

## LETTER

# Differentiation of bronchial epithelial spheroids in the presence of IL-13 recapitulates characteristic features of asthmatic airway epithelia

To the Editor,

There is a substantial need to better understand the pathophysiology of asthma to develop preventive approaches and better treatments. Preclinical models as close as possible to human *in vivo* situations are essential to fulfil these aims. 3D airway culture models, particularly airway organoids, have several advantages over traditional 2D cultures, such as mimicking organ structure, ability to generate *in vivo* relevant cell–cell interaction models, suitability for high-throughput experiments, and applications in epithelial self-assembly, morphogenesis, differentiation and repair studies.<sup>1,2</sup> A dominant endotype of asthma is characterized with an impaired airway epithelial barrier, remodelling and the involvement of type 2 inflammation with cytokines such as interleukin (IL)-4, IL-5 and IL-13.<sup>3,4</sup> In the present study, we aimed at establishing a 3D airway organoid model from primary bronchial epithelial basal cells to investigate the characteristic features of asthma, such as epithelial cell differentiation, epithelial remodelling and mucosal tight junction barrier impairment in the presence of a main pathogenetic type 2 cytokine, IL-13.

A 3D airway organoid model was developed as previously described<sup>2</sup> from commercially obtained healthy primary bronchial epithelial basal cells (Figure S1). Since this organoid model does not have self-renewal capacity and consists solely of epithelial cells, it is hereinafter referred to as the 'bronchial epithelial spheroid model'. In order to study the main features of asthma, cells were continuously stimulated with 2 ng/ml IL-13 every second day starting from Day 2 until the end of the cultures. For the control group, only fresh medium was added to the wells. Cultures were terminated on Days 8 and 16, then, light microscopy examination, mRNA isolation, paracellular flux (Day 16) and immunofluorescent staining were performed (Figure S2). Detailed methods are provided in online supplementary (Table S1–S3).

Treatment of developing spheroids with IL-13 did not affect the spheroid count per well and spheroid size (Figure 1A). However, it caused the development of thick-walled spheroids with small or no lumen resulting in a decreased ratio of 'lumen-to-total spheroid area' (Figure 1B). The differentiation process was greatly affected by the presence of IL-13, resulting in increased differentiation towards mucus-producing goblet cells with a dramatic decrease in

mRNA levels of both ciliated cell markers, dynein axonemal intermediate chain 2 (*DNAI2*) and forkhead box protein J1 (*FOXJ1*). *FOXJ1*, a master regulator of ciliogenesis, has crucial physiological roles in ciliated cell development.<sup>2,4</sup> Therefore, decreased mRNA levels of these marker genes indicate the suppression of ciliated cell differentiation. In addition, it is noteworthy that the mRNA levels of the club cell marker, secretoglobin family 1A member 1 (*SCGB1A1*) decreased in fully mature spheroids (Figure 1C) under IL-13 treatment. *SCGB1A1* has been proposed as a predictive marker for the risk of impaired lung function, such as lung epithelial damage in asthma. It is involved in tissue repair processes and has anti-inflammatory effects.<sup>5</sup> These findings are consistent with the low protein levels in bronchoalveolar lavage fluids of asthmatic individuals.<sup>5</sup> Furthermore, the decrease in the number of the club and ciliated cells by IL-13 suggests a differentiation or metaplasia towards goblet cells in bronchial epithelial spheroids. Moreover, IL-13 decreased the mRNA levels of bactericidal/permeability-increasing protein fold-containing family member A1 (*BPIFA1*). *BPIFA1* has crucial roles in reducing eosinophilic airway inflammation, relaxing airway smooth muscle and antibacterial activity against Gram-negative bacteria.<sup>6</sup> Meta-analysis of *BPIFA1* expression in epithelia obtained by bronchial brushings from asthmatic individuals shows decreased mRNA levels similar to our findings.<sup>6</sup> Confocal microscopy imaging showed a significant increase in MUC5AC intensity without detectable ciliated cells, which were identified by acetylated  $\alpha$ -tubulin staining in organoids treated with IL-13 (Figure 1D). These findings are parallel to the epithelial differentiation features of type 2 asthmatic individuals, findings in mouse models and airway epithelial cell cultures.<sup>2,4</sup>

Given the fact that the impaired epithelial barrier is one of the hallmarks of asthma,<sup>3</sup> we assessed the epithelial barrier development in spheroids. On Day 16 of the culture, fluorescein isothiocyanate (FITC)-labelled 4-kDa dextran was added to the cultures and the paracellular flux of it into the lumen of the spheroids was assessed with confocal microscopy at the end of the 24-hour incubation. EDTA was used as a positive control for epithelial barrier impairment (Figure S3). Only luminated spheroids were included in the measurements. IL-13 treatment resulted in an impaired epithelial barrier development evidenced with a high 'lumen-to-background intensity ratio' (Figure 2A)

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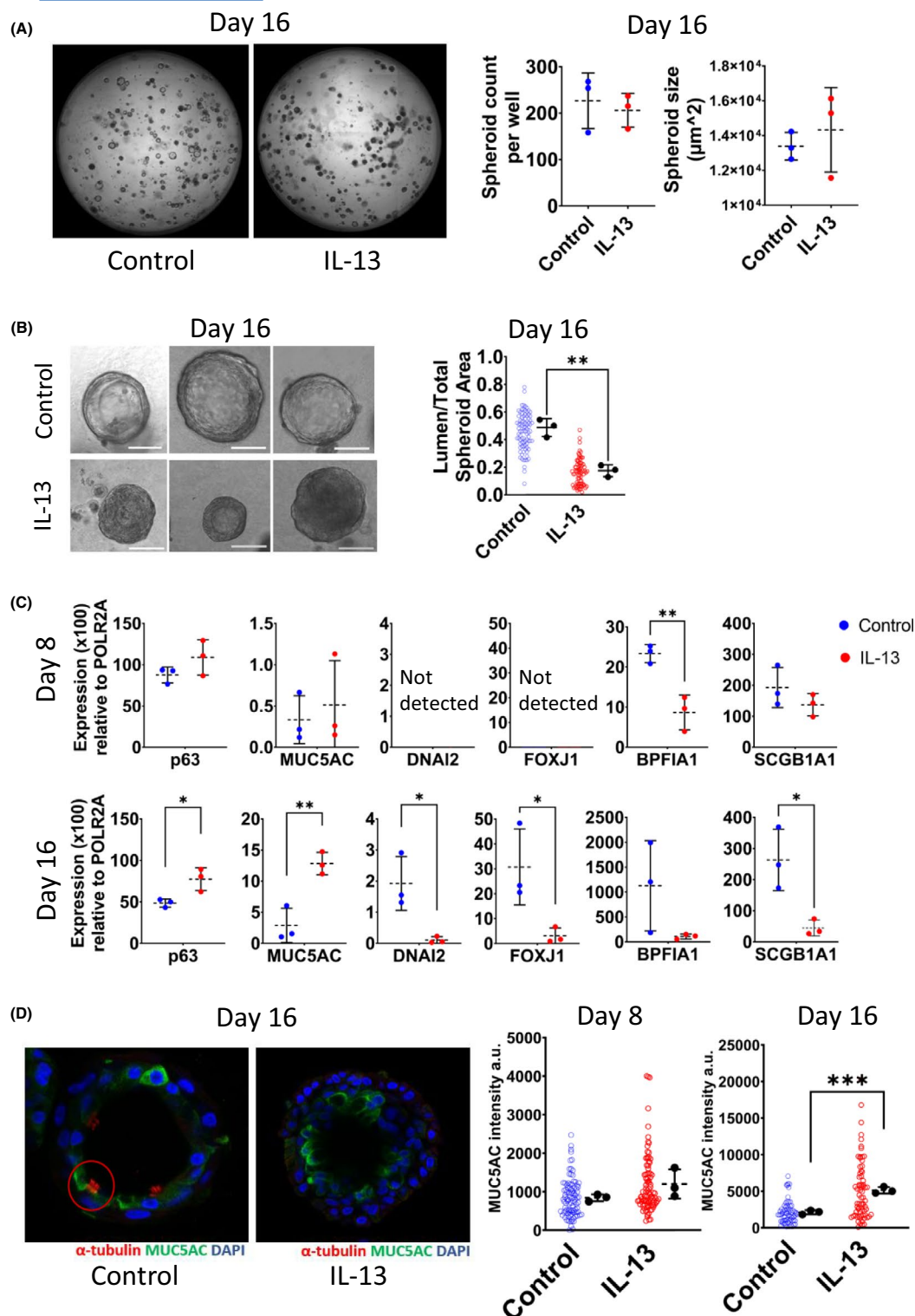


**FIGURE 1** Treatment of bronchial epithelial spheroids with IL-13 recapitulates characteristic asthmatic epithelial cell differentiation phenotype. A, Representative light microscopy images from control and IL-13-treated bronchial epithelial spheroids on Day 16. The average number of spheroids and average spheroid size per well ( $\mu\text{m}^2$ ) (right panel). B, Representative light microscopy images on Day 16. Scale bar 50  $\mu\text{m}$ . 'Lumen-to-total spheroid area' is shown on the right side. C, Quantitative RT-PCR analysis of goblet cell (MUC5AC), ciliated cell (FOXJ1 and DNAI2), basal cell (p63) club cell (SCGB1A1) markers and antimicrobial protein BPIFA1. Expression values calculated with  $2^{-(-\Delta\text{Ct})}$  formula and represented as expression (x100) relative to housekeeping gene, POLR2A. D, Immunostaining of spheroids for DNA (blue), and markers of ciliated cells (acetylated  $\alpha$ -tubulin, red) and goblet cells (MUC5AC, green). Graphical representations of MUC5AC intensity at Days 8 and 16 of treatment are shown at right. Individual spheroids were represented as hollow dots and biological replicates (n:3) as black dots. Data are presented as means  $\pm$  SDs. For statistical analysis, two-tailed unpaired Student's t-test was used. \* $p < .05$ . \*\* $p < .01$ . \*\*\* $p < .001$

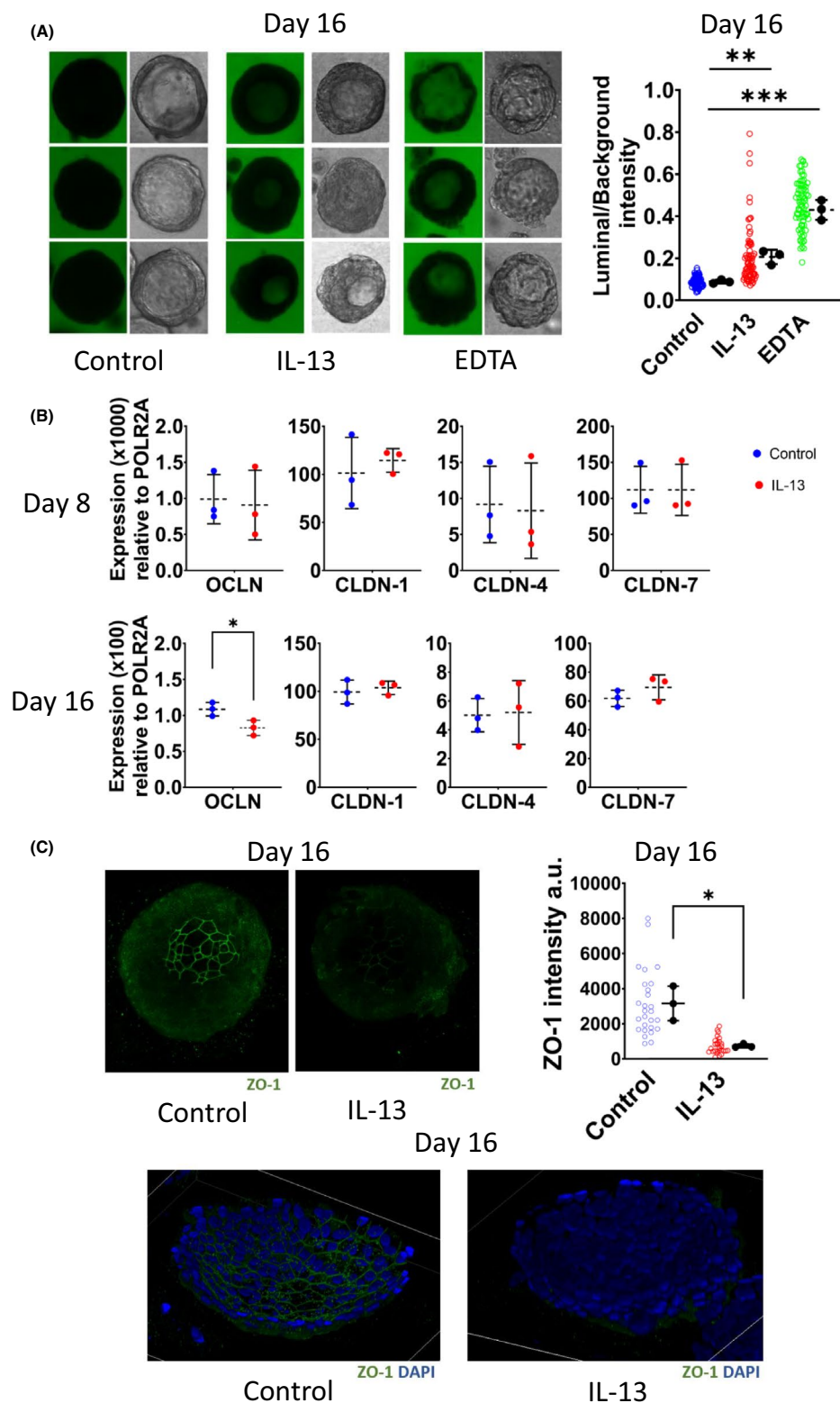


**FIGURE 2** Treatment of bronchial epithelial spheroids with IL-13 impaired epithelial barrier development. A, Representative light and fluorescence microscopy images of the paracellular flux assay performed on Day 16 of culture. Each row of the spheroid pictures represents a biological replicate. EDTA used as a positive control for epithelial barrier impairment. Individual spheroids are represented as hollow dots and biological replicates (n:3) presented as black dots (right panel). B, Quantitative RT-PCR analysis of tight junction proteins, occludin (OCLN), claudin-1 (CLDN-1), claudin-4 (CLDN-4) and claudin-7 (CLDN-7). Expression values calculated with  $2^{-\Delta\Delta Ct}$  formula and represented as expression (x100) relative to POLR2A. C, Immunostaining of spheroids for DNA (blue) and ZO-1 (green). Graphical representations of ZO-1 intensity on Days 8 and 16 of culture are shown at right. Individual spheroids were represented as hollow dots and biological replicates (n:3) as black dots. Data are presented as means  $\pm$  SDs. For statistical analysis, two-tailed unpaired Student's t-test was used. \* $p < .05$ . \*\* $p < .01$ . \*\*\* $p < .001$

and decreased occludin mRNA and ZO-1 staining intensity on Day 16 (Figures 2B,C). These results demonstrated that bronchial epithelial spheroid development under the influence of a type 2 cytokine, IL-13, is leading to an epithelial barrier disorder similar to asthma.<sup>3</sup>

In conclusion, we successfully developed an in vitro asthma model with a 3D airway organoid model termed here bronchial epithelial spheroids that recapitulates characteristic features of asthmatic airway epithelia. It demonstrates decreased ciliated and club cells and skew to a high mucus-producing and goblet cell-dominant phenotype with disrupted epithelial barrier by IL-13. These results demonstrate that IL-13 impairs the epithelial barrier by affecting both epithelial differentiation and tight junction protein expression. To the best of our knowledge, this study is the first in vitro asthma model that investigates epithelial barrier development and differentiation process in a 3D culture of primary bronchial epithelial cells with a detailed analysis including functional permeability, gene expression and immunofluorescence studies. The model is adaptable for high-throughput experiments, airway epithelial morphogenesis and development studies.

## KEYWORDS

asthma, barrier, epithelium, interleukins

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## CONFLICT OF INTEREST

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

## **Online Supplementary**

Letter to the Editor: Differentiation of bronchial epithelial spheroids in the presence of IL-13 recapitulates characteristic features of asthmatic airway epithelia

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### **1. Introduction:**

Asthma has become a crucial medical and socio-economic problem with an increasing frequency worldwide in recent decades.<sup>1</sup> There is a substantial need to better understand the pathophysiology of asthma to develop preventive approaches and better treatments. Preclinical models as close as possible to human *in vivo* situation are essential to fulfil these aims. 3D airway culture models, such as airway organoids represent a novel and promising technique. Organoids are self-organized cell populations that reflect the characteristic properties of the parent cell formed by *in vitro* proliferation and differentiation of a parent cell group in a culture microenvironment that mimics the extracellular matrix.<sup>2</sup> Airway organoids can be derived from local bronchial basal cells, adult stem cells and embryonic stem cells.<sup>2</sup> The 3D organoid culture has several advantages over traditional 2D culture, such as mimicking organ structure, ability to generate *in vivo* relevant cell-cell interaction models, suitability for high-throughput experiments, and use in epithelial self-assembly, morphogenesis, differentiation and repair studies.<sup>3</sup> Moreover, human 3D cultures are well suited to exploit CRISPR/Cas9 gene editing technology<sup>4</sup>, and are useful for investigation of cytokine/drug effects.<sup>5</sup> A model that uses bronchial basal cells to establish an airway organoid model which is termed "bronchosphere" was performed by Danahey et al.<sup>5</sup> There are also several similar nomenclatures for other types of organoids derived from tracheal airway basal cells, such as the tracheospheres.<sup>4,6</sup> In this study, we used a 3D airway organoid model termed here as bronchial epithelial spheroids, which is suitable for high-throughput experiments, and to study epithelial self-assembly, morphogenesis, differentiation, barrier and repair studies.

A dominant endotype of asthma is characterized with an impaired airway epithelial barrier, remodelling and the involvement of type 2 inflammation with cytokines such as interleukin (IL)-4, IL-5 and IL-13.<sup>1,7</sup> Both *in vivo* mouse models of asthma and *in vitro* asthma models of human airway epithelial cell culture can be generated by IL-13 stimulation alone. IL-13 stimulation of *in vitro* human airway epithelial cells results in an increased goblet cell differentiation and high MUC5AC production, inhibited ciliated cell differentiation, and impaired epithelial barrier integrity.<sup>7-10</sup> In line with these findings, targeting IL-4 and IL-13 through the IL-4 receptor-alpha with mAb is an effective treatment for asthma.<sup>11</sup> In the present study, we aimed to establish a 3D airway organoid model from primary bronchial epithelial basal cells to investigate the characteristic features of asthma, such as epithelial cell differentiation, epithelial remodelling and mucosal tight junction barrier impairment in the presence of a main pathogenetic type 2 cytokine, IL-13.

## **2. Materials and methods:**

### **2.1. Bronchial epithelial spheroid culture**

Primary human bronchial epithelial cells from healthy control subjects were purchased from Epithelix (Geneva, Switzerland) and ATCC/LGC (Promochem, Wesel, Germany). Cells in passage two were used to develop the bronchial epithelial spheroids. Before seeding the cells, 96-well plates were coated with 25% growth factor reduced extracellular matrix gel (GFR-ECM) (Sigma-Aldrich, Saint Louis, Mo) in bronchial epithelial basal medium (BEGM)-DMEM (Lonza, Basel, Switzerland) at a 1:1 ratio supplemented with 15 ng/mL all-trans retinoic acid (Sigma-Aldrich, Saint Louis, Mo) and SingleQuot Kit (Lonza) except for retinoic acid and triiodothyronine. Cells were seeded at a concentration of 2000 cells/well in 5% GFR-ECM in the same media composition described above. All bronchial epithelial spheroid cultures were maintained at 37°C, under 5% CO<sub>2</sub> and humidified conditions.

### **2.2. IL-13 treatment of developing bronchial epithelial spheroids**

Cells were continuously stimulated with 2 ng/ml recombinant human IL-13 (Peprotech, London, UK) every second day starting from day two until the end of the culture. For the control group, only medium was added to the wells (supplementary Figure 2). All bronchial epithelial spheroid cultures were maintained at 37°C, under 5% CO<sub>2</sub> and humidified

conditions. Cultures were terminated on days 8 and 16, then light microscopy examination, mRNA isolation, paracellular flux (day 16) and immunofluorescent staining were performed (supplementary Figure 2).

### **2.3. Immunofluorescence staining of primary bronchial epithelial cells**

The commercially obtained primary bronchial epithelial cells were fixed with 4% paraformaldehyde (Fluka/Sigma Aldrich, Buchs, Switzerland) for 7 min. Fixed cells were washed with ice-cold PBS then perm-blocked with 10% normal goat serum, 1% BSA, 0.1% Triton X100 in PBS pH 7.4 for 20 min. After perm-block, cells were washed with PBS and incubated with primary antibodies (Table S1) overnight at 4°C. Then, cells were washed with 0.05% Tween-PBS three times, and then incubated with the secondary antibodies (goat anti-rabbit IgG-AF488, Life technologies and goat anti-mouse IgG-AF546, Life technologies) 45 min at room temperature. As the final step, cells were washed with 0.05% Tween-PBS three times and stored until confocal microscopy assessment in PBS. Samples were analyzed with confocal microscopy (Zeiss LSM780, Oberkochen, Germany).

### **2.4. Immunofluorescence staining of bronchial epithelial spheroids**

Bronchial epithelial spheroids were harvested with ice-cold PBS and fixed with 4% paraformaldehyde (Fluka/Sigma Aldrich, Buchs, Switzerland) for 45 min. Fixed spheroids were washed with ice-cold PBS then perm-blocked with 1% DMSO, 1% goat serum, 1% BSA, 0.5% tritonX100 for 60 min. After perm-block, spheroids were washed with 0.1% BSA/PBS and incubated with primary antibodies (Table S2) overnight at 4 °C. After incubation, spheroids were washed with wash buffer (0.1% BSA and 0.1% tritonX100 in PBS), and then incubated with the secondary antibodies (goat anti-rabbit IgG-AF488, Life technologies and goat anti-mouse IgG-AF546, Life technologies) overnight at 4°C. As final step the spheroids washed with wash buffer and stored until confocal microscopy assessment in 0.1% BSA/PBS. Samples were analysed with confocal microscopy (Zeiss LSM780, Oberkochen, Germany). For differentiation analysis (staining of MUC5AC and acetylated  $\alpha$ -tubulin) minimum of 20 organoids were analysed per condition from each subject. From each spheroid three images were taken at 10  $\mu$ M intervals and processed in ImageJ/Fiji software separately. For zonula occludens-1 (ZO-1) staining minimum of 10 spheroids were analysed per condition from

each subject. Twelve images were taken from each spheroid at 0.9  $\mu$ M intervals then images were combined with maximum intensity projection and processed in ImageJ/Fiji software.

## **2.5. Paracellular flux assay**

For paracellular flux assay glass-bottomed 96-well plates were used. On day 16 fluorescein isothiocyanate (FITC)-labelled 4-kDa dextran (0.5 mg/mL, Sigma-Aldrich) was added to the culture mediums and incubated for 24 hours. 10 mM EDTA added to the culture medium was used as a positive control for epithelial barrier disruption. At the end of the incubation images were taken from 25-30 spheroids with confocal microscopy (Zeiss LSM780, Oberkochen, Germany) and processed with ImageJ/Fiji software. Fluorescence within a spheroid was measured by focusing on the entire luminal area inside the spheroid. The representative background fluorescence was estimated from four different areas surrounding the organoid for each of the images. The luminal intensity of each spheroid was divided by the background intensity to calculate the "lumen-to-background intensity ratio".

## **2.6. Image processing with ImageJ/Fiji**

ImageJ/Fiji software was used for image processing. The average spheroid count per well and average spheroid sizes were calculated with analyse particles tool after subtracting background and setting the threshold. The lower limit for particle diameter was set to 50  $\mu$ M. For calculating "lumen-to-total spheroid area ratio" the spheroid lumen and total spheroid area were calculated separately. Intensity analysis for MUC5AC was done by averaging the raw intensity density of three image planes taken from each spheroid. Intensity analysis for ZO-1 the maximum intensity projection images were used of each individual spheroid for measuring raw intensity density.

## 2.7. mRNA isolation and quantitative RT-PCR

Bronchial epithelial spheroids were harvested with ice-cold PBS and total mRNA was isolated with RNeasy Micro Kit (Qiagen, Hilden, Germany). RNA was reverse transcribed into cDNA by using a RevertAid RT kit containing random hexamers (Thermo Fisher Scientific). Quantitative RT-PCR was performed on a QuantStudio 7 Flex (Applied Biosystems, Life Technologies) by using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). Sequences of primers are listed in Table S3. *POLR2A* was used as the housekeeping gene. We interpret the quantitative RT-PCR data with the  $2^{(-\Delta Ct)}$  ( $\Delta Ct = Ct$  of gene of interest -  $Ct$  of *POLR2A*) method and represented here as expressions (x100) relative to housekeeping gene, *POLR2A*.

## 2.8. Data analysis

Data were analysed with GraphPad Prism software (Version 9; GraphPad Software, La Jolla, Calif). Differences between independent variables were evaluated by two-tailed unpaired Student's t-test. Differences between the different time points of the same biological replicate were evaluated by two-tailed paired Student's t-test. The results were considered as significant at P values of  $< 0.05$ ,  $< 0.01$ , and  $< 0.001$ .

## 3. Establishment of bronchial epithelial spheroid model

Before starting the bronchial epithelial spheroid cultures, we investigated the basal cell characteristic of the cells by immunofluorescence staining with basal cell markers, p63 and ITGA6 (supplementary Figure 1A). The immunostaining revealed that a high proportion of these cells are showing basal cell features. This data shows that 3D airway cell culture can be performed with these cells. With these primary bronchial epithelial basal cells, a human bronchial epithelial spheroid model was established. Upon culturing cells according to the previously described methods for 16 days, basal cells self-assemble, and form fully differentiated bronchial epithelial spheroids with a mean diameter of 130  $\mu m$  (supplementary Figure 1B). Lumen formation was initiated between days 10 and 12 and functional beating cilia became visible after day 12 (supplementary Movie 1-2). The resulting micro-scale spheroid structures, composed of functional

secretory cells like mucin-producing goblet cells (MUC5AC) and club cells (SCGB1A1), ciliated cells (DNAI2, FOXJ1), and basal cells (p63). The detection of cell differentiation markers at day 8 of cell culture and their increase between days 8 and 16 demonstrates the differentiation of basal cells into ciliary cells, goblet cells, and club cells within the bronchial epithelial spheroids. (supplementary Figure 1C). It should be noted that goblet cell differentiation begins in the early stages of spheroid development, before luminal formation, as evidenced by immunostaining and detectable MUC5AC mRNA values at day 8 of culture. Therefore, there was no significant increase in MUC5AC mRNA expression between days 8 and 16 of culture. Similarly, at day 8 of culture, club cell differentiation was demonstrated by detectable SCGB1A1 mRNA expression. After 16 days bronchial epithelial spheroids were also proved to be fully differentiated by assessing the markers for ciliated cells (acetylated  $\alpha$  tubulin) and goblet cells (MUC5AC) via immunohistochemistry (supplementary Figure 1D-E). Although 3D airway cultures established from basal cells can be termed as organoids in the literature, some authors suggest that such organoids that do not have self-replicating feature should be called spheroids. Since this organoid model does not have self-renewal capacity and consists solely of epithelial cells, it is hereinafter referred to as “bronchial epithelial spheroid model”.

#### **4. Additional discussion**

At the end of two weeks of IL-13 treatment, we found increased expression of the goblet cell marker, MUC5AC, mRNA and confocal staining. At the same time, there was no ciliated cells detected by both light microscopy examination and immunofluorescent staining of alpha-tubulin in IL-13 treatment condition. In addition, ciliated cell marker mRNA (FOXJ1 and DNAI2) values were low when compared with control. Although, the precise mechanism of goblet cell increase was not examined in our study, these results suggest that either IL-13 stimulation reprograms basal cell differentiation towards goblet cell but or induce metaplasia of other cell types such as ciliated and club cells to goblet cells. IL-13 treatment also dampened expression of the two important antimicrobial proteins, SCGB1A1 and BPIFA1.<sup>7</sup> These findings are parallel with decreased SCGB1A1 levels in bronchoalveolar lavage

fluids of children with asthma.<sup>12</sup> Moreover, recent findings emphasized the importance of BPIFA1 in the development of asthma.<sup>13</sup>

In this study, the outcome of epithelial barrier development was evaluated with a functional test, the paracellular flux assay.<sup>14-16</sup> The working principle of the test is based on the passage of FITC-labelled 4-kDa dextran into the spheroid lumen. If the barrier is intact the flow of the newly added FITC-labelled 4-kDa dextran into the spheroid lumen will be no or low. Conversely, a disrupted barrier will not prevent FITC-labelled 4-kDa dextran from entering the lumen and this will be resulting in a high “lumen-to-background intensity ratio” compared to a spheroid with a healthy barrier. Due to healthy epithelial barrier development in the control bronchial epithelial spheroid group, FITC-labelled 4-kDa dextran influx into the spheroid lumen was low. Therefore, it resulted in a low FITC-labelled 4-kDa dextran fluorescence intensity in the lumen. IL-13 treatment resulted in a high “lumen-to-background intensity ratio” compared to control due to impaired epithelial barrier development.

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236 **TABLE S1.** Primary antibodies for immunostaining of primary bronchial epithelial cells

| Antigen | Clone    | Supplier  | Dilution |
|---------|----------|-----------|----------|
| ITGA-6  | MP4F10   | Abcam     | 1:250    |
| p63     | Poly6190 | Biolegend | 1:33     |

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239 **TABLE S2.** Primary antibodies for immunostaining of bronchial epithelial spheroids

| Antigen                      | Clone      | Supplier     | Dilution |
|------------------------------|------------|--------------|----------|
| MUC5AC                       | EPR16904   | Abcam        | 1:200    |
| Acetylated $\alpha$ -tubulin | 6-11B-1    | Abcam        | 1:500    |
| ZO-1                         | Polyclonal | ThermoFisher | 1:250    |

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242 **TABLE S3.** Primer sequences for quantitative PCR (Indicated as 5'-3')

| Gene    | Forward primer                    | Reverse primer                    |
|---------|-----------------------------------|-----------------------------------|
| MUC5AC  | GGA ACT GTG GGG ACA GCT CTT       | GTC ACA TTC CTC AGC GAG GTC       |
| SCGB1A1 | CGA GCT TTC AGC GTG TCA TC        | GCC TCA TAA CTG GAG GGT GT        |
| BPIFA1  | TGG AAA ACC TTC CGC TCC TG        | ACT TTT CCA AGC AGT CCC CC        |
| p63     | CAG GAA GAC AGA GTG TGC TGG T     | AAT TGG ACG GCG GTT CAT CCC T     |
| DNAI2   | CCG CAT TTG GTC TGA AGA CAG C     | GTT CCG TCC ATC CTG GTG GTA A     |
| FOXJ1   | TTC AAT CCG CCA CAA CCT GT        | CTC CCG AGG CAC TTT GAT GA        |
| OCLN    | GAC CTG AAT GGG TAC ATG TGT GTA A | GGA ATT CTC ACA ACA CCA ACT GAA G |
| CLDN-1  | TCT GGC TAT TTT AGT TGC CAC AG    | AGA GAG CCT GAC CAA ATT CGT       |
| CLDN-4  | TGG GGC TAC AGG TAA TGG G         | GGT CTG CGA GGT GAC AAT GTT       |
| CLDN-7  | GGG CAT GAA GTG CAC GCG CT        | CGG CAA GAC CTG CCA CGA TG        |
| POLR2A  | TTG TAT CCG TAC CCA CAG CA        | CAT GAT CAG CTC CCC ATT CT        |

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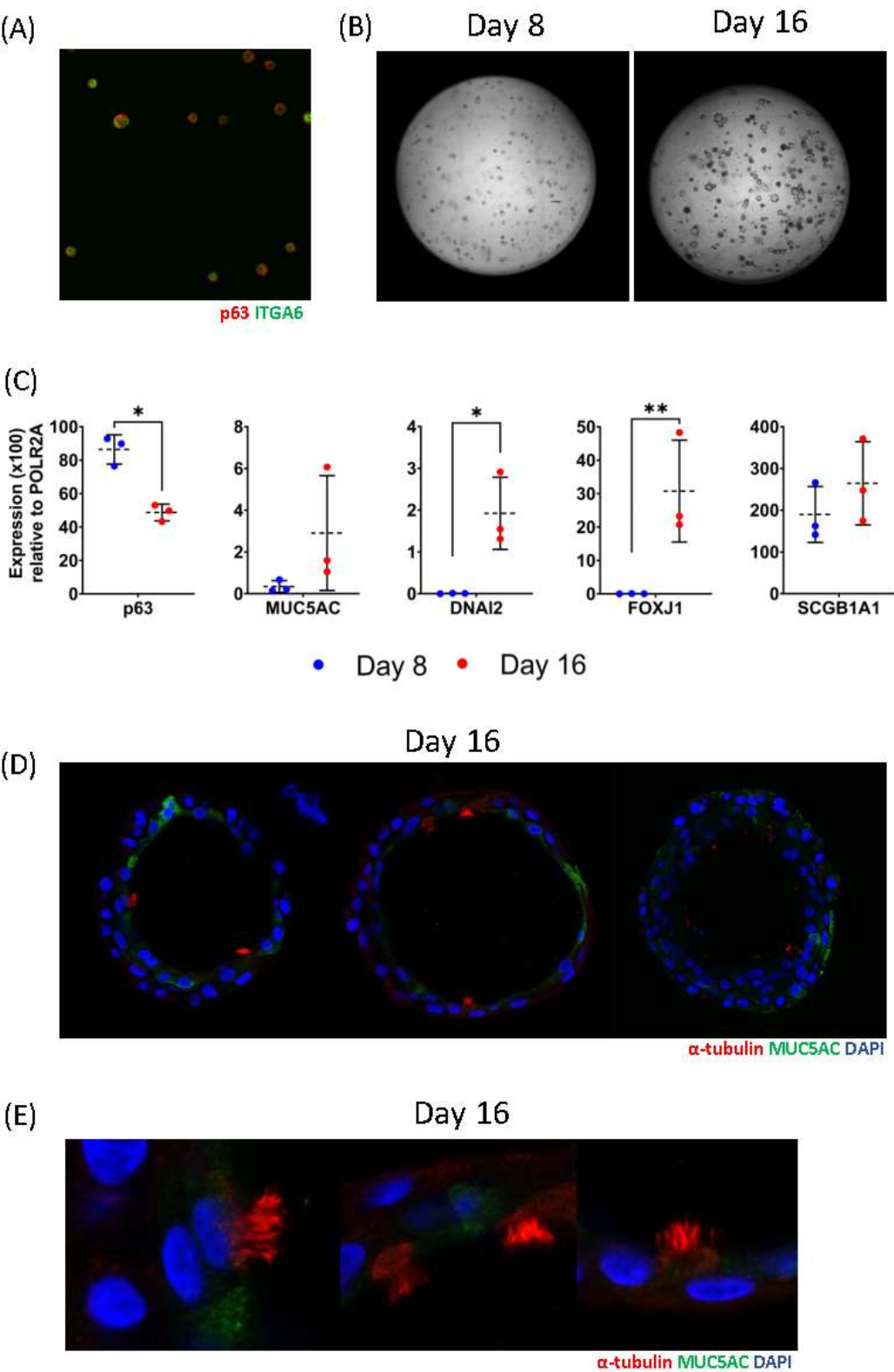


**Figure S1.** Establishment of 3D airway organoid model from primary bronchial epithelial basal cells. **A**, Immunofluorescence staining of primary bronchial epithelial cells that were used for the bronchial epithelial spheroid culture with basal cell markers, p63 (red) and ITGA6 (green). **B**, Representative light microscopy images from days 8 and 16 of bronchial epithelial spheroid cultures. **C**, Quantitative RT-PCR analysis of differentiation markers on days 8 and 16 of culture. Shown here are the goblet cell marker (MUC5AC), ciliated cell markers (FOXJ1 and DNAI2), basal cell marker (p63), and club cell marker (SCGB1A1). Expression values were calculated with  $2^{-\Delta Ct}$  formula and represented as expressions expression (x100) relative to POLR2A in arbitrary units. Data are presented as means +/- SDs. **D-E**, Immunofluorescence staining of mature bronchial epithelial spheroids (day 16) for acetylated alpha-tubulin (red), MUC5AC (green) and DNA (blue). For statistical analysis two-tailed paired Student's t-test was used. \*P < 0.05. \*\*P < 0.01. \*\*\*P < 0.001.

(A)

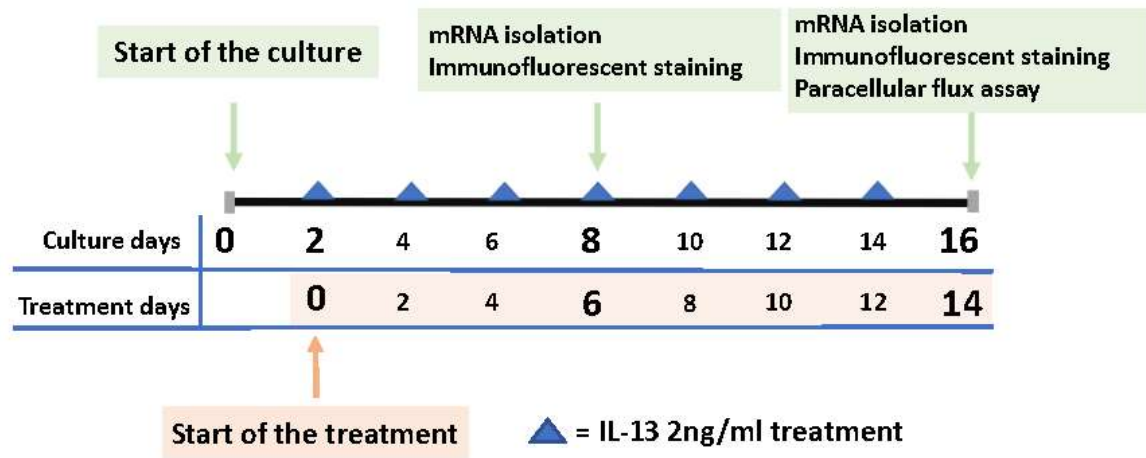
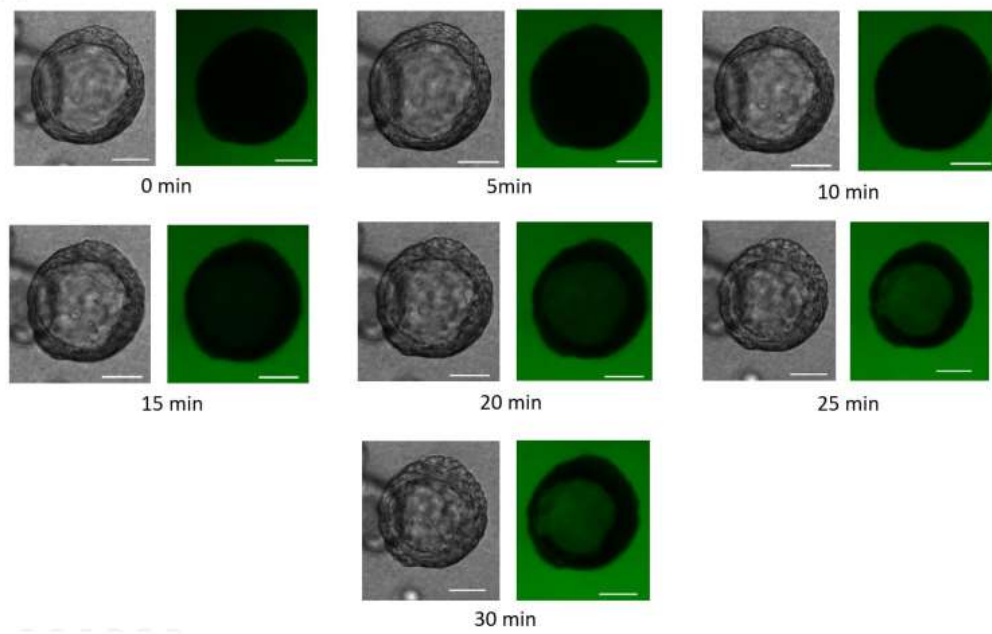


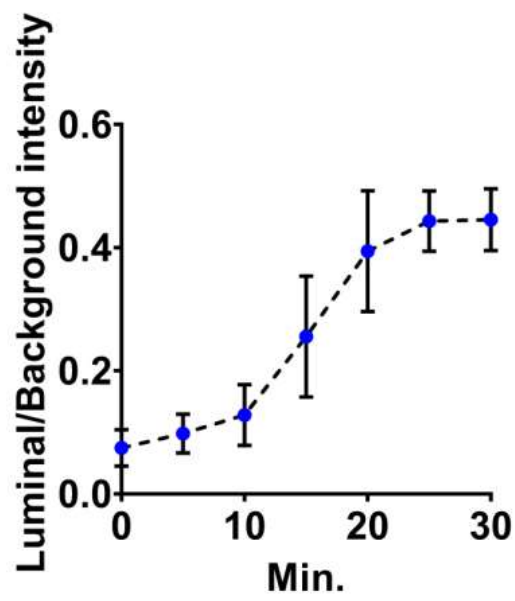
Fig. S2 Pat et al.

**Figure S2.** Schematic representation of the experimental protocol

(A)



(B)



264 **Figure S3.** EDTA treatment as a positive control for epithelial barrier impairment. **A,**  
265 Representative light and fluorescence microscopy images of treatment of mature bronchial  
266 epithelial spheroids with 10 mM EDTA for 30 minutes. **B,** Increase of lumen-to-background  
267 intensity ratio during 30 minutes of 10 mM EDTA treatment.

268

269 **Movie S1.** Timelapse video of beating cilia in fully formed bronchial epithelial spheroids (day  
270 16), related to supplementary Figure 1

271 **Movie S2.** Timelapse video of beating cilia in fully formed bronchial epithelial spheroids (day  
272 16), related to supplementary Figure 1