Air-exposed
Day: 0
7
13
19
Daily exposure to CS
CS Cessation
FOXJ1 mRNA normalized expression
MUC5B mRNA normalized expression
SCGB1A1 mRNA normalized expression
SLPI mRNA normalized expression
BPIFA1 (sPLUNC) mRNA normalized expression
BPIFB1 (PLUNC) mRNA normalized expression
PIGR mRNA normalized expression
Time (days)
b)
Unexposed
AIR
CS
Air-exposed
Un-exposed
Daily exposure to CS

Figure 7

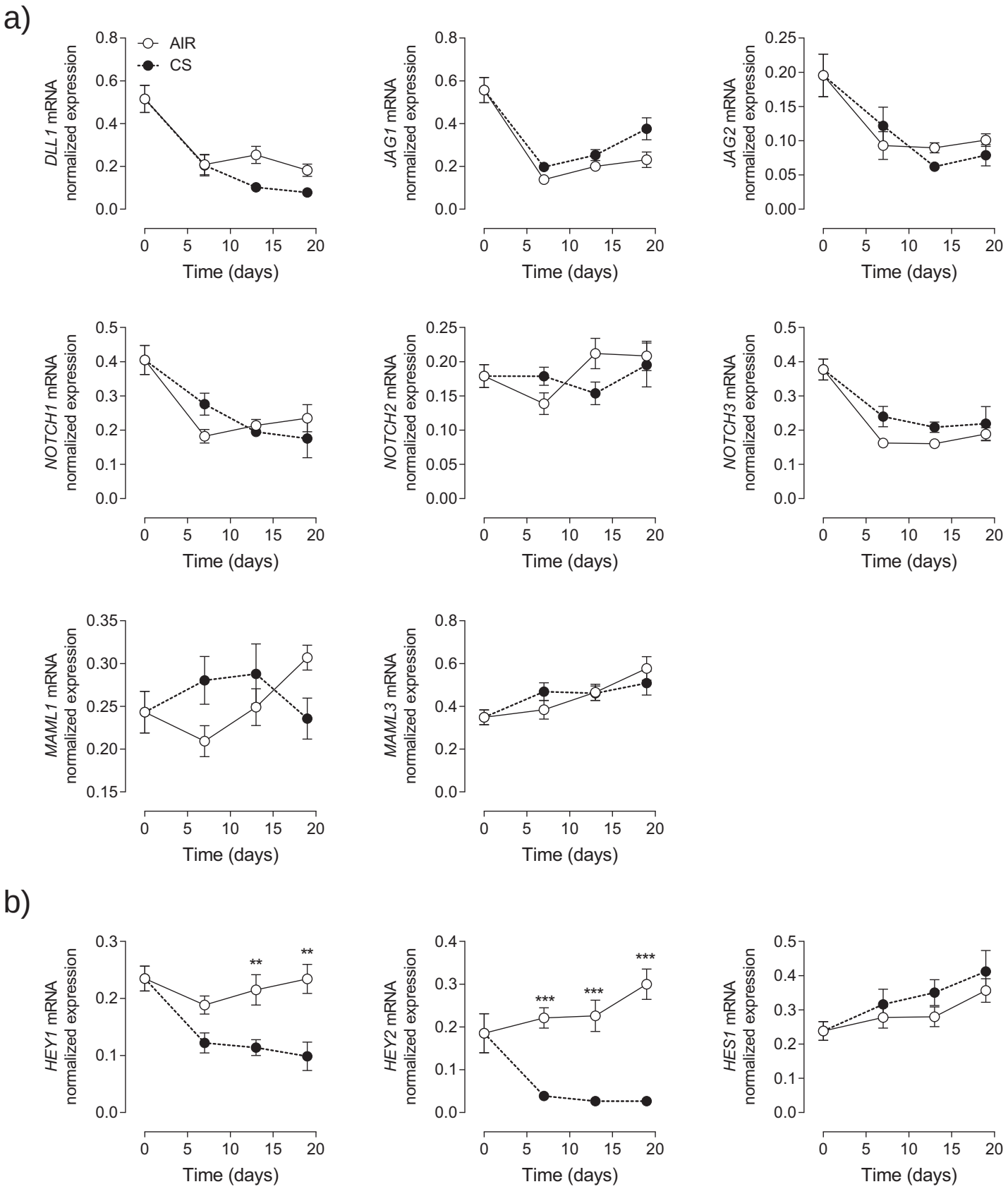
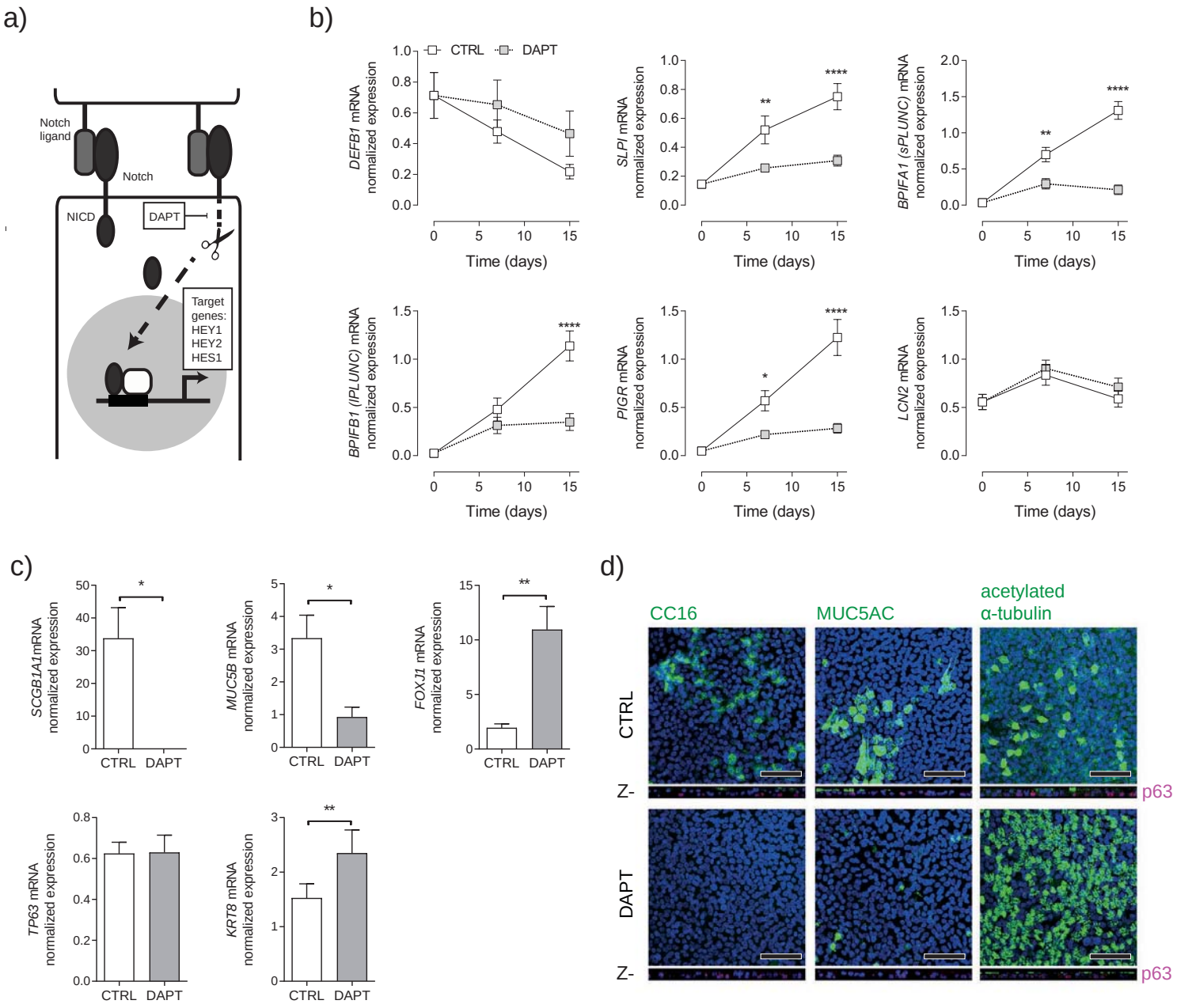

a)
AIR
CS
DLL1 mRNA normalized expression
JAG1 mRNA normalized expression
JAG2 mRNA normalized expression
NOTCH1 mRNA normalized expression
NOTCH2 mRNA normalized expression
NOTCH3 mRNA normalized expression
MAML1 mRNA normalized expression
MAML3 mRNA normalized expression
Time (days)
b)
HEY1 mRNA normalized expression
HEY2 mRNA normalized expression
HES1 mRNA normalized expression
**
***

Figure 8


a)
Notch ligand
Notch
NICD
DAPT
Target genes: HEY1 HEY2 HES1
b)
CTRL
DAPT
DEFB1 mRNA normalized expression
SLPI mRNA normalized expression
BPIFA1 (sPLUNC) mRNA normalized expression
BPIFB1 (lPLUNC) mRNA normalized expression
PIGR mRNA normalized expression
LCN2 mRNA normalized expression
Time (days)
c)
SCGB1A1 mRNA normalized expression
MUC5B mRNA normalized expression
FOXJ1 mRNA normalized expression
TP63 mRNA normalized expression
KRT8 mRNA normalized expression
CTRL
DAPT
d)
CC16
MUC5AC
acetylated α-tubulin
CTRL
DAPT
Z-
p63

ONLINE DATA SUPPLEMENT

SUPPLEMENTAL METHODS

Collection of cells

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

RNA isolation, cDNA synthesis and qPCR

Cells were lysed using lysis buffer from Promega, Leiden, the Netherlands. Next, RNA was extracted using the Maxwell tissue RNA extraction kit (Promega) and quantified using the Nanodrop ND-1000 Spectrophotometer (Nanodrop technologies, Wilmington, DE). cDNA

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

Confocal microscopy

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

Antibacterial activity assay

Direct antimicrobial activity was assessed in cultures of ALI-PBEC that were exposed daily to whole cigarette smoke or air controls for 13 days, followed by replacement with antibiotics-free cell culture medium for an additional 48 h period. *Moraxella catarrhalis*

strain LUH2760 and *Klebsiella pneumoniae* strain LUH2754 were cultured in Tryptic Soy broth (TSB) while shaking overnight at 37^0 C. Next, the overnight cultures were transferred into fresh TSB medium (1/50 dilution) and incubated for 4 h at 37°C -while shaking- to obtain mid log-phase-growing bacteria. Bacterial concentrations of log-phase cultures were determined by $OD_{600\ nm}$ measurements, pre-diluted in PBS and final dilution was made in antibiotics-free cell culture medium. Twenty µl of bacterial suspension was added on the apical surface of the cells at a concentration of ~$6x10^5$/ml CFU/ml for *M. catarrhalis* and ~$1x10^4$ CFU/ml for *K. pneumoniae* and incubated at 37°C, 5% CO_2 for 2 h. Hereafter, membranes containing the cells with bacteria were dissected from the inserts and placed into tubes containing sterile glass beads and 1% TSB in PBS. Next cells were disrupted by using a minilys personal homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) for 2 times 30 s and kept on ice in between. Serial dilutions of both bacterial suspensions were plated on Tryptic Soy Sheep blood (TSS) agar plates (Biomerieux, Zaltbommel, The Netherlands), and incubated overnight at 37°C to assess surviving bacteria by CFU determination.

ELISA

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

Trans-epithelial electrical resistance

Epithelial barrier integrity of ALI-PBEC cultures was determined during cell differentiation by measuring the trans-epithelial electrical resistance (TEER) using the MilliCell-ERS

(Millipore, Bedford, MA). TEER values were shown as Ω*cm^2 and calculated as TEER = (measured value – background value) *surface transwell insert in cm^2.

Transcytosis assay

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

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

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

SUPPLEMENTAL RESULTS

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

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

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

References

1. Amatngalim GD, van Wijck Y, de Mooij-Eijk Y, Verhoosel RM, Harder J, Lekkerkerker AN, Janssen RA, Hiemstra PS. Basal cells contribute to innate immunity of the airway epithelium through production of the antimicrobial protein RNase 7. *J Immunol* 2015: 194(7): 3340-3350.
2. van Wetering S, van der Linden AC, van Sterkenburg MA, Rabe KF, Schalkwijk J, Hiemstra PS. Regulation of secretory leukocyte proteinase inhibitor (SLPI) production by human bronchial epithelial cells: increase of cell-associated SLPI by neutrophil elastase. *Journal of investigative medicine : the official publication of the American Federation for Clinical Research* 2000: 48(5): 359-366.
3. Gohy ST, Detry BR, Lecocq M, Bouzin C, Weynand BA, Amatngalim GD, Sibille YM, Pilette C. Polymeric immunoglobulin receptor down-regulation in chronic obstructive pulmonary disease. Persistence in the cultured epithelium and role of transforming growth factor-beta. *Am J Respir Crit Care Med* 2014: 190(5): 509-521.

SUPPLEMENTAL TABLES

sTable 1. Primer sequences.

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

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

sTable 2. Antibodies used for confocal imaging

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

SUPPLEMENTAL FIGURE LEGENDS

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

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

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

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

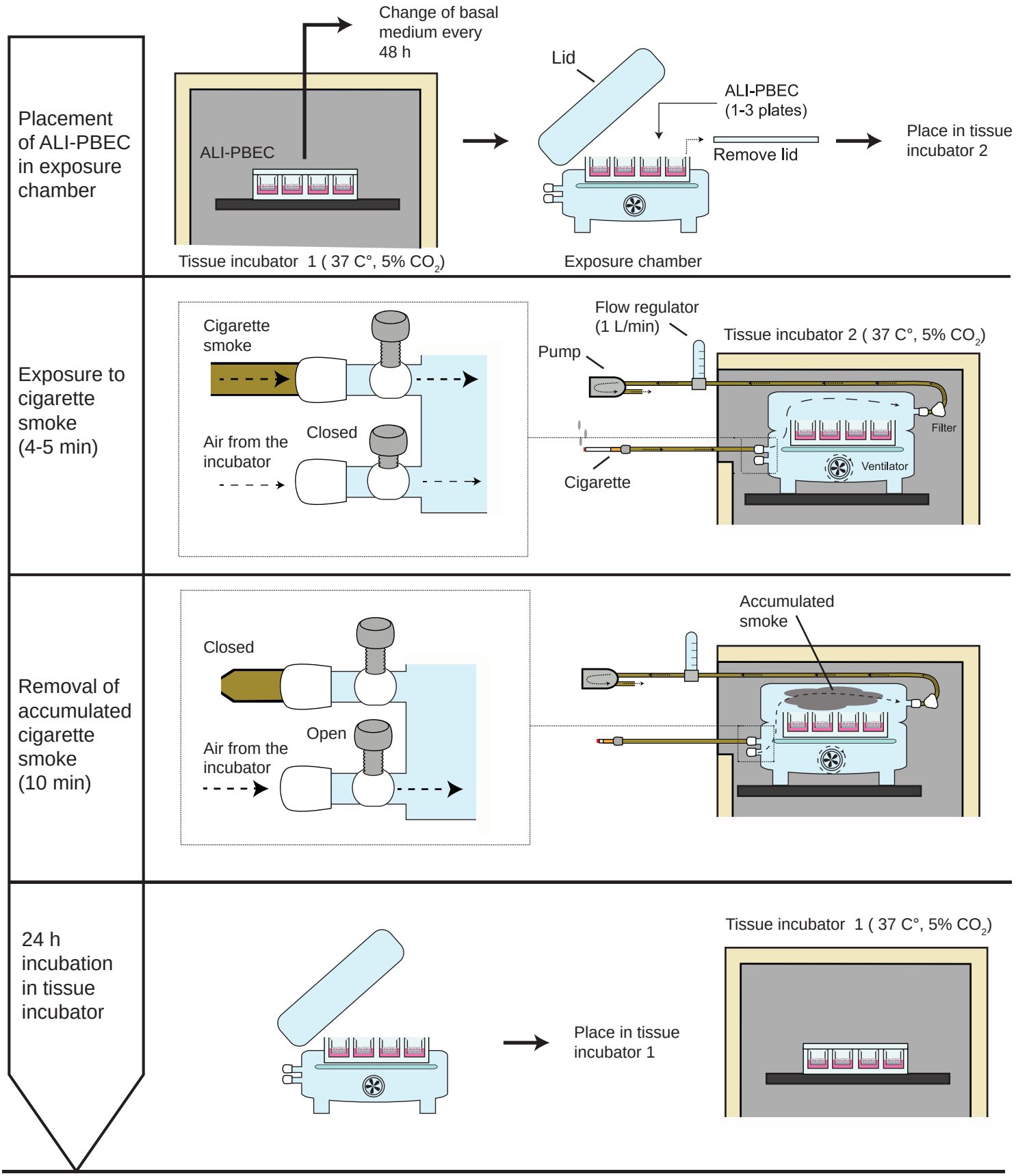
s**Figure 3. Persistence of cigarette smoke-induced changes in airway epithelial HDP expression and cellular composition.**

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

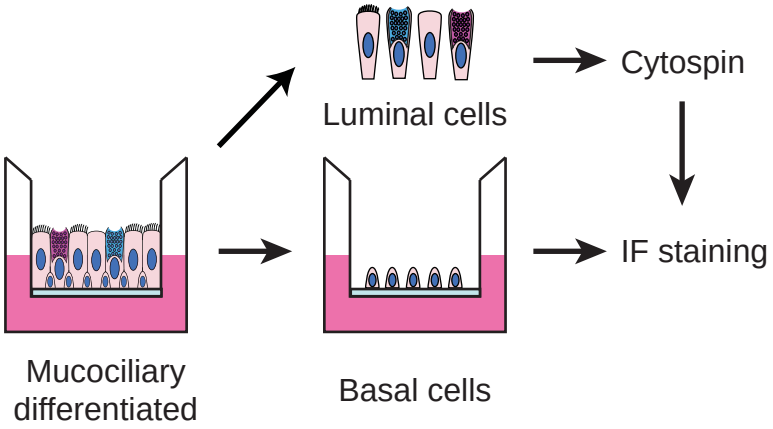
sFigure 4. Chronic cigarette smoke exposure of airway epithelial cell cultures does not lead to cell toxicity

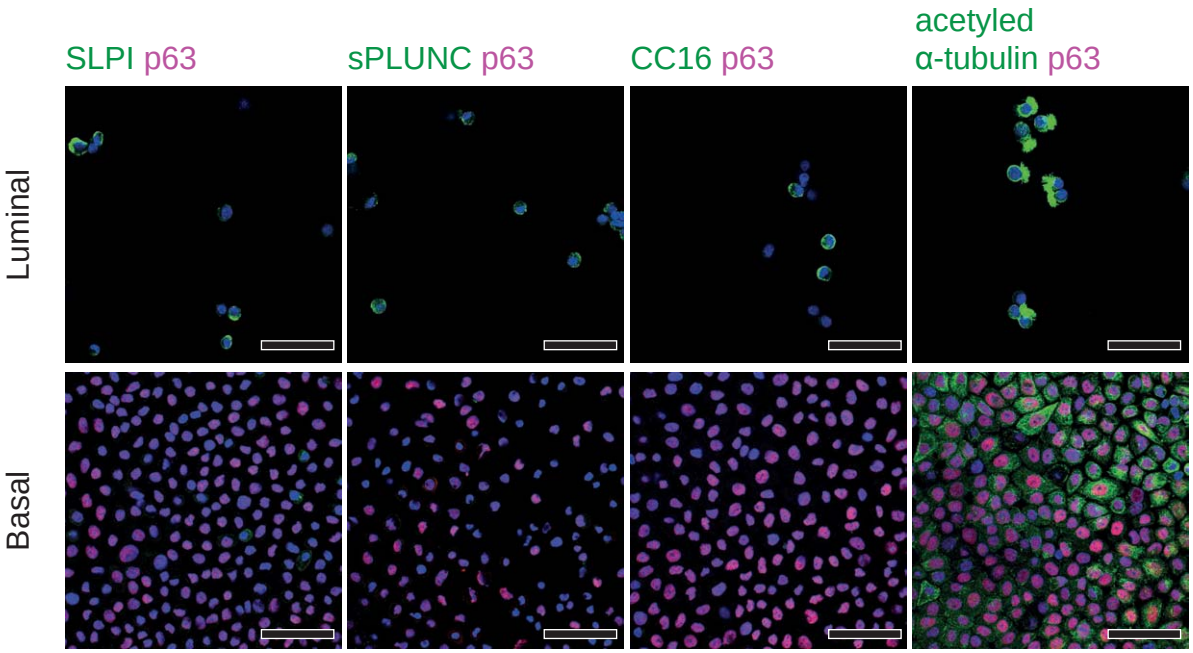
Air-liquid interface cultures of primary bronchial epithelial cells (ALI-PBEC) were daily exposed to whole cigarette smoke (CS) or air as a control (AIR) during differentiation for 13-19 consecutive days. (A) Each day trans-epithelial electrical resistance (TEER) measurements were performed ~18 h after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

sFigure 1


Placement of ALI-PBEC in exposure chamber
Change of basal medium every 48 h
ALI-PBEC
Tissue incubator 1 (37 C°, 5% CO2)
Lid
ALI-PBEC (1-3 plates)
Remove lid
Exposure chamber
Place in tissue incubator 2
Exposure to cigarette smoke (4-5 min)
Cigarette smoke
Air from the incubator
Closed
Flow regulator (1 L/min)
Pump
Tissue incubator 2 (37 C°, 5% CO2)
Filter
Ventilator
Cigarette
Removal of accumulated cigarette smoke (10 min)
Closed
Open
Air from the incubator
Accumulated smoke
24 h incubation in tissue incubator
Place in tissue incubator 1
Tissue incubator 1 (37 C°, 5% CO2)
Procedure is repeated for 13-19 days

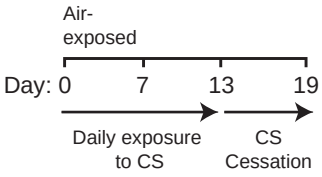
sFigure 2

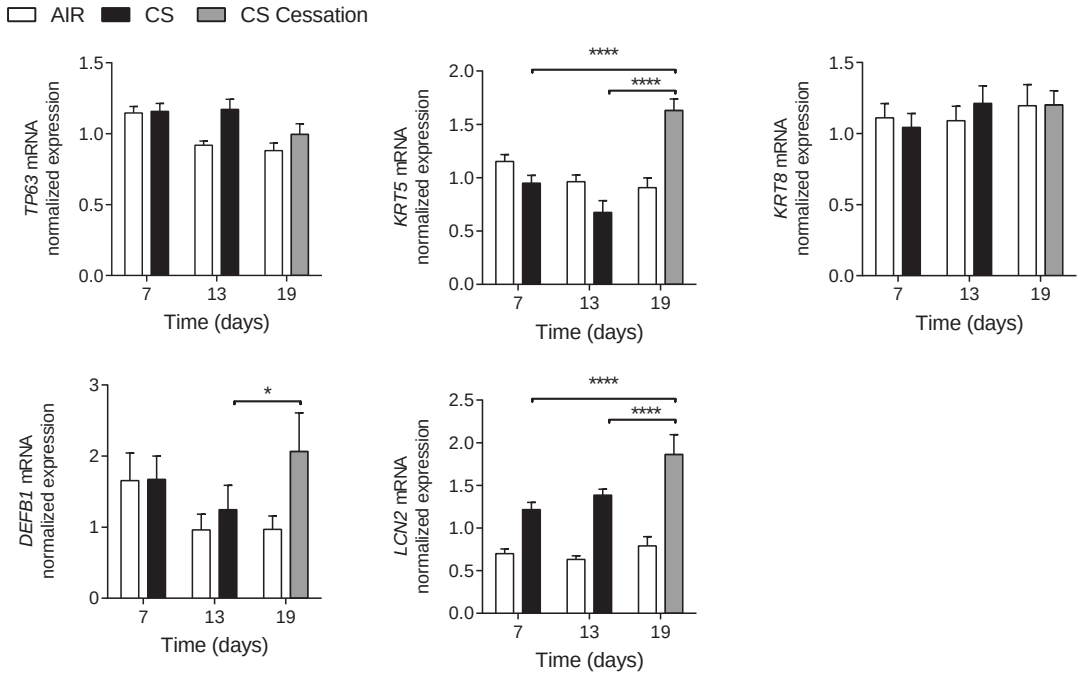

Luminal cells
Cytospin
IF staining
Mucociliary differentiated
Basal cells


SLPI p63
sPLUNC p63
CC16 p63
acetyled α-tubulin p63
Luminal
Basal

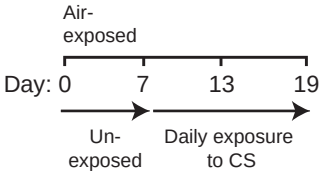
sFigure 3

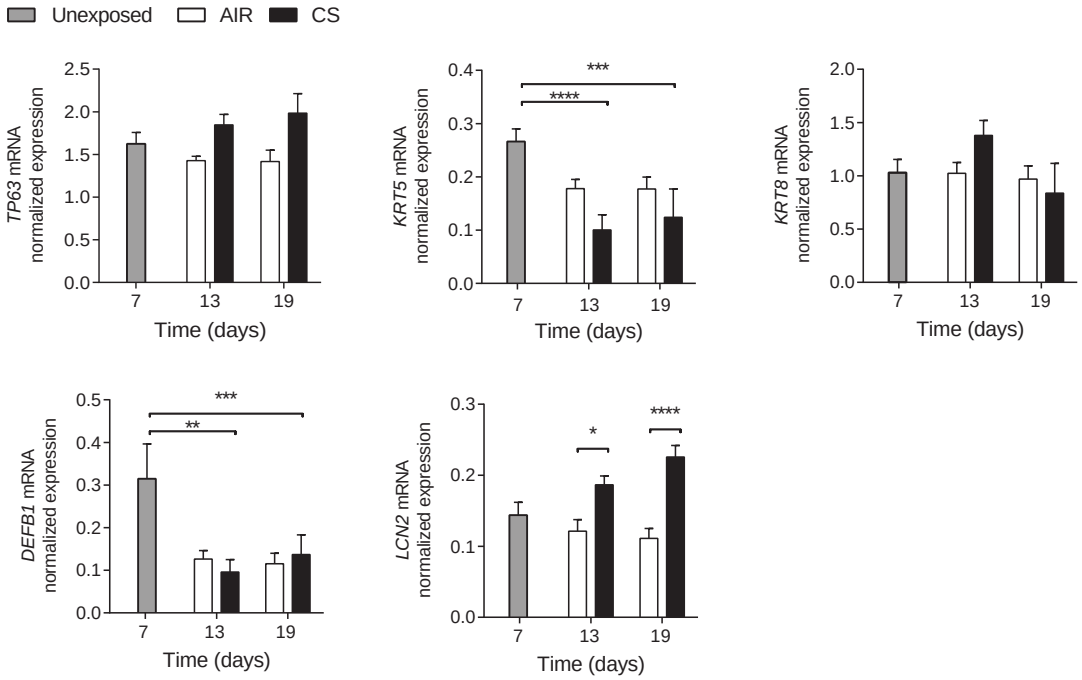
a)


Air-
exposed
Day: 0
7
13
19
Daily exposure
to CS
CS
Cessation

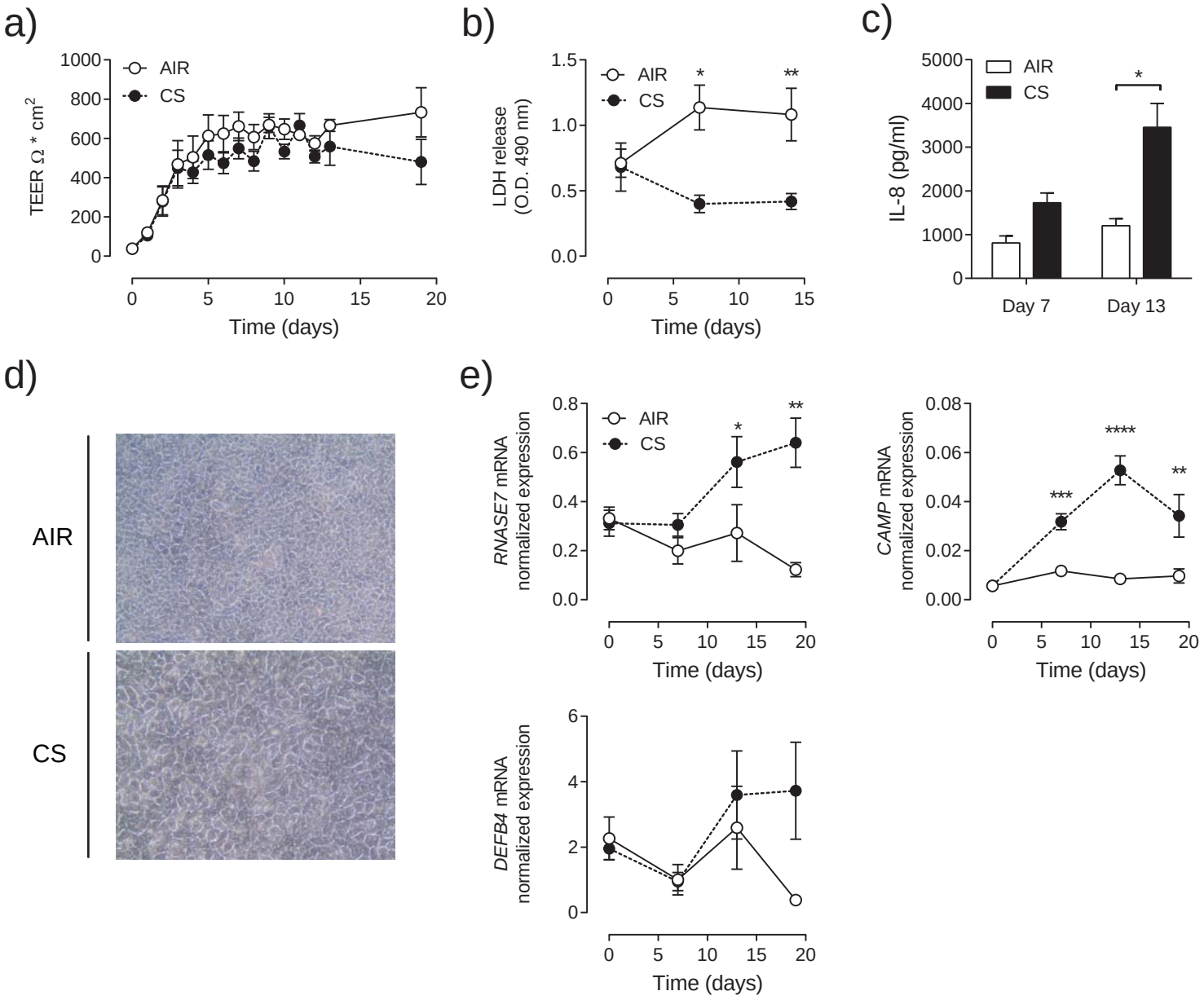

AIR
CS
CS Cessation
TP63 mRNA normalized expression
KRT5 mRNA normalized expression
KRT8 mRNA normalized expression
DEFB1 mRNA normalized expression
LCN2 mRNA normalized expression
Time (days)
****
*

b)


Air-
exposed
Day: 0
7
13
19
Un-
exposed
Daily exposure
to CS


Unexposed
AIR
CS
TP63 mRNA normalized expression
KRT5 mRNA normalized expression
KRT8 mRNA normalized expression
DEFB1 mRNA normalized expression
LCN2 mRNA normalized expression
Time (days)
***
****
**
*

sFigure 4


a)
TEER Ω * cm2
AIR
CS
Time (days)
b)
LDH release (O.D. 490 nm)
AIR
CS
Time (days)
c)
IL-8 (pg/ml)
AIR
CS
Day 7
Day 13
d)
AIR
CS
e)
RNASE7 mRNA normalized expression
AIR
CS
Time (days)
CAMP mRNA normalized expression
Time (days)
DEFB4 mRNA normalized expression
Time (days)