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SUPPORTING INFORMATION

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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.^{1,2} 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.^{3,4} 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 described² 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.^{2,4} Therefore, decreased mRNA levels of these marker

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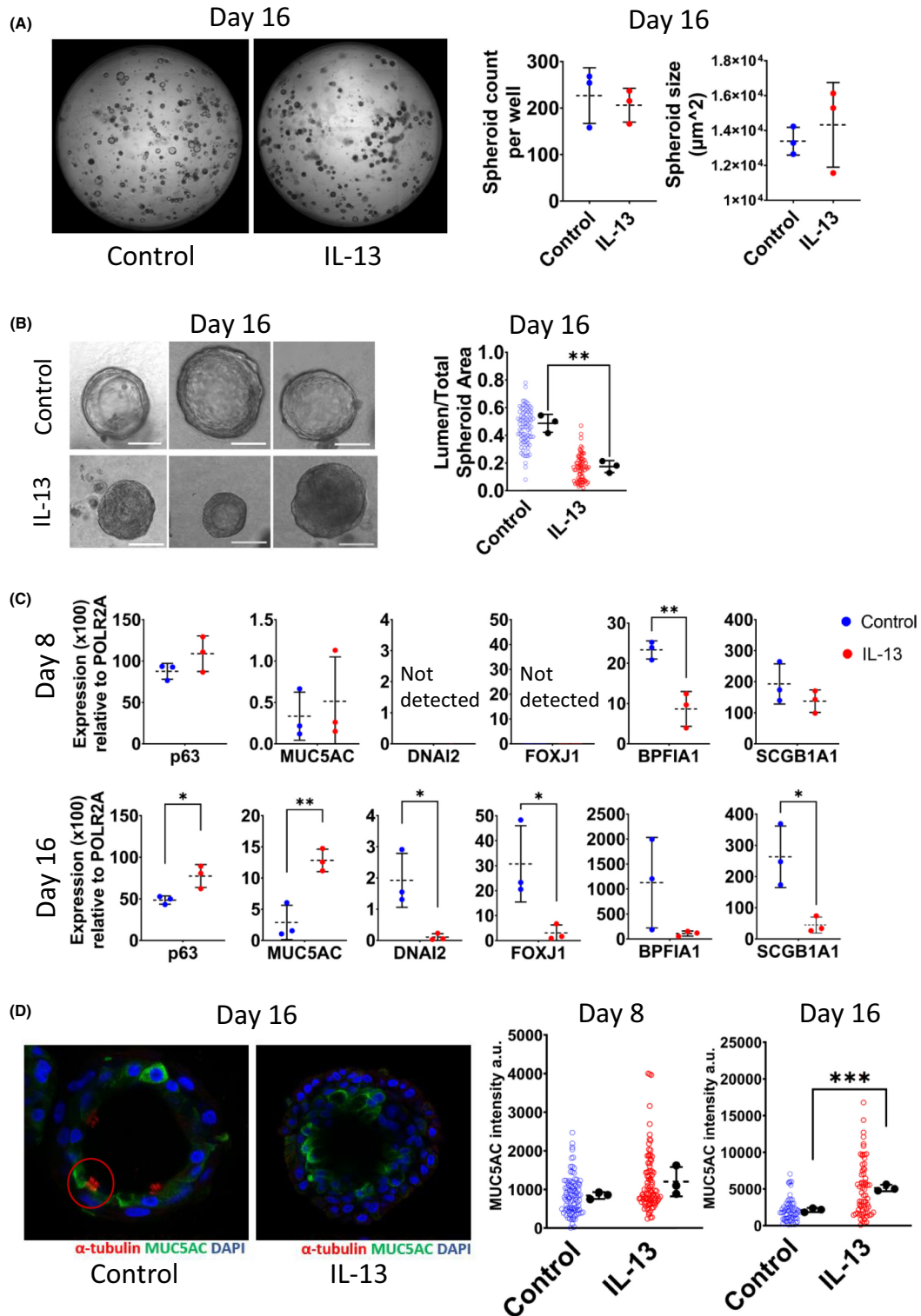


FIGURE 1 Treatment of bronchial epithelial spheroids with IL-13 recapitulates characteristic asthmatic epithelial cell differentiation phenotype. A, Representative light microscopy images from control and IL-13-treated bronchial epithelial spheroids on Day 16. The average number of spheroids and average spheroid size per well (μm^2) (right panel). B, Representative light microscopy images on Day 16. Scale bar 50 μm . '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 α -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. * $p < .05$. ** $p < .01$. *** $p < .001$

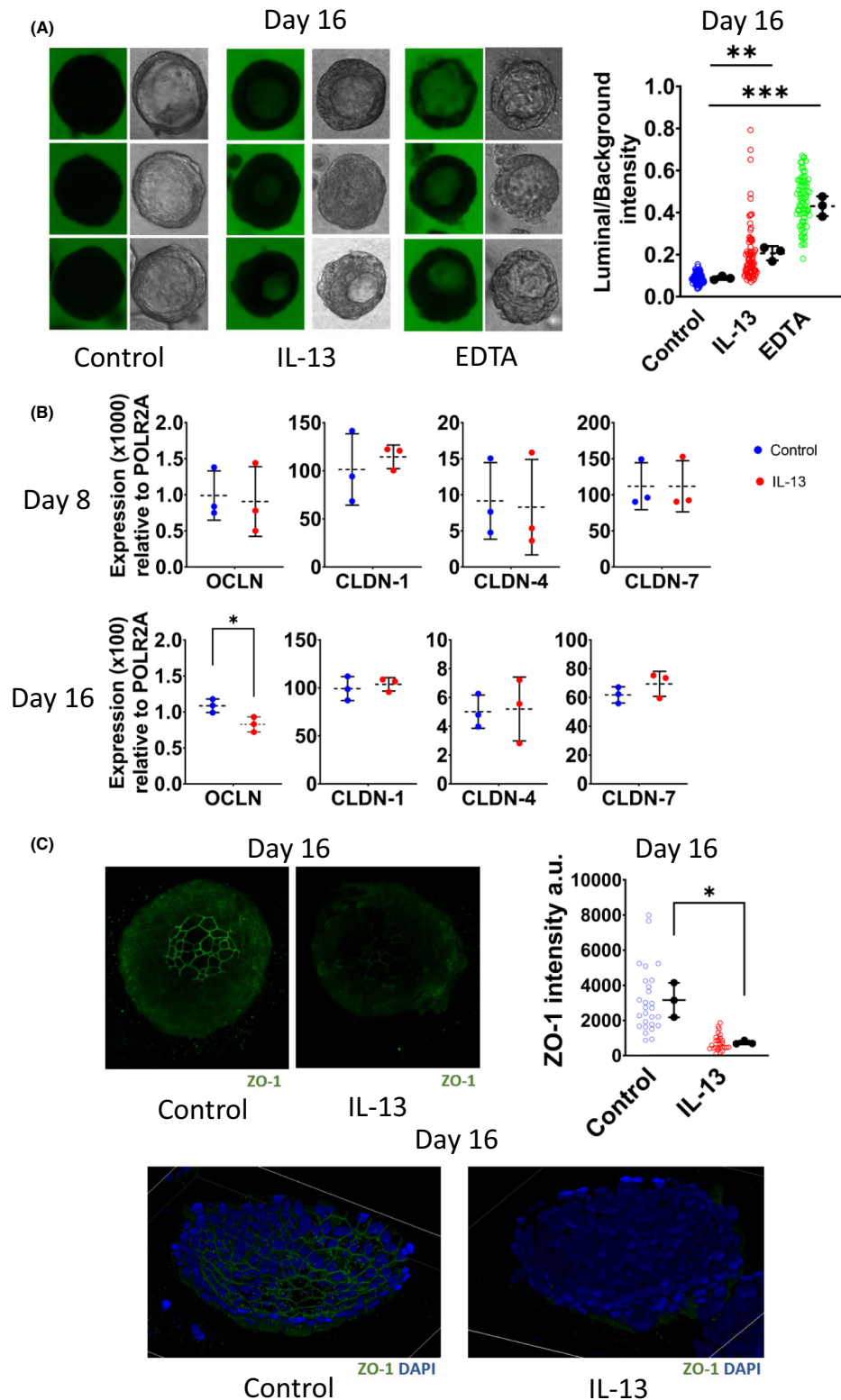


FIGURE 2 Treatment of bronchial epithelial spheroids with IL-13 impaired epithelial barrier development. A, Representative light and fluorescence microscopy images of the paracellular flux assay performed on Day 16 of culture. Each row of the spheroid pictures represents a biological replicate. EDTA 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 ($\times 100$) 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

genes indicate the suppression of ciliated cell differentiation. In addition, it is noteworthy that the mRNA levels of the club cell marker, secretoglobulin 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.⁵ These findings are consistent with the low protein levels in bronchoalveolar lavage fluids of asthmatic individuals.⁵ 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.⁶ Meta-analysis of BPIFA1 expression in epithelia obtained by bronchial brushings from asthmatic individuals shows decreased mRNA levels similar to our findings.⁶ Confocal microscopy imaging showed a significant increase in MUC5AC intensity without detectable ciliated cells, which were identified by acetylated α -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.^{2,4}

Given the fact that the impaired epithelial barrier is one of the hallmarks of asthma,³ 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) 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.³

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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Prevalence and risk factors of chronic urticaria in China: A nationwide cross-sectional study

To the Editor,

Chronic urticaria (CU) is a common disease imposing substantial burdens.¹ Despite the huge population, little information is available on the epidemic of CU in China. We aimed to provide reliable data for the prevalence of CU and associated factors in Chinese adults.

Based on 298 disease surveillance points of China Chronic Diseases and Risk Factors Surveillance, a nationally representative sample of Chinese adults were selected using a multistage stratified cluster sampling scheme. Sociodemographic characteristics, lifestyles, self-reported chronic diseases, and metabolic indicators were collected through questionnaire and laboratory testing, and latitude of disease surveillance points was extracted from Baidu Map. CU was defined as wheals, angioedema or both for more than 6 weeks in the preceding 12 months.² Risk factors of CU were examined by multivariable regression and the dose-response relationships with metabolic indicators were further tested. More details of methods and analyses are in the Appendix S1.

Overall, 184,509 adults aged 18 years or older participated in the survey from August 2018 to June 2019. After excluding people without information on CU or major risk factors, 184,326 participants were included and their characteristics are presented in Table S1. The weighted prevalence of CU was estimated at 2.6% (95% CI 2.4%–2.8%), with relatively higher estimate in north region (Figure S1). The odds of CU was positively associated with increased age (odds ratio 1.07, 95% CI 1.04–1.10), female sex (1.52, 1.40–1.65), rural locality (1.17, 1.06–1.29), increased latitude (1.02, 1.01–1.02), ever smoking (1.17, 1.07–1.27), improper sleep duration (1.60, 1.45–1.76 for <5 h; 1.30, 1.13–1.49 for >10 h), household fuel use (1.14, 1.03–1.27), diabetes (1.08, 1.01–1.16), dyslipidemia (1.13, 1.06–1.20), cardiovascular disease (1.63, 1.50–1.77), and atopy (4.67, 4.29–5.08), while was negatively associated with higher education (0.87, 0.80–0.96, and 0.87, 0.76–0.99 for high school and college

or above, respectively), higher household income per capita (0.80, 0.73–0.87, and 0.76, 0.70–0.83 for middle and high tertile groups, respectively), and physical inactivity (0.85, 0.79–0.92) (Table 1).

In stratified analyses, the associations with household fuel use and overweight/obese were only seen in rural area (odds ratio 1.18, 95% CI 1.05–1.32) and women (1.10, 1.02–1.19), respectively. Additional spline regression analyses demonstrated nonlinear relationships of glycated hemoglobin (HbA1c), total cholesterol, and high-density lipoprotein cholesterol (HDLc) with CU risk, with higher estimates at both ends (Figure 1).

Within the range of worldwide prevalence (0.1%–3.4%),³ our estimated prevalence (2.6%) together with the huge population base of China makes CU a major public health challenge. Interestingly, associations between CU and metabolic disorders were found, which could be explained by some common pathobiological pathways, including pro-inflammatory state, increased oxidative stress, and activation of the coagulation system. The U-shaped relationships between HbA1c, cholesterol, and HDLc, and CU suggested that low HbA1c, low cholesterol, and high HDLc are not as good as supposed previously. Indeed, low HbA1c has been reported to increase the risk of all-cause mortality,⁴ which might be explained by malnutrition, pro-inflammatory state, and autoimmune activation.⁵ Moreover, the significant U-shaped association of HDLc with all-cause mortality has also been observed and challenging the protective role of high HDLc levels.⁶ These findings suggest that the clinical control targets of metabolism indicators including glucose and lipid may warrant further exploration. Other risk factors of CU are discussed in the Appendix S1. Despite the large sample size of our study, the association magnitudes were generally small, and thus, the replication of our findings in other population is needed.

This is the largest and most comprehensive population-based Chinese epidemiological study of CU. Smoking cessation, indoor air