ORIGINAL ARTICLE
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Aberrant epithelial differentiation by cigarette smoke dysregulates respiratory host defence

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Loss of highly expressed host defence proteins as a result of cigarette smoke-induced airway epithelial remodelling http://ow.ly/Q6Jr30iR6Jg

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

Localisation 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 exposed daily 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.

Expression of the polymeric immunoglobulin receptor, secretory leukocyte protease inhibitor and long and short PLUNC (palate, lung and nasal epithelium clone protein) was restricted to luminal cells and exposure of differentiating PBECs 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 transepithelial transport of IgA and bacterial killing.

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 chronic obstructive pulmonary disease to respiratory infections.

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Introduction

Respiratory infections and microbial colonisation 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 β-defensin (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 (RNase)7, LL-37 and hBD-2 is low during homeostasis, but can be induced by inflammatory mediators, microbial products and upon injury of epithelial cells, and thus contributes 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 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, characterised 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 polarised in the epithelium, and if so, how cigarette smoke-induced remodelling of the airway epithelium affects their expression. We hypothesised that cigarette smoke-induced alterations in epithelial cell differentiation result in a decreased expression of proteins that contribute to respiratory HDPs, which may render the host more susceptible to infection.

Methods

Cell culture

Primary bronchial epithelial cells (PBECs) were obtained from tumour-free resected lung tissue at the Leiden University Medical Center (Leiden, the Netherlands), as described in the online supplementary material, and cultured as described [11]. PBECs were cultured at the air–liquid interface (ALI) for 13–19 days (figure 1a). 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, Amsterdam, the Netherlands) in PBS for 10 min on ice and washed afterwards in ice-cold PBS. The remaining basal epithelial cell fractions on the transwell inserts were either lysed in RNA lysis buffer (Promega, Leiden) or fixed with 1% paraformaldehyde (Millipore) in PBS for 10 min on ice and washed afterwards with ice-cold PBS. Next, cells were stained as described in the online supplementary material with antibodies described in online supplementary table S2.

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 using 3R4F reference cigarettes (University of Kentucky, Lexington, KY, USA). Cigarette smoke exposure was repeated daily as described in [11], in the figure legends of figure 1 and online supplementary figure S1 and illustrated in

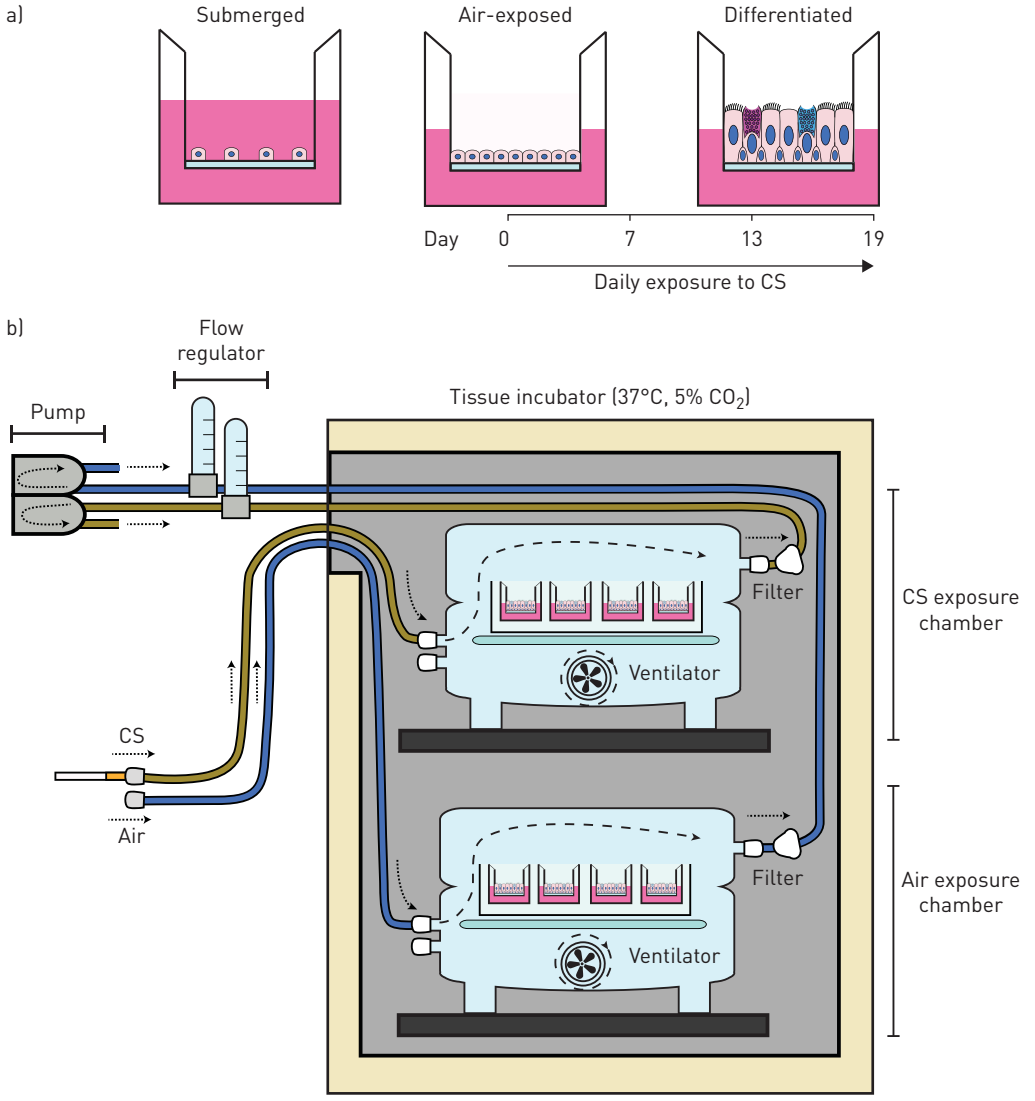

FIGURE 1 Cell culture set-up and cigarette smoke (CS) exposure of primary bronchial epithelial cells (PBECs) differentiated at the air–liquid interface. a) PBECs were seeded on coated transwells and cultured in submerged conditions until confluent. At day 0, cultures were air-exposed and cultured for an additional 13–19 days to allow mucociliary differentiation. b) Each day, starting at day 0, cultures were exposed to CS by placing them in an exposure chamber that was infused with either CS 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. ~4 h before each CS exposure the apical surface of the cultures was washed to remove mucus. Basal medium was changed every other day. CO_2: carbon dioxide.

figure 1b and online supplementary figure S1. Briefly, cells were exposed in modified hypoxic chambers for 4–5 min to either cigarette smoke from one cigarette or to room air, after which smoke was removed by ventilation with air over 10 min and cells were subsequently placed back in the incubator overnight. ~18–20 h later, ALI-PBEC were washed apically with PBS and 4 h thereafter 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, complementary DNA synthesis and quantitative PCR

Methods are described in the online supplementary material with primers described in online supplementary table S1.

Confocal microscopy

Cells were fixed on transwell inserts in 1% paraformaldehyde (Millipore) in PBS for 10 min on ice and washed afterwards with ice-cold PBS. Next, cells were stained as described in the online supplementary material with antibodies described in online supplementary table S2.

Transcytosis experiment

Methods are described in the online supplementary material.

Antibacterial activity assay

Methods are described in the online supplementary material

ELISA and transepithelial electrical resistance

Methods are described in the online supplementary material.

Inhibition of differentiation by DAPT

At day 0, PBECs 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, La Jolla, CA, USA). Data are shown as mean±SEM and significance was tested using 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 polarised distribution in airway epithelial cell cultures

In this study we have focused on a set of proteins and peptides that are important for respiratory host defence. These HDPs were selected based on their constitutive and/or high expression by airway epithelial cells during homeostasis, *i.e.* SLPI, s/lPLUNC, pIgR, hBD-1 and LCN2. First, we investigated whether expression of these proteins was polarised in the airway epithelial cultures. To this end, we prepared luminal and basal epithelial cell-enriched fractions of well-differentiated PBECs, cultured at the ALI (figure 2a). 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) (figure 2a), and by immunofluorescence staining for p63 (basal cells), CC16 (club cells) and acetylated α-tubulin (ciliated cells) (online supplementary figure S2). Further analysis of these fractions showed that the luminal cell-enriched fraction expressed significantly higher levels of *BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *SLPI* (figure 2a). In contrast, *LCN2* and *DEFB1* expression did not differ between the luminal and basal cell-enriched fraction (figure 2a). The luminal cell-specific expression of SLPI and sPLUNC was further confirmed using confocal imaging, in which the staining of both proteins did not colocalise with $p63^+$ basal cells, but was highly present at the apical side of the PBEC culture and in the luminal cell-enriched fraction (figure 2b and online supplementary figure S2).

Chronic cigarette smoke exposure of airway epithelial cell cultures reduces expression of respiratory HDPs

Next, we 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 (figure 1 and online supplementary figure S1). Gene expression analysis showed that *DEFB1* (hBD-1) mRNA levels decreased during differentiation, but were not affected by cigarette smoke exposure (figure 3a). Conversely, expression of *SLPI*, *BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *PIGR* strongly increased during differentiation, and this increase was significantly prevented by cigarette smoke (figure 3a). In contrast, gene expression of *LCN2* (lipocalin 2) was increased by cigarette smoke exposure during differentiation (figure 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 (figure 3b). Indeed, hBD-1 levels reduced over the time of differentiation in the apical wash, but were not significantly affected by chronic cigarette smoke exposure, whereas SLPI levels were significantly lower in chronic cigarette smoke-exposed cell cultures (figure 3b). Next, we performed immunofluorescence staining of the airway epithelial cell cultures and found strongly reduced presence of SLPI-, sPLUNC- and pIgR-positive cells in chronic cigarette smoke-exposed epithelium compared to air controls (figure 3c). These results confirmed selective impairment of specific respiratory HDPs by chronic cigarette smoke exposure during airway epithelial differentiation.

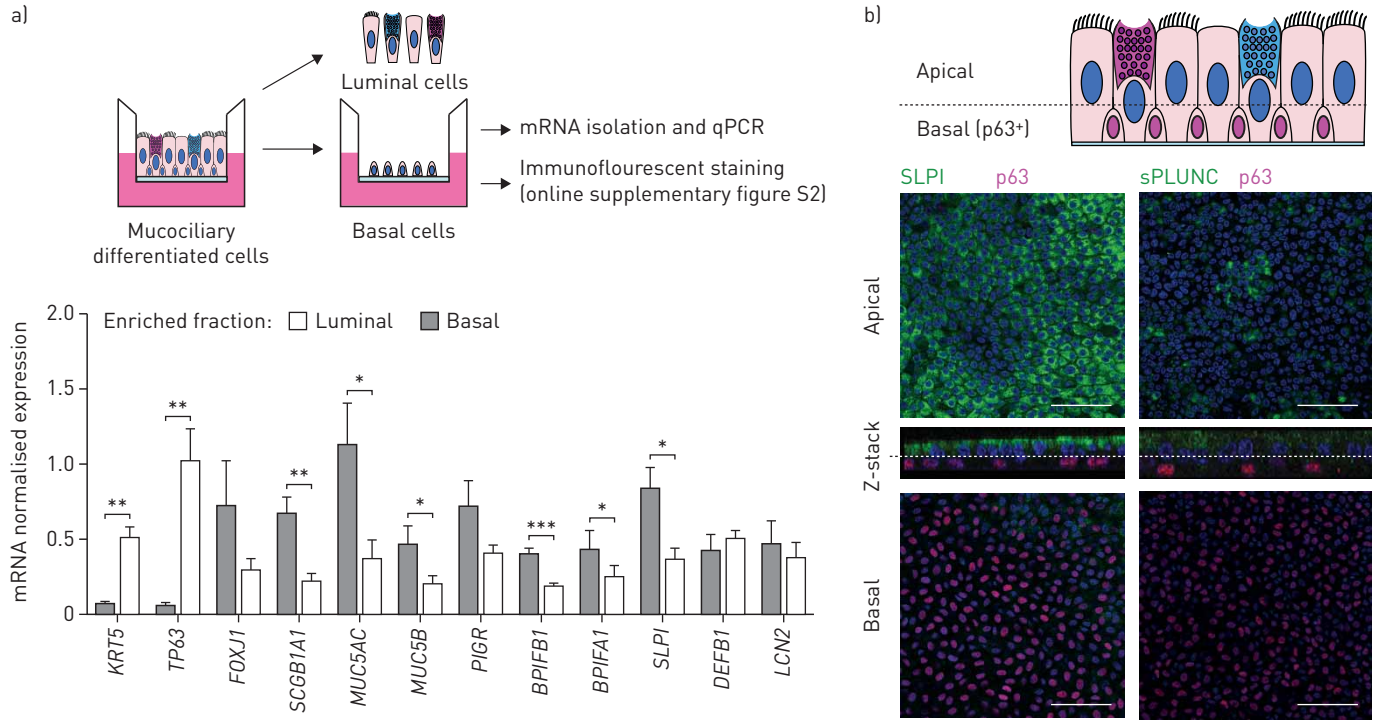

FIGURE 2 Respiratory host defence proteins display a polarised distribution in air–liquid interface (ALI) cultures of primary bronchial epithelial cells (PBECs). a) PBECs were seeded on coated transwells and cultured in submerged conditions until confluent. At day 0, cultures were air-exposed and cultured at the ALI. After 3–4 weeks of differentiation luminal and basal cell-enriched fractions were separated followed by RNA isolation, complementary DNA synthesis and quantitative (q)PCR analysis. Data are shown as target gene expression normalised for the geometric mean expression of the reference genes ATP synthase, hydrogen transporting, mitochondrial F1 complex, β-polypeptide (*ATP5B*), β2-microglobulin (*B2M*) and ribosomal protein L13a (*RPL13A*); n=5–7 different donors. Statistical significance was tested using a paired t-test. *: p<0.05, **: p<0.01, ***: p<0.001. b) Confocal images to visualise polarised distribution of secretory leukocyte protease inhibitor (SLPI) and short palate, lung and nasal epithelium clone protein (sPLUNC) in differentiated ALI-PBEC culture 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 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining (blue). Z-stacks and images of the apical and basal side of stained cells were made by confocal imaging. Images shown are representative for results obtained with cells from four different donors. Scale bars=50 µm.

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 whether the strong reduction in *SLPI*, *BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *PIGR* expression levels in the cigarette smoke-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 cigarette smoke-exposed cultures (figure 4a). Furthermore, we analysed bacterial killing by chronic cigarette smoke-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 cigarette smoke-exposed PBEC cultures when compared to air-exposed cultures for both pathogens (figure 4b). These data indicate that various host defence mechanisms are functionally impaired in cigarette smoke-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 whether chronic cigarette smoke exposure affected differentiation of ALI-PBECs 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*), which is expressed by intermediate/committed progenitor epithelial cells [13], was not affected by cigarette smoke (figure 5a). In contrast, expression of the specialised 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 cigarette smoke (figure 5a). Confocal imaging confirmed the aberrant epithelial

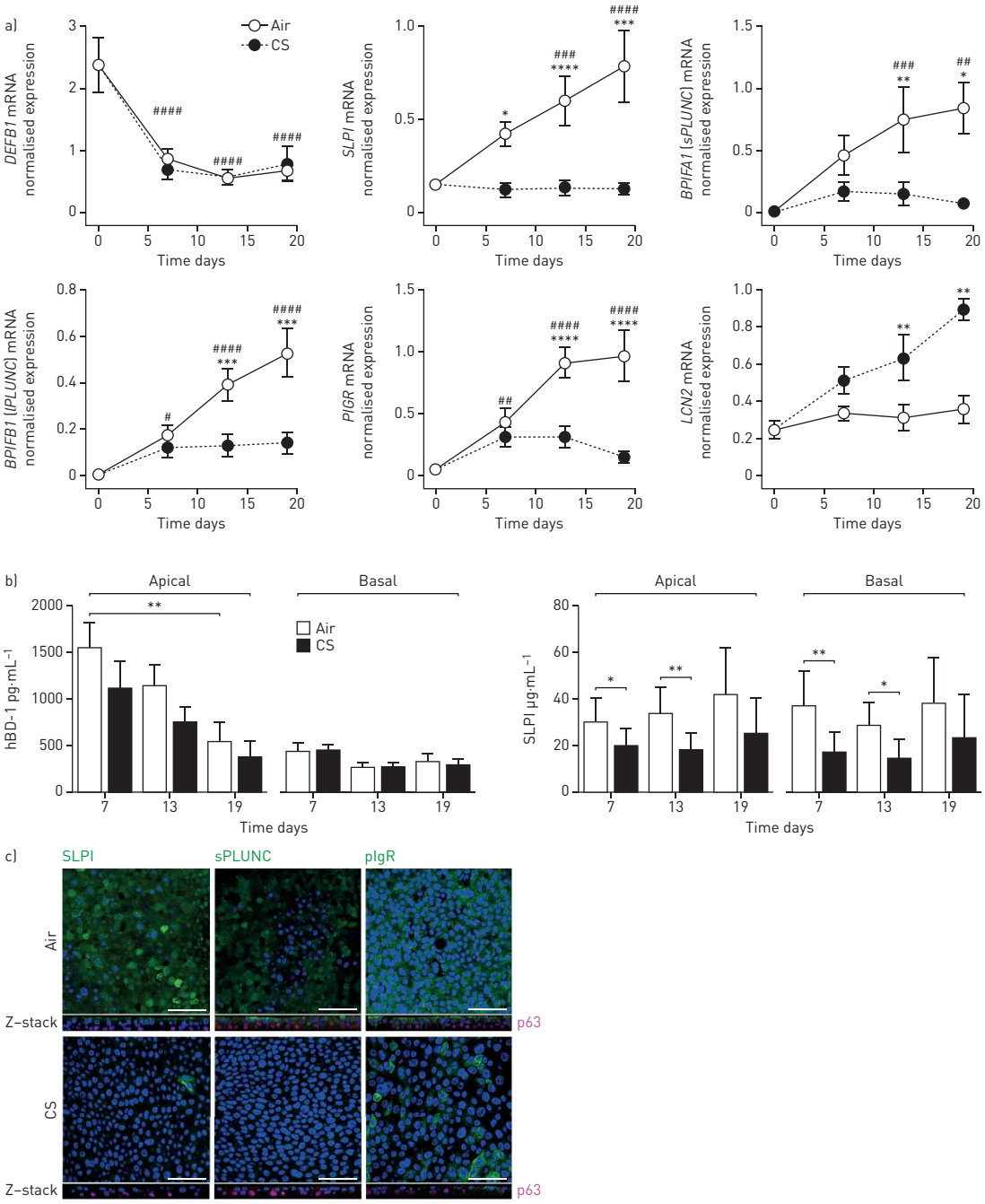

FIGURE 3 Chronic cigarette smoke exposure of air–liquid interface (ALI) cultures of primary bronchial epithelial cells (PBECs) lowers the expression of luminal cell-restricted host defence proteins. a) ALI-PBECs were exposed daily to whole cigarette smoke (CS) or air as a control (Air) during differentiation for 13–19 consecutive days. Cells were lysed at several points during this time course and RNA was isolated followed by complementary DNA synthesis to assess gene expression of *DEFB1* (human β-defensin-1 (hBD-1)), *SLPI* (secretory leukocyte protease inhibitor), *BPIFA1* (short palate, lung and nasal epithelium clone protein (*sPLUNC*)), *BPIFB1* (long palate, lung and nasal epithelium clone protein (*lPLUNC*)), *PIGR* (polymeric immunoglobulin receptor) and *LCN2* (lipocalin 2). Data are presented as target gene expression normalised for the geometric mean expression of the reference genes ATP synthase, hydrogen-transporting, mitochondrial F1 complex, β polypeptide (*ATP5B*), β2-microglobulin (*B2M*) and ribosomal protein L13a (*RPL13A*). Days 0, 7 and 13 n=8 different donors; 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 days 7, 13 and 19 and unexposed cultures at day 0. b) ELISA for hBD-1 and SLPI was performed on the apical wash and basal medium of these cultures. Days 7 and 13, n=8 different donors and day 19 n=4 different donors. Statistical differences on day 7 and day 13 (not day 19) was tested using a paired two-way ANOVA to compare air and CS. *: p<0.05, **: p<0.01. c) ALI-PBECs were differentiated for 2–3 weeks in which they were daily exposed to CS or air as a control. Subsequently, the cells were fixed in 1% paraformaldehyde and stained using primary antibodies against SLPI, sPLUNC and pIgR (all green staining) in combination with 4′,6-diamidino-2-phenylindole (DAPI) to stain the nuclei (blue staining). Images shown are representative for results obtained with cell cultures from four different donors. Scale bars=50 µm.

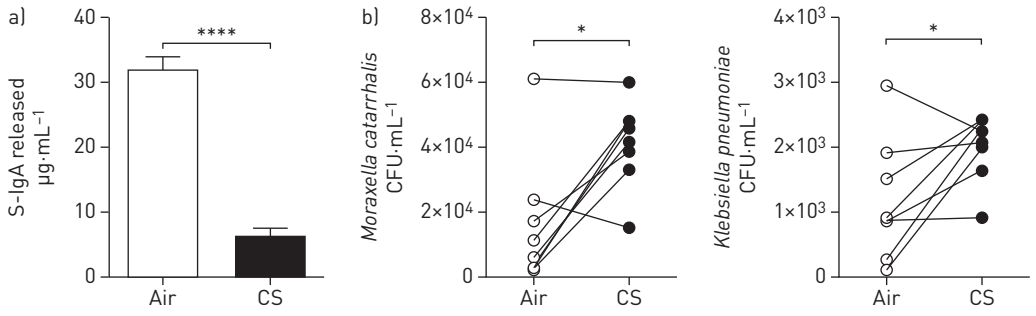

FIGURE 4 Chronic cigarette smoke exposure of air–liquid interface (ALI) cultures of primary bronchial epithelial cells (PBECs) impairs host defence activities. a) ALI-PBECs were exposed daily to whole cigarette smoke (CS) or air as a control (Air) during differentiation for 13 consecutive days. After 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 recognise S-IgA and not d-IgA). n=10 different donors. b) After 13 days of chronic CS exposure, ALI-PBECs 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 CFU·mL^{-1}. n=8 different donors. Significance was determined using a paired t-test. *: p<0.05, ****: p<0.0001.

differentiation in cigarette smoke-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 cigarette smoke-exposed cultures, while cytokeratin-8 (CK-8)$^+$ and p63$^+$ cells remained unchanged between air- and cigarette smoke-exposed cultures (figure 5b).

Reversibility of cigarette smoke-induced effects on HDP expression

To assess the persistence of the cigarette smoke-induced reduction in *SLPI*, *BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *PIGR* expression levels and its effect on cellular composition, we allowed the cultures to recover from 13 days of cigarette smoke exposure by culturing the cells for an additional 6 days without cigarette smoke exposure. Chronic cigarette smoke-exposed cultures were able to (partly) recover from the lack of end-stage differentiation, since all specialised luminal cell markers, except for *SCGB1A1* (club cells), significantly increased in expression (figure 6a). Furthermore, in addition, *SLPI*, *BPIFA1* (sPLUNC), *BPIFB1* (lPLUNC) and *PIGR* showed enhanced expression compared to day 13 (figure 6a). Additionally, *KRT5* (basal cell marker), and *DEFB1* and *LCN2* increased upon cigarette smoke cessation, whereas *TP63* and *KRT8* were unaffected (online supplementary figure S3A). 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 whether the effects observed after chronic cigarette smoke exposure can be obtained when exposing an already partly differentiated epithelium to chronic cigarette smoke, we performed a separate experiment. In this experiment, we first allowed the cultures to differentiate for 1 week, after which we started chronic cigarette smoke exposure for an additional 12 days. Here we found similar effects of cigarette smoke exposure on ALI-PBEC cultures regarding cell-type specific markers and HDP expression compared to cigarette smoke exposure starting from day 0 (figure 6b and online supplementary figure S3B).

Notch signalling inhibition impairs HDP expression during differentiation

Our results so far showed an impaired end-stage differentiation into specialised luminal epithelial cells in cigarette smoke-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 cigarette smoke-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 cigarette smoke exposure did not influence gene expression of Notch ligands, receptors or transcriptional co-activators in these cultures (figure 7a). However, the Notch signalling target genes, *HEY1* and *HEY2*, were significantly reduced by chronic cigarette smoke exposure, while *HES1* was not (figure 7b), indicating that chronic cigarette smoke 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

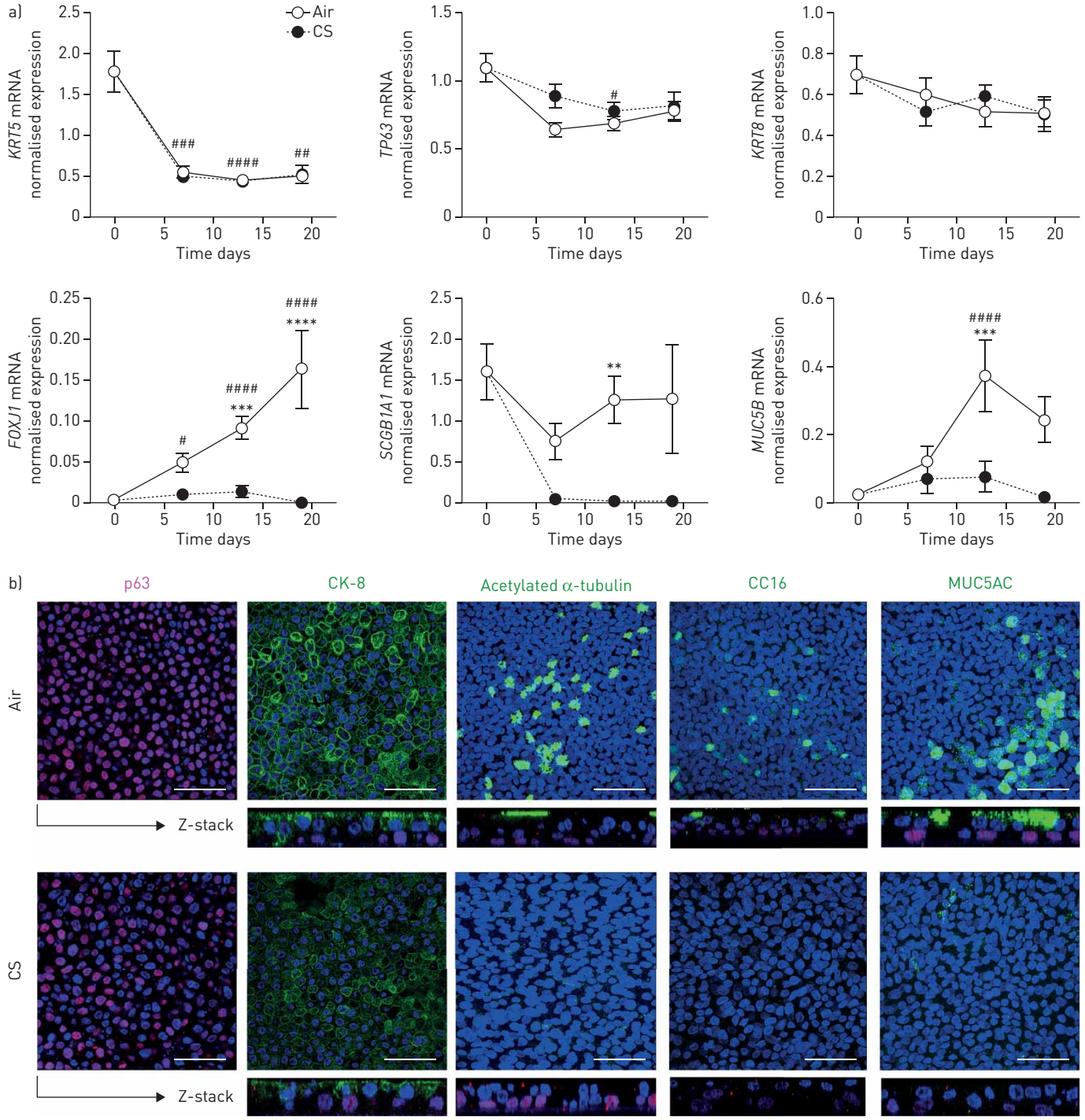

FIGURE 5 Chronic cigarette smoke (CS) exposure of air–liquid interface (ALI) cultures of primary bronchial epithelial cells (PBECs) changes cellular composition. a) ALI-PBECs were exposed during differentiation for 13–19 consecutive days to whole CS. Cells were lysed at several time points and RNA was isolated followed by complementary DNA synthesis, to assess gene expression of basal cell markers cytokeratin-5 (*KRT5*) and *TP63*, of early progenitor cell marker cytokeratin-8 (*KRT8*) and of specialised cell markers *FOXJ1* (ciliated cells), *SCGB1A1* (club cells) and *MUC5B* (goblet cells). Data are shown as target gene expression normalised for the geometric mean expression of the reference genes ATP synthase, hydrogen-transporting, mitochondrial F1 complex, β-polypeptide (*ATP5B*), β2-microglobulin (*B2M*) and ribosomal protein L13a (*RPL13A*). Days 0, 7 and 13 n=8 donors; 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 days 7, 13 and 19 and unexposed cultures at time 0. b) ALI-PBECs were differentiated for 2–3 weeks and exposed daily to CS. Subsequently, the cells were fixed in 1% paraformaldehyde and stained using primary antibodies against basal cells (p63) (red), in combination with primary antibodies against cytokeratin-8 (CK-8), acetylated α-tubulin (ciliated cells), CC16 (club cells) and MUC5AC (goblet cells) (green); 4′,6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei (blue). Z-stacks and images of the apical and basal side of stained cells were obtained using confocal imaging. Images shown are representative for results obtained with cells from four different donors (CK-8, n=3 different donors). Scale bars=50 µm.

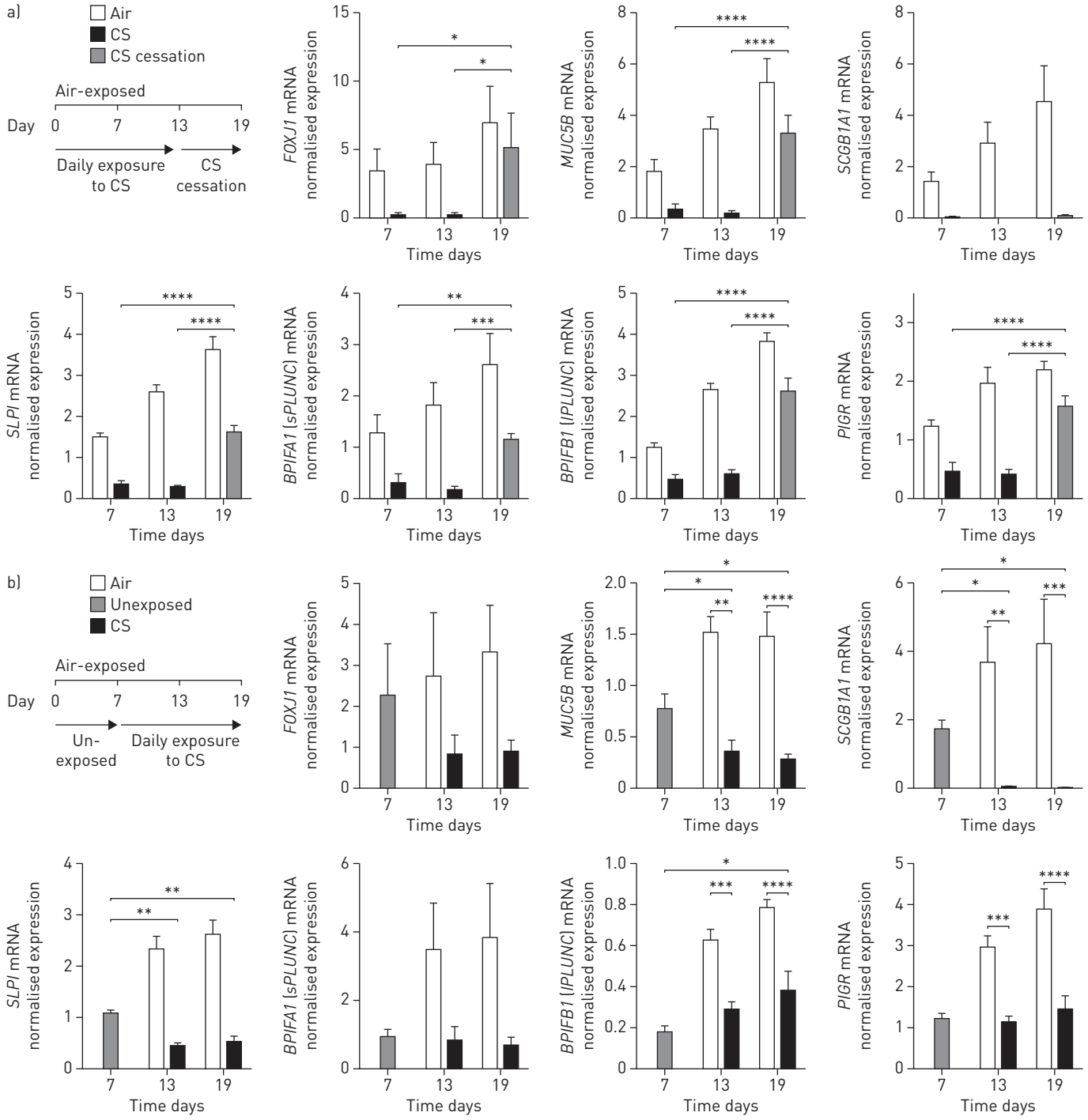

FIGURE 6 Cigarette smoke (CS)-induced impairment of host defence proteins and differentiation markers are partly persistent upon CS cessation. a) Air–liquid interface (ALI) cultures of primary bronchial epithelial cells (PBECs) were exposed during differentiation for 13 consecutive days to whole CS after 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 complementary DNA 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* (secretory leukocyte protease inhibitor), *BPIFA1* (short palate, lung and nasal epithelium clone protein (sPLUNC)), *BPIFB1* (long palate, lung and nasal epithelium clone protein (lPLUNC)) and *PIGR* (polymeric immunoglobulin receptor). Data are presented as target gene expression normalised for the geometric mean expression of the reference genes ATP synthase, hydrogen-transporting, mitochondrial F1 complex, β-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. b) ALI-PBECs 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 analysed similarly to (a). Data are shown as target gene expression normalised 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.

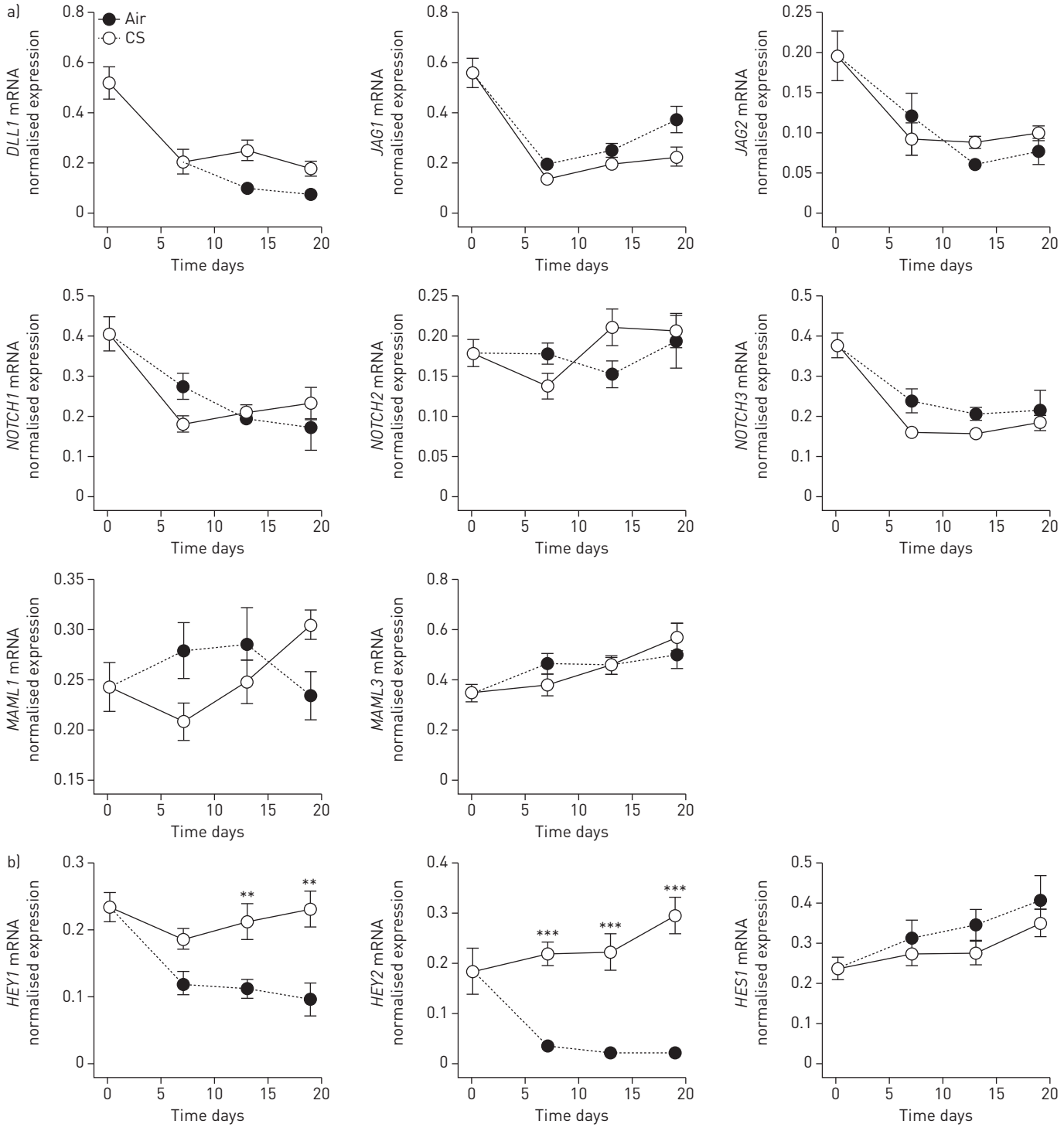

FIGURE 7 Chronic cigarette smoke (CS) exposure of air–liquid interface (ALI) cultures of primary bronchial epithelial cells (PBECs) results in selective impairment of Notch signalling. a) After 2 weeks of differentiation and daily cigarette smoke exposure, ALI-PBECs were lysed, RNA was isolated and complementary DNA synthesised. Subsequent quantitative (q)PCR 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 normalised for the geometric mean expression of ATP synthase, hydrogen-transporting, mitochondrial F1 complex, β-polypeptide (*ATP5B*), β2-microglobulin (*B2M*) and ribosomal protein L13a (*RPL13A*). n=8 different donors. b) Subsequent qPCR analysis was performed on the Notch signalling target genes *HEY1*, *HEY2* and *HES1*. Data are presented as target gene expression normalised 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.

signalling (figure 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 (figure 8b). Furthermore, DAPT-exposed cultures showed a skewing of cell differentiation away from a secretory phenotype towards an increase in ciliated cells (figure 8c) that was also confirmed by confocal imaging (figure 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 exposed daily to cigarette smoke during differentiation as a result of an impaired end-stage differentiation of specialised 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 cigarette smoke-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 recognised as an important contributor to chronic inflammatory lung diseases [15, 16].

The cellular composition of the ALI-PBEC cultures changed drastically upon chronic cigarette smoke-exposure. While presence of intermediate CK-8$^+$ cells (also called early, intermediate or committed progenitor epithelial cells [13, 17]) was not affected by chronic cigarette smoke exposure, the specialised luminal cell markers were reduced in chronic cigarette smoke-exposed cultures. These results indicate that specifically end-stage differentiation seems impaired by cigarette smoke exposure. The effects of chronic cigarette smoke exposure were also observed when the cells were first allowed to differentiate for 1 week in absence of cigarette smoke. Furthermore, upon cessation of cigarette smoke 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 cigarette smoke on club cell differentiation or the regulation of *SCGB1A1* gene expression. This is underscored by a study showing that expression of the club cell-protein CC16 (*SCGB1A1*) is reduced in COPD patients and in cigarette smoke-exposed mice. Loss of CC16 was correlated with increased severity of the disease and cigarette smoke-induced pulmonary inflammation was lower in mice overexpressing CC16 [18].

Cytotoxicity is unlikely to have a major contribution to the cigarette smoke-induced effects on the ALI-PBEC cultures since we detected no difference in transepithelial electrical resistance (TEER) as a measure of barrier function during the course of differentiation between cigarette smoke- and air-exposed controls. We previously observed transient decreases in TEER after acute single cigarette smoke exposures, normalising after 24 h [11]. In our chronic cigarette smoke set-up we measured TEER 18–20 h after the previous cigarette smoke exposure, which may explain why we did not observe significant differences in TEER between air- and cigarette smoke-exposed cultures. Previous studies have shown decreases in TEER by cigarette smoke, but often use cigarette smoke extract (CSE) and not whole cigarette smoke [19, 20]. CSE has a different composition and concentration to the whole cigarette smoke 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. Lactate dehydrogenase release was not increased in our cigarette smoke-exposed cultures, while cellular size appeared larger in the cigarette smoke-exposed cultures for some donors. Finally, the cigarette smoke-exposed cultures produced higher amounts of interleukin-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 supplementary material and in online supplementary figure S4.

Unexpectedly, we did not observe goblet cell hyperplasia in cultures that were exposed to cigarette smoke, shown previously in smokers [21], guinea pigs [22], rats [23] and in cell lines [23, 24] or PBECs exposed to CSE [19]. However, Brekman *et al.* [25] observed a decline in goblet cells markers in PBECs continuously exposed to CSE. Data are therefore conflicting and dependent on the type of cell culture used. Obviously, in our primary differentiated cultures, whole cigarette smoke exposure alone is insufficient to induce goblet cell hyperplasia within 19 days. We strongly consider that the findings in patients are probably explained by a secondary effect of the cigarette smoke-induced inflammation (which is obviously incompletely represented in our *in vitro* model). For example, neutrophil recruitment as a consequence of the cigarette smoke-induced inflammation and subsequent release of proteases and other molecules may help to explain goblet cell formation in patients. In addition, the presence of other cell types besides neutrophils, such as macrophages, seem important for goblet cell hyperplasia. This has been suggested in the literature [26–29]. Furthermore, several studies suggest that various other factors that are

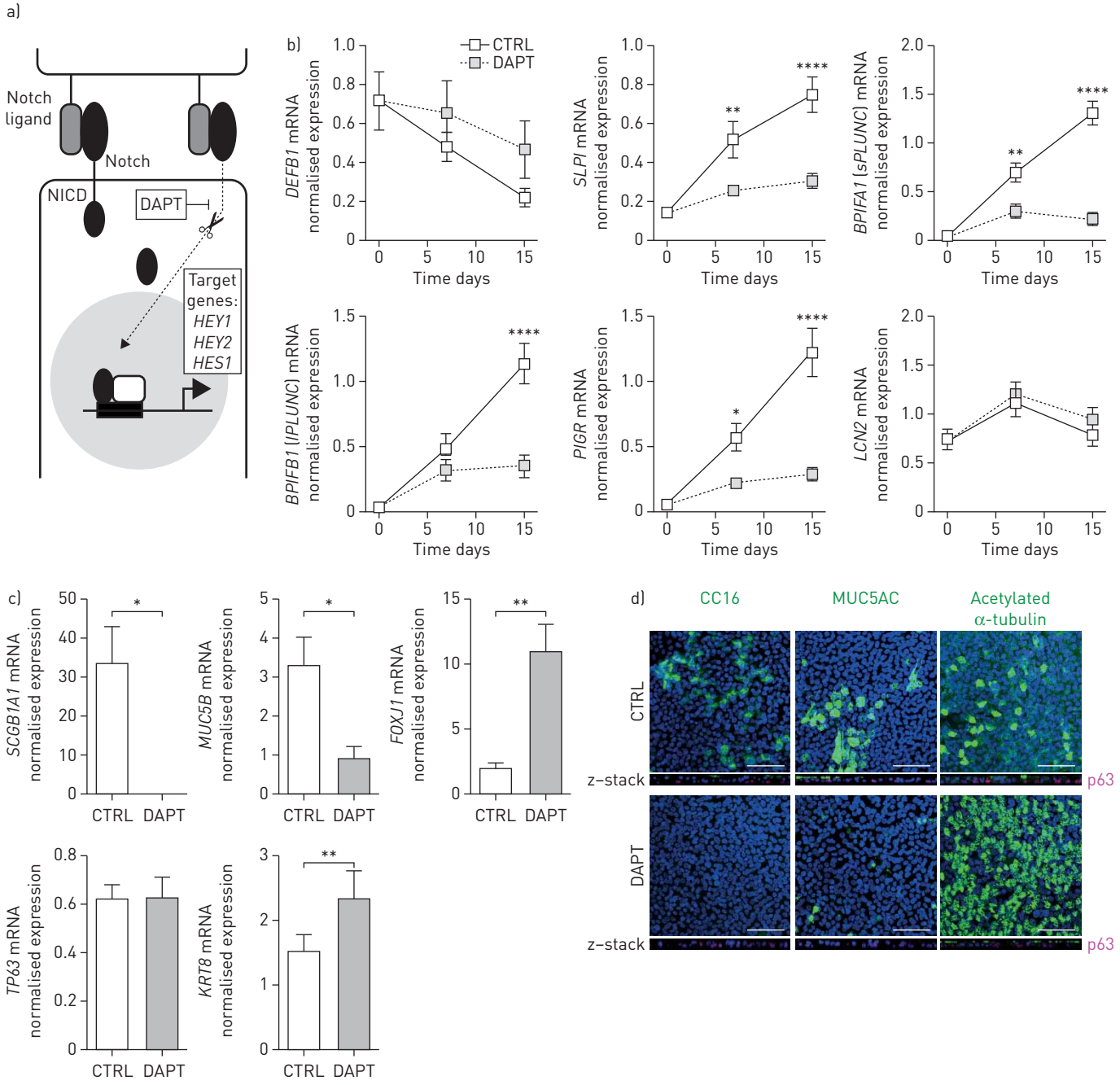

FIGURE 8 DAPT inhibits host defence protein expression in air–liquid interface (ALI) cultures of primary bronchial epithelial cells (PBECs). a) Mechanism of action of the Notch inhibitor DAPT, a γ-secretase inhibitor that prevents proteolytic cleavage of the Notch intracellular domain (NCID). b) PBECs 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 days 0, 7, and 15 cells were lysed, RNA was isolated and complementary DNA synthesised. Subsequent quantitative (q)PCR analysis was performed to assess expression of respiratory defence proteins and epithelial cell-specific genes such as *DEFB1* (human β 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 normalised for the geometric mean expression of the reference genes ATP synthase, hydrogen-transporting, mitochondrial F1 complex, β-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<0.05, **: p<0.01, ****: p<0.0001 between control (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-PBECs 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) (green); 4′,6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei (blue). Images shown are representative for results obtained with cells from three different donors. Scale bars=50 µm.

involved in COPD pathogenesis, including bacterial and viral infections, might have an important role in promoting goblet cell hyperplasia [30, 31].

Whereas previous studies have shown that cigarette smoke reduces the presence of ciliated cells [19, 25, 32], so far cigarette smoke-induced airway epithelial remodelling has not yet been 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.* [33] showed that expression of SLPI was significantly reduced in damaged bronchial epithelium of COPD patients compared to non-COPD individuals, and Gohy *et al.* [10] showed reduced levels of pIgR in the lungs of patients with COPD, compared to healthy controls; however, this was not seen in smokers with normal lung function. Finally, reduced levels of PLUNC were detected in bronchial brushes performed in current smokers compared to never-smokers [34].

We observed impaired antibacterial activity of the cigarette smoke-exposed airway epithelial cultures against *M. catarrhalis* and *K. pneumoniae*. In addition, we evaluated direct antibacterial activity of the chronic cigarette smoke-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 cigarette smoke-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 nuclear factor-κB activation and modulates macrophage functions [37–39]. sPLUNC is involved in the regulation of the epithelial sodium channel, 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 cigarette smoke exposure on components of the Notch signalling pathway and Notch target genes, and found that cigarette smoke 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 cigarette smoke 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 HDPs that are increased upon differentiation. Whereas chronic cigarette smoke 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 than 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 cigarette smoke-exposed cultures suggests involvement of altered Notch signalling in the cigarette smoke-induced effects. However, alterations in other signalling pathways might also be involved in the observed effects of cigarette smoke, such as those involving epithelial growth factor receptor [32], transforming growth factor-β [25], Wnt [43] and bone morphogenetic protein [44]. It is likely that a more systems/-omics approach has the potential to elucidate in detail all effects of chronic cigarette smoke 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 colonisation 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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