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Original Article

Role of basal cells in nasal polyp epithelium in the pathophysiology of eosinophilic chronic rhinosinusitis (eCRS)

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Abbreviations:
cBC, cultured Basal-like cells; cEC, cultured Epithelial cells; CRS, chronic rhinosinusitis; eCRS, eosinophilic chronic rhinosinusitis; NeCRS, non-eosinophilic chronic rhinosinusitis; NHBE, normal human bronchial epithelial; NP, nasal polyp; UT, uncinate tissue

ABSTRACT

Background: Basal cell hyperplasia is commonly observed in nasal polyp epithelium of eosinophilic chronic rhinosinusitis (eCRS). We examined the function and mechanisms of basal cell hyperplasia in the pathophysiology of eCRS.
Methods: We found that normal human bronchial epithelial (NHBE) cells obtained basal cell characteristics when cultured with PneumaCult™-Ex Plus Medium. Most of the cells passaged three times expressed basal cell surface markers CD49f and CD271 by flow cytometry, and basal cell nuclear marker p63 by immunohistochemical staining. We named these NHBE cells with basal cell characteristics cultured Basal-like cells (cBC), and NHBE cells cultured with BEGM™ cultured Epithelial cells (cEC). The characteristics of cBC and cEC were examined and compared by RNA sequencing, RT-PCR, ELISA, and cell proliferation studies.
Results: RNA sequencing revealed that cBC showed higher gene expression of thymic stromal lymphopoietin (TSLP), IL-8, TLR3, and TLR4, and lower expression of PAR-2 compared with cEC. The mRNA expression of TSLP, IL-8, TLR3, and TLR4 was significantly increased in cBC, and that of PAR-2 was significantly increased in cEC by RT-PCR. Poly(I:C)-induced TSLP production and LPS-induced IL-8 production were significantly increased in cBC. IL-4 and IL-13 stimulated the proliferation of cBC. Finally, the frequency of p63-positive basal cells was increased in nasal polyp epithelium of eCRS, and Ki67-positive proliferating cells were increased in p63-positive basal cells.
Conclusions: Type 2 cytokines IL-4 and IL-13 induce basal cell hyperplasia, and basal cells exacerbate type 2 inflammation by producing TSLP in nasal polyp of eCRS.

Introduction

Chronic rhinosinusitis (CRS) is a heterogeneous disease and can be classified into two major subtypes: CRS with nasal polyp (CRSwNP) and CRS without nasal polyp (CRSsNP). In the United States and European countries, the majority of CRSwNP cases exhibit eosinophilic and type 2 inflammation, whereas CRSsNP is characterized by type 1/type 3 inflammation.[1–3] The endotypes of inflammation in CRSwNP differ between European and Asian countries.[4] Half of CRSwNP cases in Japan exhibit type 1/type 3 inflammation,[5] and CRSwNP patients in other East Asian countries present both type 2 and type 1/type 3 inflammation. For the diagnosis of eosinophilic CRS (eCRS) with type 2 inflammation, a novel scoring system and algorithm were established in Japan based on the assessment of bilateral disease, nasal polyp formation, ethmoid sinus-predominant CT shadows, and blood eosinophilia.[6] eCRS is clinically characterized by marked eosinophilia in nasal polyps, higher morbidity of bronchial asthma, and a higher risk of recurrence after surgery.

Basal cell hyperplasia was commonly observed in nasal polyp epithelium of eCRS. Basal cells are progenitor cells and have an ability to differentiate into various cell types, such as secretory cells and ciliated cells.[7] They play important roles in epithelial barrier function, and inflammatory tissue remodeling in airway mucosa.[8] Recent studies using flow cytometry and histochemical examination have revealed that the frequency of basal cells was significantly

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increased in nasal polyps from patients with CRSwNP, whereas those of secretory cells and ciliated cells were decreased.[9,10] By comparing the transcriptomes of non-polyp and polyp basal cells, elevated expression of transcripts was recognized in polyp basal cells involved in extracellular matrix remodeling and chemoattraction of effector cells.[9] Some of these upregulated genes are IL-4/IL-13 responsive, and IL-4/IL-13 induced the expansion and differentiation of basal progenitor cells.[10]

Thymic stromal lymphopoietin (TSLP) is expressed in epithelial cells of nasal mucosa, and contributes to both innate and adaptive type 2 inflammation[11,12] by stimulating the production of type 2 cytokines IL-4 and IL-13 from Th2 cells and group 2 innate lymphoid cells (ILC2).[13] We previously revealed the elevated expression of TSLP in epithelial cells of nasal polyps from patients with eCRS compared with those from patients with non-eCRS (NeCRS).[14] A recent study using single-cell RNA sequencing revealed that TSLP was expressed in basal cells of ethmoid sinus mucosa, and basal cells may be a major source of TSLP in nasal epithelium.[9] These results suggest that basal cell hyperplasia and basal cell-derived TSLP may play important roles in the pathophysiology of type 2 inflammation in eCRS.

In the present study, we found that normal human bronchial epithelial (NHBE) cells obtained basal cell characteristics when cultured with PneumaCult™-Ex Plus Medium. PneumaCult™-Ex Plus Medium is a serum-free culture medium that supports greater expansion of primary human airway epithelial cells compared with bronchial epithelial growth medium (BEGM™).[15] We named these NHBE cells with basal cell characteristics cultured Basal-like cells (cBC) and NHBE cells cultured with BEGM™ cultured Epithelial cells (cEC). To elucidate the function and mechanism of basal cell hyperplasia in eCRS, (1) RNA sequencing data of cBC and cEC were analyzed, (2) mRNA expression in cBC and cEC was examined by RT-PCR, (3) airborne antigen-induced production of TSLP from cBC and cEC was compared, and (4) the frequency of p63-positive basal cells and Ki67-positive proliferating activity were examined using immunofluorescence staining of nasal polyps.

Methods

Study subjects

Thirty-seven patients with nasal or paranasal sinus diseases were recruited from Shiga University of Medical Science hospital according to a protocol approved by the Institutional Review Board of Shiga University of Medical Science (approval number R2021-132). Informed consent was obtained from all participants. All experiments were conducted according to the Declaration of Helsinki on biomedical research involving human subjects. Sinonasal tissues, such as uncinate tissue (UT), and nasal polyp (NP), were obtained during endoscopic sinus surgery (ESS). None of the patients had been treated with systemic corticosteroids for at least four weeks before the surgery. The diagnosis of CRS was made based on clinical, endoscopic, and radiographic criteria as described in the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS 2020).[1] The CRSwNP cases were classified into eCRS and non-eCRS (NeCRS) according to a published clinical scoring system (JESREC score).[6] Table 1 presents a detailed list of the patient characteristics. Control subjects include patients with benign sinus tumors (6 cases), sinus cystic lesions (2 cases), and thyroid eye disease (1 case). The diagnosis of asthma was made based on the criteria of the Global Initiative of Asthma.[16] The JESREC score was calculated with the following point breakdown: bilateral disease sites (3 points); nasal polyp (2 points); CT shadow, ethmoid $\geq$ maxillary sinus (2 points); and blood eosinophilia, 2–5% (4 points), 5–10% (8 points), or more than 10% (10 points). Patients with scores higher than 11 points and mucosal eosinophilia higher than 70 per high-power field (400× magnification) were defined as eCRS.[6]

Reagents

We purchased the following antigens and materials: house dust mite (HDM) and *Alternaria alternata* (*Alternaria*) (Greer Laboratories, Lenoir, NC, USA), protease from *Staphylococcus aureus* (*S. aureus*) (Abnova, Taipei, Taiwan), polyinosinic-polycytidylic acid (Poly(I:C)) (InvivoGen, San Diego, CA, USA), lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Sigma–Aldrich, St. Louis, MO, USA), and human recombinant IL-4 and IL-13 (FUJIFILM Wako Pure Chemical, Osaka, Japan).

Cell culture

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza (Lonza, Basel, Switzerland), and were cultured with PneumaCult™-Ex Plus Medium (STEMCELL Technologies, Vancouver, Canada) to confluence in a 75 cm^2 flask (Corning, Corning, NY, USA). Then, the NHBE cells were passaged twice with PneumaCult™-Ex Plus Medium or bronchial epithelial growth medium (BEGM™) (Lonza, Basel, Switzerland) in a 75 cm^2 flask. Primary nasal epithelial (PNE) cells were collected from the NPs of eCRS patients by curettage with a Rhino-Probe (Arlington Scientific, Inc., Springville, UT, USA), and were passaged twice with PneumaCult™-Ex Plus Medium or BEGM™, respectively.

Table 1
Patient characteristics..

Subject characteristics	UT		NP	
	Control	CRSsNP	NeCRS	eCRS
Subject number	9 (8M/1F)	10 (5M/5F)	8 (6M/2F)	10 (4M/6F)
Age (years)	60.0 (47.0-76.5)	49.0 (37.0-65.0)	40.0 (37.5-55.5)	53.0 (48.0-66.0)
Asthma	0	3	2	9
Blood eosinophils (%)	3.20 (1.50-4.65)	1.90 (0.90-4.10)	3.45 (1.50-6.50)	9.25 (8.10-9.90) *
Lund-Mackay CT score		2.0 (1.0-3.0)	10.0 (6.0-14.5)	14.5 (9.0-21.0)

UT, uncinate tissue; NP, nasal polyp; CRSsNP, chronic rhinosinusitis without nasal polyp; eCRS, eosinophilic chronic rhinosinusitis; NeCRS, non-eosinophilic chronic rhinosinusitis; F, female; M, male. The clinical data are expressed as median and interquartile range. *$P<0.05$.

The cultured NHBE cells and cultured PNE cells were cultured again with PneumaCult™-Ex Plus Medium or BEGM™ in a 24-well tissue culture plate (Corning) to 80% confluence. After incubating in each medium without supplements and steroids (starving) for 24 h, the cells were stimulated for 24 h with HDM (100 μg/mL), *Alternaria* (400 μg/mL), protease from *S. aureus* (1 μg/mL), Poly(I:C) (10 μg/mL), or LPS (1 μg/mL). Then, the supernatant was collected for ELISA. The cells were used for RT-PCR or RNA sequencing after starving for 30 h.

For the cell proliferation study, the cultured NHBE cells were seeded (1×10^4 cells/well) and cultured in a 24-well tissue culture plate for 4 days with PneumaCult™-Ex Plus Medium or BEGM™ by adding cytokines (TNF-α, IFN-γ, IL-1β, IL-4, IL-5, IL-13, calprotectin, S100A8, S100A9, amphiregulin, or IL-17) every 2 days. The cells were dissociated using an Animal Component-Free Cell Dissociation Kit (STEMCELL Technologies) and counted with an improved Neubauer cell counting chamber (SLGC, Saitama, Japan).

Flow cytometry

The cultured NHBE cells were preincubated with Fc-Block (BD Bioscience, Franklin Lakes, NJ, USA) for 10 min at room temperature after staining with a Zombie NIR™ Fixable Viability Kit (BioLegend, San Diego, CA, USA) to assess cell viability. The cells were stained with APC anti-human CD271 (clone ME20.4, BioLegend), and FITC anti-human CD49f (clone GoH3, BioLegend) for 30 min at 4 °C. As a control, cells were stained with individual isotype antibodies. After washing, the cells were resuspended in cell stain buffer (BioLegend) and analyzed with a BD LSR Fortessa X-20 (BD Bioscience) using FlowJo v10 by TreeStar (BD Bioscience).

Immunofluorescence staining

Cultured NHBE cells were seeded and cultured on Lab-Tek™ II Chamber Slides (ThermoFisher, Waltham, MA, USA). The slides were fixed and permeabilized by Cytofix/Cytoperm reagents (BD Bioscience) for 20 min at room temperature, and then washed with BD Perm/Wash buffer for 30 min at room temperature. Fixed cells were blocked with Normal Serum Block (Biolegend) for 1 h. For immunofluorescence, the cells were incubated overnight at 4 °C with 1:250 purified mouse anti-human TP63 antibody (clone W15093A, Biolegend) or purified mouse IgG2b, κ isotype ctrl (clone MG2b-57, Biolegend). After three washes, the cells were incubated for 45 min with 1:500 Alexa Fluor 647 goat anti-mouse IgG (minimal x-reactivity) antibody (clone Poly4053, Biolegend) and mounted in Vectashield® mounting medium with DAPI (Vector Laboratories, Newark, CA, USA). Fluorescent cell images were visualized using a confocal laser scanning microscope TCS SP8 X (Leica, Wetzlar, Germany).

Nasal tissues were fixed in 4% formaldehyde in 0.1 M of phosphate-buffered saline (PBS) for 2 h at 4 °C and immersed in 0.1 M of PBS containing 15% sucrose for 24 h at 4 °C. Then nasal tissues were embedded in paraffin and cut into 4 μm-thick sections. After deparaffinization, slides were quenched for 10 min in 1 mg/mL sodium borohydride in PBS. After heat-induced antigen retrieval with antigen retrieval solution pH 9 (NICHIREI BIOSCIENCE INC., Tokyo, Japan), slides were blocked with Normal Serum Block (Biolegend) for 60 min. The slides were incubated overnight at 4 °C with primary antibodies or isotype controls in a humidified chamber. The primary antibodies used in this study were 1:500 purified mouse anti-human TP63 antibody (clone W15093A, Biolegend), 1:200 rat anti-human Ki67 monoclonal antibody (SoIA15, ThermoFisher), and 1:100 rabbit anti-human TSLP antibody (ab47943, Abcam, Cambridge, UK). The isotype controls were purified mouse IgG2b, κ isotype ctrl (clone MG2b-57, Biolegend) and purified rat IgG2a, κ isotype ctrl (clone RTK2758, Biolegend), and purified (azide-free) rabbit polyclonal isotype ctrl (clone Poly29108, Biolegend). After three washes with 0.05% Tween 20 in PBS, the slides were incubated for 45 min with 1:500 secondary antibodies and 1:200 Hoechst 33342 solution (FUJIFILM Wako Pure Chemical). The secondary antibodies were Alexa Fluor 647 goat anti-mouse IgG (minimal x-reactivity) antibody (clone Poly4053, Biolegend), Alexa Fluor 555 goat anti-rat IgG (minimal x-reactivity) antibody (clone Poly4054, Biolegend), and Alexa Fluor 488 donkey anti-rabbit IgG (minimal x-reactivity) antibody (clone Poly4064, Biolegend). The slides were washed three times and mounted with VECTASHIELD PLUS antifade mounting medium (Vector Laboratories). Fluorescent images were visualized using a confocal laser scanning microscope TCS SP8 X (Leica). Five random views per sample were used to measure the area of the epithelium and the number of stained cells per area.

RNA sequencing

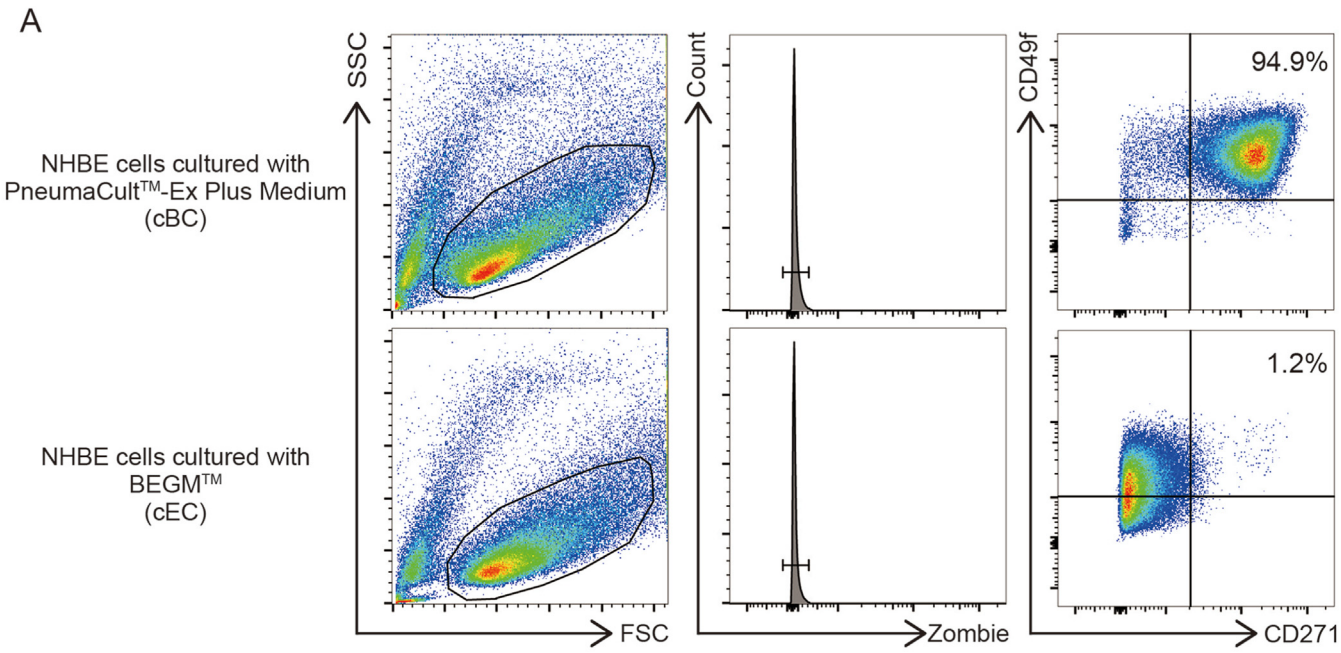
Total RNA of cultured NHBE cells was purified with a NucleoSpin® RNA Plus XS (Macherey–Nagel, Düren, Germany). RNA sequence libraries were prepared using the TruSeq stranded mRNA library prep kit (Illumina, San Diego, CA, USA) and sequenced using the Illumina platform at Macrogen Japan Corp. DEG analysis was performed on a comparison pair. Statistical analysis was performed using fold change (fc), nbinomWaldTest using DESeq2 per comparison pair. Significant results were selected on conditions of $|fc| \geq 2$ & nbinomWaldTest raw p-value <0.05.

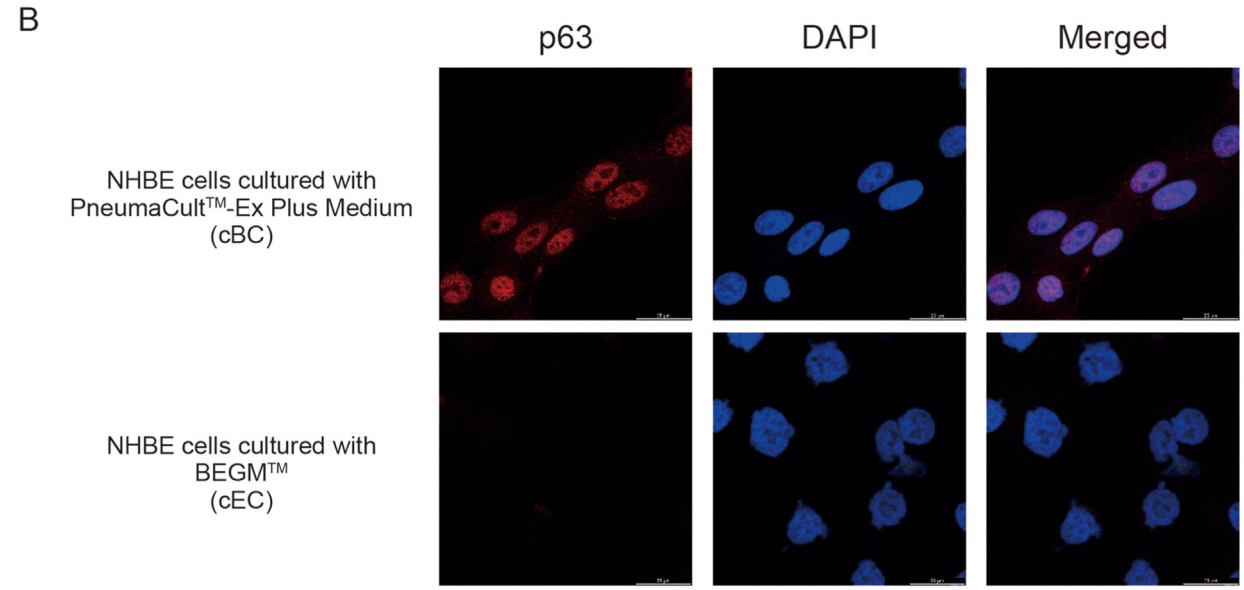
Real-time RT-PCR

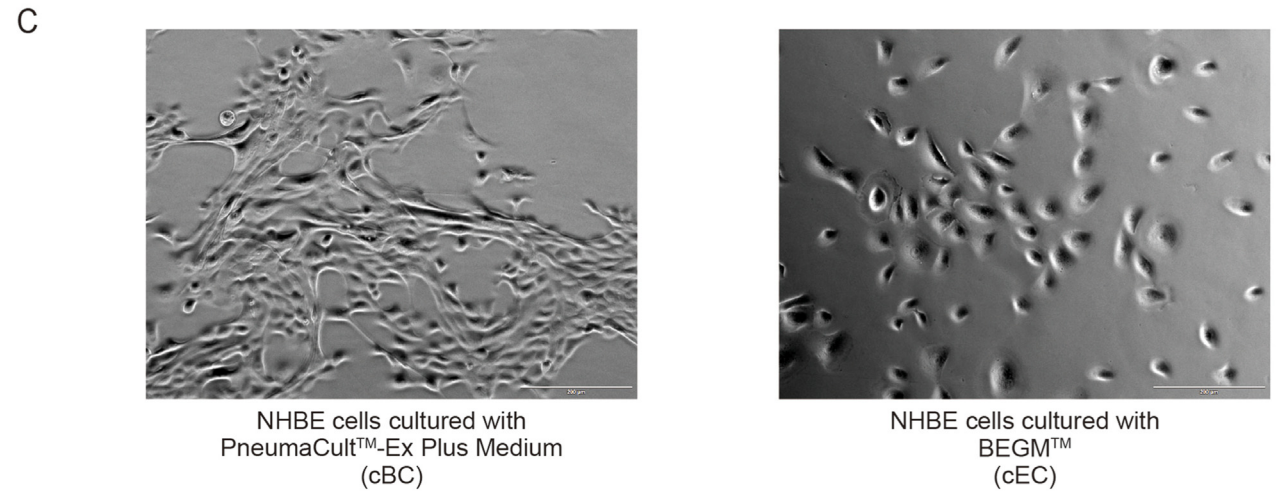
cDNA was synthesized from a purified RNA sample using a transcriptor high fidelity cDNA synthesis kit (Roche, Basel, Switzerland). The light-cycler 480 real-time PCR system (Roche) amplifies and detects specific products. The real-time RT-PCR mixture contained 5 μL of cDNA, 10 μL of LightCycler 480 Probe Master (Roche), 0.2 μL of probes, and 0.1 μL of two primer sets: TSLP (forward primer: CCAGGCTATTCGGAAACTCA; reverse primer: AATTGTGACACTTGTTCCAGACA), toll-like receptor 3 (TLR3) (forward primer: ATCTTCCAATTGCGTGAAAAC; reverse primer: TGGATATCTTTGCCAATTCATCT), protease-activated receptor (PAR)-2 (forward primer: GTGGCACCATCCAAGGAAC; reverse primer: CAG ATGCAGAAAACTCATCCACAG), IL-8 (forward primer: GAGCACTCCATAAGGCACAAA; reverse primer: ATGGTTCCTTCCGGTGGT), toll-like receptor 4 (TLR4) (forward primer: CTCTCCTGCGTGAG ACCAG; reverse primer: TCCATGCATTGATAAGTAATATTAGGA), IL-33 (forward primer: TGGAAGAACACAGCAAGCAA; reverse primer: TCATTTGAGGGGTGTTGAGA) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward primer: AGCCACATCGCTCAGACAC; reverse primer: GCCCAATACGACCAAATCC). The reaction mixture was topped up to a final volume of 20 μL with sterile water. The real-time RT-PCR protocol was as follows: denaturation by a hot start at 95 °C for 5 min, followed by 50 cycles of a three-step program (denaturation at 95 °C for 10 s and annealing at 60 °C for 20 s and extension at 72 °C for 10 s). Transcription was normalized to that of GAPDH in each sample and expressed as relative expression compared with control.

ELISA

The collected cell supernatants were analyzed for TSLP and IL-8 (R&D Systems, Minneapolis, MN, USA) by ELISA. The detection limits of the TSLP and IL-8 assays were 31.2 pg/mL.


A
NHBE cells cultured with PneumaCult™-Ex Plus Medium (cBC)
NHBE cells cultured with BEGM™ (cEC)
SSC
FSC
Count
Zombie
CD49f
CD271
94.9%
1.2%


B
p63
DAPI
Merged
NHBE cells cultured with PneumaCult™-Ex Plus Medium (cBC)
NHBE cells cultured with BEGM™ (cEC)


C
NHBE cells cultured with PneumaCult™-Ex Plus Medium (cBC)
NHBE cells cultured with BEGM™ (cEC)

Statistical analyses

All the data are show as mean ± standard error of the mean (SEM) from the indicated number of replicates. Two-sided differences between the two samples were analyzed with the Mann–Whitney U test or Wilcoxon signed-rank test. Data from more than three-group comparisons were evaluated by Kruskal–Wallis one-way analysis. In our statistical analysis, we applied the Bonferroni correction to adjust for multiple testing. Values of P <0.05 were considered significant.

Results

NHBE cells cultured with PneumaCult™-Ex Plus Medium or BEGM™

Primary NHBE cells were cultured with PneumaCult™-Ex Plus Medium and passaged twice with PneumaCult™-Ex Plus Medium or BEGM™. We found that NHBE cells cultured with PneumaCult™-Ex Plus Medium expressed basal cell characteristics. Most of the NHBE cells (94.9%) cultured with PneumaCult™-Ex Plus Medium expressed basal cell surface markers, CD49f and CD271, by flow cytometry, while the NHBE cells cultured with BEGM™ hardly expressed them (1.2%) (Fig. 1A). Immunofluorescence staining revealed that the NHBE cells cultured with PneumaCult™-Ex Plus Medium expressed the basal cell nuclear marker p63 (Fig. 1B, Supplementary Fig. 1A and B). Morphologically, the NHBE cells cultured with PneumaCult™-Ex Plus Medium showed a sheet-like microscopic appearance compared with those cultured with BEGM™ (Fig. 1C). We named these NHBE cells with basal cell characteristics cultured Basal-like cells (cBC), and NHBE cells cultured with BEGM™ cultured Epithelial cells (cEC).

RNA sequencing analysis of cBC and cEC

To analyze the gene expression of cBC and cEC, five samples each were used for RNA sequencing. We examined the expression of epithelial-cell derived cytokines and receptors related to type 1 inflammation, IL-18[17]; type 2 inflammation, TSLP, IL-33, IL-25, TLR3, F2RL1 (PAR-2), P2RY2[18–21]; type 3 inflammation, IL-22RA1, IL-10RB[22]; and neutrophilic inflammation, CXCL8 (IL-8), TLR4.[23–25] Among these genes, IL-18, TSLP, IL-33, TLR3, F2RL1 (PAR-2), P2RY2, IL-22RA1, IL-10RB, CXCL8 (IL-8), and TLR4 were detected, and cBC showed significantly higher expression of TSLP, TLR3, P2RY2, CXCL8 (IL-8), and TLR4, and significantly lower expression of IL-33, F2RL1 (PAR-2), and IL-22RA1 compared to cEC. There was no significant difference in the expression of IL-18 and IL-10RB between cBC and cEC. The MA plot is shown in Figure 2, based on the result of differentially expressed genes (DEG).

The mRNA expression of TSLP, IL-8, TLR3, TLR4, and PAR-2

The results of RNA sequencing were confirmed by RT-PCR. The mRNA expression of TSLP, CXCL8 (IL-8), TLR3, and TLR4 was significantly increased in cBC, and that of F2RL1(PAR-2) was significantly increased in cEC (Fig. 3A).

Production of TSLP and IL-8

TSLP production from cEC was induced by HDM, *Alternaria*, protease from *S. aureus*, and the TLR3 ligand Poly(I:C). Poly(I:C)-induced

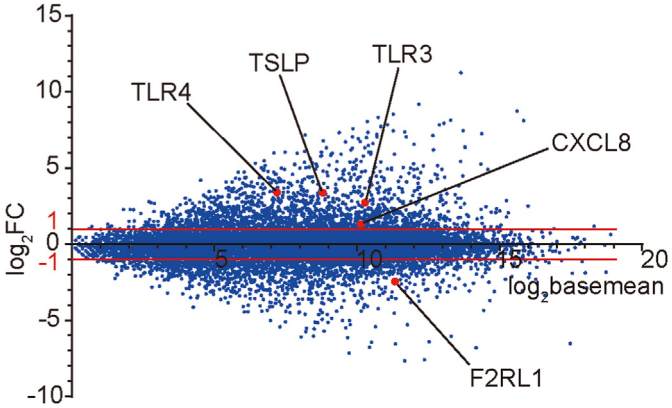


Fig. 2. MA plot between cultured Basal-like cells (cBC) and cultured Epithelial cells (cEC), X-axis: mean of normalized counts, Y-axis: log2 fold change. Genes with log2 fc >1 indicate that the genes are significantly upregulated, and genes with log2 fc <1 indicate that the genes are significantly downregulated in cBC compared to cEC.

TSLP production from cBC was significantly increased compared with that from cEC, whereas HDM, *Alternaria*, and protease from *S. aureus* did not induce TSLP production from cBC (Fig. 3B).

IL-8 production was induced by *Alternaria*, protease from *S. aureus*, and Poly(I:C) from both cBC and cEC. Poly(I:C)-induced IL-8 production from cBC was significantly increased compared with that from cEC. The TLR4 ligand LPS stimulated IL-8 production from cBC, whereas LPS did not induce IL-8 production from cEC (Fig 3C).

Cell proliferation

IL-4 (0.5 ng/mL) and IL-13 (5 ng/mL) stimulated the cell proliferation of cBC, but did not stimulate that of cEC (Fig. 3D and Supplementary Fig. 1C and D). Other inflammatory cytokines, IL-5, TNF-α, IFN-γ, IL-1β, calprotectin, S100A8, S100A9, amphiregulin, and IL-17, had no effects on cell proliferation of cBC (data not shown).

Cultured PNE cells from NPs of eCRS patients

We named primary nasal epithelial (PNE) cells from NPs of eCRS patients cultured with PneumaCult™-Ex Plus Medium cultured nasal Basal-like cells (cNBC) and those cultured with BEGM™ cultured nasal Epithelial cells (cNEC). The mRNA expression of TSLP, CXCL8(IL-8), TLR3, and TLR4 was significantly increased in cNBC compared with cNEC (Fig. 4A).

TSLP production from cNEC was induced by HDM, *Alternaria*, protease from *S. aureus*, and Poly(I:C). Poly(I:C)-induced TSLP production from cNBC was significantly increased compared with that from cNEC, whereas HDM, *Alternaria*, and protease from *S. aureus* did not induce TSLP production from cNBC (Fig. 3B).

IL-8 production was induced by HDM, *Alternaria*, Poly(I:C), and LPS from both cNEC and cNBC. Poly(I:C)-induced IL-8 production from cNBC was significantly increased compared with that from cNEC (Fig. 4C).

Immunofluorescence staining of nasal tissues with basal cell nuclear marker (p63), proliferating marker (Ki67), and TSLP

Immunofluorescence staining of UT from control subjects and patients with CRSsNP, and NP from patients with NeCRS and eCRS

Fig. 1. A: Gating strategy to identify basal cell surface markers (CD49f and CD271) in NHBE cells cultured with PneumaCult™-Ex Plus Medium or BEGM™. **B:** Immunofluorescence staining with basal cell nuclear marker p63 in NHBE cells cultured with PneumaCult™-Ex Plus Medium or BEGM™. Scale bars indicate 25 μm. **C:** Microscopic cell images of NHBE cells cultured with PneumaCult™-Ex Plus Medium or BEGM™. Scale bars indicate 200 μm.

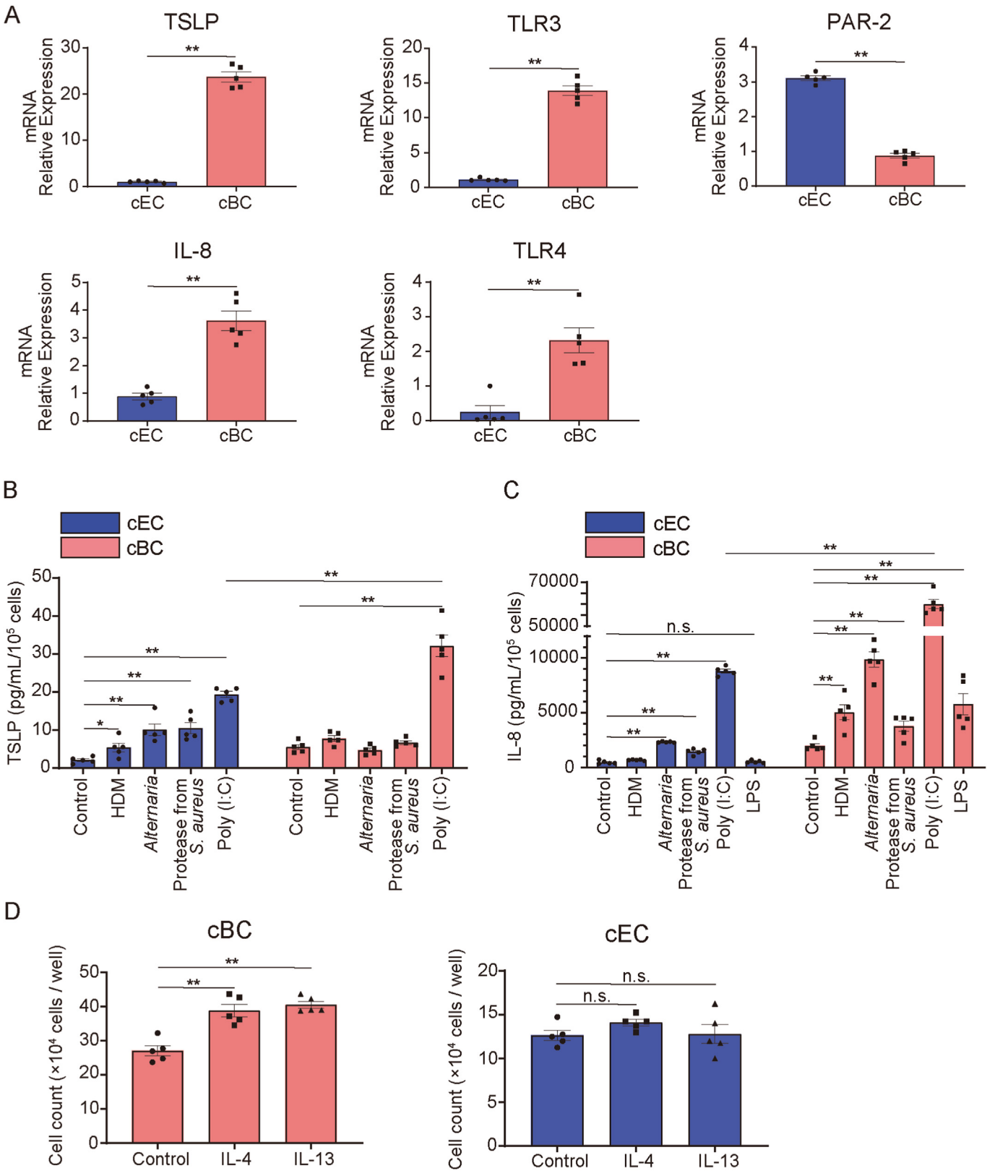


Fig. 3. A: mRNA expression of TSLP, TLR3, PAR-2, IL-8, and TLR4 in cultured Basal-like cells (cBC) and cultured Epithelial cells (cEC). $^{*}P < 0.05$, $^{**}P < 0.01$; n = 5. **B, C:** Effects of 24 h incubation with HDM (100 μg/mL), *Alternaria* (400 μg/mL), protease from *S. aureus* (1 μg/mL), Poly (I:C) (10 μg/mL), and LPS (1 μg/mL) on the production of TSLP **(B)** and IL-8 **(C)** from cBC and cEC. $^{*}P < 0.05$, $^{**}P < 0.01$; n = 5. **D:** Effects of IL-4 (0.5 ng/mL) and IL-13 (5 ng/mL) on the cell proliferation of cBC and cEC. The cells were seeded (1×10^4 cells/well) and cultured for four days. $^{**}P < 0.01$; n = 5.

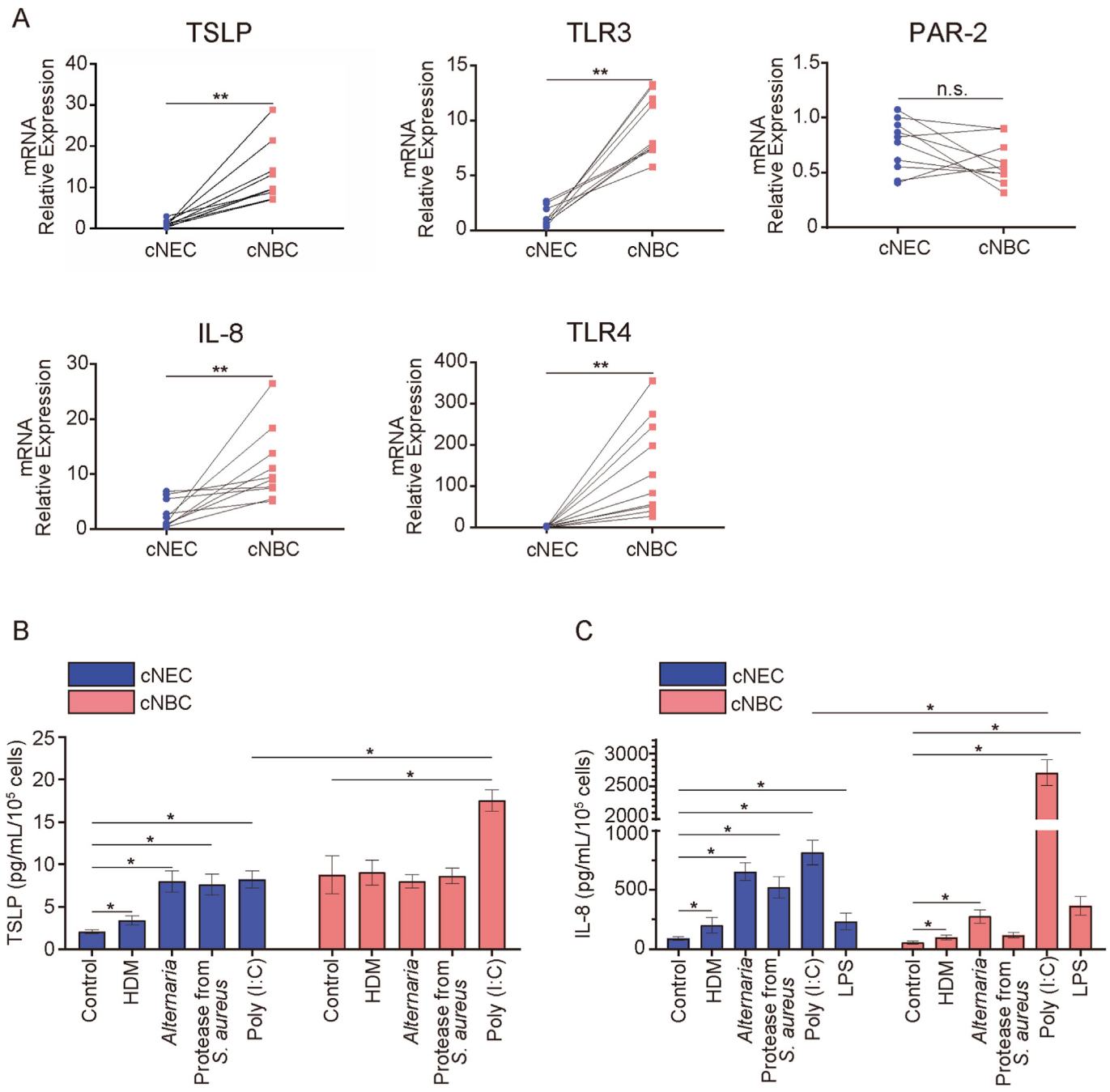


Fig. 4. A: mRNA expression of TSLP, TLR3, PAR-2, IL-8, and TLP4 in cultured nasal Basal-like cells (cNBC) and cultured nasal Epithelial cells (cNEC). Two samples per patient were used in the experiment. $^{**}P < 0.01$; n = 5. **B, C:** Effects of 24 h incubation with HDM (100 μg/mL), *Alternaria* (400 μg/mL), protease from *S. aureus* (1 μg/mL), Poly (I:C) (10 μg/mL), and LPS (1 μg/mL) on the production of TSLP **(B)** and IL-8 **(C)** for 24 h from cNBC and cNEC. Two samples per patient were used in the experiment. $^{*}P < 0.05$, $^{**}P < 0.01$; n = 4.

are shown in Figure 5A and Supplementary Fig. 1E–H. The p63-positive basal cells were increased in NP from patients with eCRS. The Ki67-positive proliferating cells were increased in basal cells of NP from eCRS patients. TSLP was consistently expressed in the basal cell layer of UT from CRSsNP patients and NP from eCRS and NeCRS patients.

The quantitative study of p63-and Ki67-positive cells revealed that the frequency of p63-positive basal cells was increased (Fig. 5B), and the Ki67-positive proliferating cells were increased in basal cells of NP from patients with eCRS (Fig. 5C). These results indicate that the type 2 inflammation induces basal cell hyperplasia, and basal cells exacerbate the type 2 inflammation by producing TSLP in NP of eCRS.

Discussion

In the present study, cultured NHBE cells with basal cell characteristics (cultured Basal-like cells: cBC) were used to examine the function of basal cells in eCRS compared with cultured Epithelial cells (cEC). We found that cBC showed increased gene expression of TSLP, IL-8, TLR3, and TLR4, and decreased expression of PAR-2 by RNA sequencing. The mRNA expression of TSLP, IL-8, TLR3, and TLR4

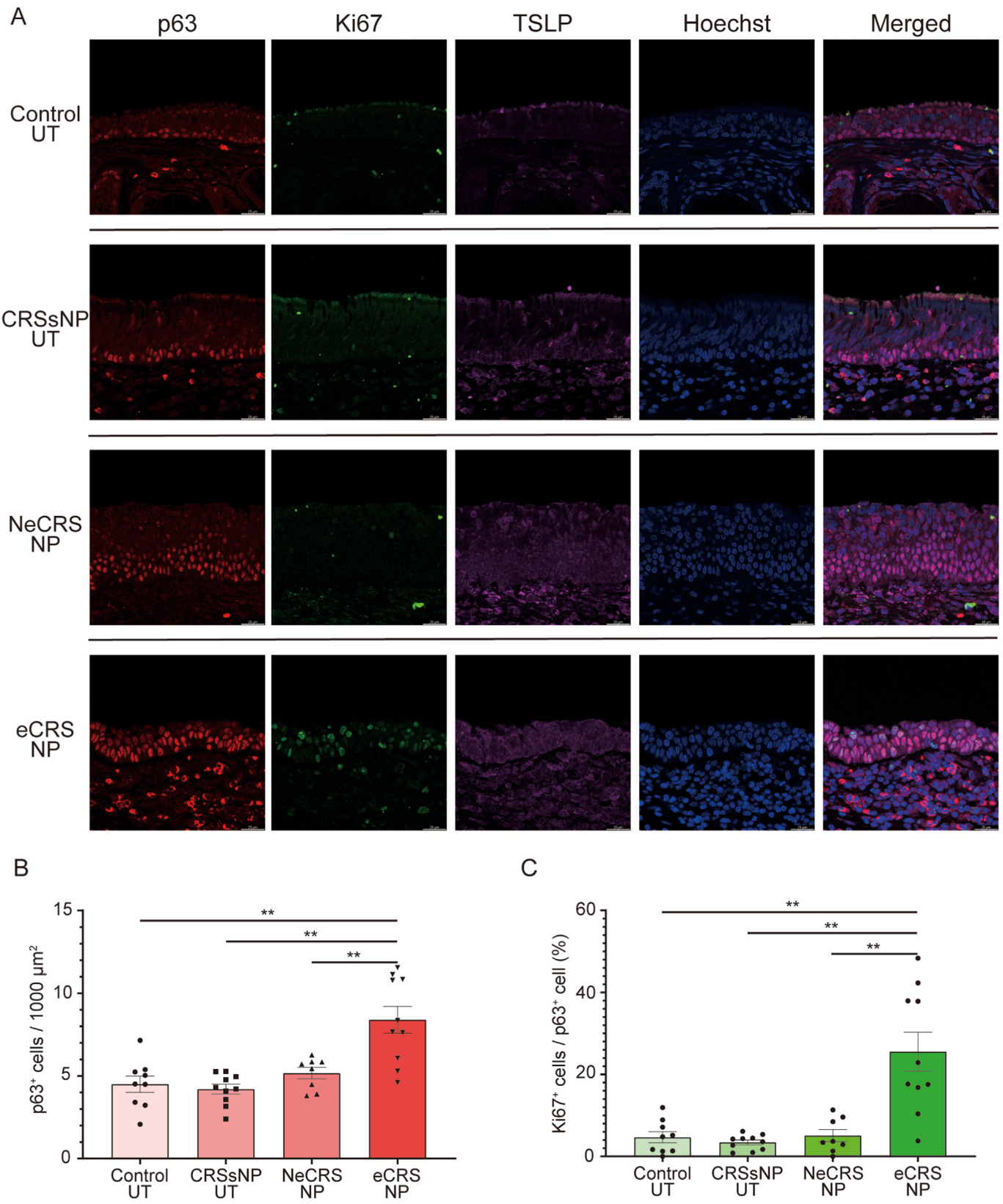


Fig. 5. A: Immunofluorescence staining with p63 (red), Ki67 (green), TSLP (magenta) in the uncinate tissue (UT) from healthy controls and patients with CRSsNP, and nasal polyp (NP) from patients with NeCRS and eCRS. Nuclei were counterstained with Hoechst (blue). Scale bars indicate 25 μm. **B, C:** Quantitative analysis of p63-positive cells in 1000 μm^2 **(B)** and the ratio of Ki-67-positive cells in p63-positive cells **(C)** in uncinate tissue (UT) and nasal polyp (NP). $^{**}P < 0.01$; n = 9 control, n = 10 CRSsNP, n = 8 NeCRS, n = 10 eCRS.

were significantly increased, and that of PAR-2 was significantly decreased. Poly(I:C)-induced TSLP production and LPS-induced IL-8 production from cBC were significantly increased, and IL-4 and IL-13 stimulated the proliferation of cBC. The frequency of p63-positive basal cells was increased in NP epithelium of eCRS, and Ki67-positive proliferating cells were increased in p63-positive basal cells. This is the first report to examine the function and mechanism of basal cell hyperplasia in the pathophysiology of eCRS. Basal cells exacerbate type 2 inflammation by producing TSLP, and type 2 cytokines IL-4 and IL-13 stimulate basal cell hyperplasia in NP of eCRS.

PneumaCult™-Ex Plus Medium is a serum-free medium originally used as an expansion medium for airway epithelial cells in the preparatory stage of air-liquid interface 3D cultures. It has been reported that NHBE cells cultured with PneumaCult™-Ex Plus Medium rapidly proliferated and form cobblestone-shaped clustered colonies compared with NHBE cells cultured with BEGM™.[15] We found that these NHBE cells cultured with PneumaCult™-Ex Plus Medium obtained basal cell characteristics. Most of the cultured cells (94.9%) expressed the basal cell surface markers CD49f and CD271 by flow cytometry, and immunofluorescence staining revealed the expression of a basal cell nuclear marker, p63. Morphologically, these cells form a microscopic sheet-like appearance. We named these cultured NHBE cells with basal cell characteristics cultured Basal-like cells (cBC), and examined the function of cBC cells in comparison to NHBE cells cultured with BEGM™ (cultured Epithelial cells: cEC).

The mRNA expression of TSLP was significantly upregulated in cBC, and immunofluorescence staining showed that TSLP was consistently expressed in the basal layer of UT and NP from patients with CRS. These results are consistent with the previous study using single-cell RNA sequencing showing that TSLP expression was restricted to basal cells in sinonasal mucosa of CRS patients.[9] TSLP is an important cytokine for type 2 inflammation, and TSLP production was induced by viruses, protease-containing allergens, and type 2 cytokines IL-4 and IL-13 in airway epithelial cells.[20] We previously reported that TSLP expression was upregulated in nasal polyp epithelium of eCRS,[14] and Poly(I:C) or *Alternaria* stimulated the TSLP production from airway epithelial cells.[18]

IL-33, an epithelial-derived cytokine for type 2 inflammation, may play important roles in the pathophysiology of eCRS,[14] and basal cells were reported to be a major source of IL-33 in nasal epithelium of CRSwNP.[9] However, RNA sequencing showed that the expression of IL-33 was downregulated in cBC compared with cEC. A recent study revealed that the stability of IL-33 reduced over increasing passage number of cultured epithelial cells.[26] In the present study, cBC and cEC were examined at passage 4, and cNBC and cNEC were examined at passage 2. The mRNA expression of IL-33 was not different between cNBC and cNEC, whereas it was decreased in cBC compared with cEC (date not shown). It is possible that the increased passaging may affect the decreased mRNA expression of IL-33 in cBC.

In the present study, the expression of IL-8, TLR3, and TLR4 was upregulated, and that of PAR-2 was downregulated in cBC. The TLR3 ligand Poly(I:C) significantly stimulated TSLP production, and the TLR4 ligand LPS stimulated IL-8 production from cBC. eCRS is a heterogenous disease mainly characterized by eosinophilic and type 2 inflammation, but neutrophilic and type 1/type 3 inflammation are also involved in the pathophysiology. IL-8, a neutrophil chemotactic factor, is a key mediator for neutrophil recruitment for neutrophilic and type 1 inflammation, and a recent study showed that neutrophilic inflammation was associated with severe type 2 inflammatory CRSwNP.[27] TLR3 and TLR4 play important roles in virus- and bacteria-induced airway inflammation, and eCRS is known to be exacerbated by viral and bacterial infection. These results indicate that the elevated expression of TLR3 and TLR4 in basal cells may contribute to the type 2 and type 1/type 3 inflammation in eCRS, by stimulating the production of TSLP and IL-8.

The type 2 cytokines IL-4 and IL-13 are produced by Th2 cells, mast cells, and ILC2, and are important in the pathophysiology of eCRS, including epithelial barrier dysfunction, tissue remodeling, mucus production, and IgE responses.[28] The mRNA expression of IL-4 and IL-13 was elevated in NP of CRS patients,[29,30] and they impaired epithelial barrier function with decreased expression of tight junction proteins.[31–33] A recent study demonstrated that IL-4 and IL-13 prevented basal cell differentiation through an insulin receptor substrate (IRS)-dependent signaling pathway that is overexpressed in CRSwNP. IL-4 and IL-13 induced the basal cell remodeling that drives the accumulation of stem cells with proinflammatory capacity.[10] In the present study, IL-4 and IL-13 stimulated the cell proliferation of cBC, but did not affect that of cEC. Immunohistochemical study demonstrated that the frequency of basal cells and Ki67-positive proliferating basal cells was increased in NP of eCRS. It has been reported that Ki67-positive proliferating cells were increased in the epithelium from patients with CRSwNP or severe asthma[34–36]; however, this is the first study to examine the proliferating activity of basal cells. These results are coincident with previous studies showing that Ki67-positive proliferating cells were increased in the basal layer of the skin of atopic dermatitis.[37–39]

Our study indicates that type 2 cytokines IL-4 and IL-13 induce basal cell hyperplasia in NP of eCRS, and proliferated basal cells are a major source of TSLP, which exacerbates type 2 inflammation by inducing the production of IL-4 and IL-13, forming a vicious cycle that further induces basal cell hyperplasia. Recently, novel biologicals targeting biomarkers for type 2 inflammation, i.e., IL-4/IL-13, IL-5, IgE, and TSLP, have been developed and were clinically effective for patients suffering from refractory CRSwNP or uncontrolled asthma.[40–43] Treatment with an anti-IL-4Ra antibody (dupilumab) that inhibits both IL-4 and IL-13 signaling, or anti-TSLP (tezepelumab) may be effective for eCRS by inhibiting the vicious cycle caused by basal cell hyperplasia.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.alit.2024.04.001.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

KK contributed to the research, collection of clinical specimens, and writing the manuscript; HK contributed to the study design and manuscript writing; IT and KN cooperated in the collection of clinical specimens and study design; KM, TM, HA, YK, TN, and SS contributed to the study design; TS contributed to the study design and writing the manuscript. All authors read and approved the final manuscript.

References

1. Fokkens WJ, Lund VJ, Hopkins C, Hellings PW, Kern R, Reitsma S, et al. European Position Paper on Rhinosinusitis and Nasal Polyps 2020. *Rhinology* 2020;**58**:1–464.
2. Stevens WW, Schleimer RP, Kern RC. Chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol Pract* 2016;**4**:565–72.
3. Klingler AI, Stevens WW, Tan BK, Peters AT, Poposki JA, Grammer LC, et al. Mechanisms and biomarkers of inflammatory endotypes in chronic rhinosinusitis without nasal polyps. *J Allergy Clin Immunol* 2021;**147**:1306–17.

4. Zhang N, Van Zele T, Perez-Novo C, Van Bruaene N, Holtappels G, DeRuyck N, et al. Different types of T-effector cells orchestrate mucosal inflammation in chronic sinus disease. *J Allergy Clin Immunol* 2008;**122**:961–8.
5. Fujieda S, Imoto Y, Kato Y, Ninomiya T, Tokunaga T, Tsutsumiuchi T, et al. Eosinophilic chronic rhinosinusitis. *Allergol Int* 2019;**68**:403–12.
6. Tokunaga T, Sakashita M, Haruna T, Asaka D, Takeno S, Ikeda H, et al. Novel scoring system and algorithm for classifying chronic rhinosinusitis: the JESREC Study. *Allergy* 2015;**70**:995–1003.
7. Rock JR, Randell SH, Hogan BL. Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling. *Dis Model Mech* 2010;**3**: 545–56.
8. Yan Y, Gordon WM, Wang DY. Nasal epithelial repair and remodeling in physical injury, infection, and inflammatory diseases. *Curr Opin Otolaryngol Head Neck Surg* 2013;**21**:263–70.
9. Ordovas-Montanes J, Dwyer DF, Nyquist SK, Buchheit KM, Vukovic M, Deb C, et al. Allergic inflammatory memory in human respiratory epithelial progenitor cells. *Nature* 2018;**560**:649–54.
10. Wang X, Hallen NR, Lee M, Samuchiwal S, Ye Q, Buchheit KM, et al. Type 2 inflammation drives an airway basal stem cell program through insulin receptor substrate signaling. *J Allergy Clin Immunol* 2023;**151**:1536–49.
11. Zhou B, Headley MB, Aye T, Tocker J, Comeau MR, Ziegler SF. Reversal of thymic stromal lymphopoietin-induced airway inflammation through inhibition of Th2 responses. *J Immunol* 2008;**181**:6557–62.
12. Jessup HK, Brewer AW, Omori M, Rickel EA, Budelsky AL, Yoon BR, et al. Intradermal administration of thymic stromal lymphopoietin induces a T cell- and eosinophil-dependent systemic Th2 inflammatory response. *J Immunol* 2008;**181**:4311–9.
13. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 2002;**3**:673–80.
14. Kouzaki H, Matsumoto K, Kato T, Tojima I, Shimizu S, Shimizu T. Epithelial cell-derived cytokines contribute to the pathophysiology of eosinophilic chronic rhinosinusitis. *J Interferon Cytokine Res* 2016;**36**:169–79.
15. Rayner RE, Makena P, Prasad GL, Cormet-Boyaka E. Optimization of normal human bronchial epithelial (NHBE) cell 3D cultures for in vitro lung model studies. *Sci Rep* 2019;**9**:500.
16. Reddel HK, Bacharier LB, Bateman ED, Brightling CE, Brusselle GG, Buhl R, et al. Global Initiative for Asthma Strategy 2021: Executive summary and rationale for key changes. *J Allergy Clin Immunol Pract* 2022;**10**:S1–18.
17. Nakanishi K, Tsutsui H, Yoshimoto T. Importance of IL-18-induced super Th1 cells for the development of allergic inflammation. *Allergol Int* 2010;**59**: 137–41.
18. Kouzaki H, O'Grady SM, Lawrence CB, Kita H. Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. *J Immunol* 2009;**183**:1427–34.
19. Kouzaki H, Iijima K, Kobayashi T, O'Grady SM, Kita H. The danger signal, extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and innate Th2-type responses. *J Immunol* 2011;**186**:4375–87.
20. Kato A, Favoreto S, Avila PC, Schleimer RP. TLR3- and Th2 cytokine-dependent production of thymic stromal lymphopoietin in human airway epithelial cells. *J Immunol* 2007;**179**:1080–7.
21. Bartemes KR, Kita H. Dynamic role of epithelium-derived cytokines in asthma. *Clin Immunol* 2012;**143**:222–35.
22. Hirose K, Ito T, Nakajima H. Roles of IL-22 in allergic airway inflammation in mice and humans. *Int Immunol* 2018;**30**:413–8.
23. Baggiolini M, Clark-Lewis I. Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Lett* 1992;**307**:97–101.
24. Poltorak A, Smirnova I, He X, Liu MY, Van Huffel C, McNally O, et al. Genetic and physical mapping of the Lps locus: identification of the toll-4 receptor as a candidate gene in the critical region. *Blood Cells Mol Dis* 1998;**24**:340–55.
25. Rudack C, Steinhoff M, Mooren F, Buddenkotte J, Becker K, von Eiff C, et al. PAR-2 activation regulates IL-8 and GRO-alpha synthesis by NF-kappaB, but not RANTES, IL-6, eotaxin or TARC expression in nasal epithelium. *Clin Exp Allergy* 2007;**37**:1009–22.
26. Reeves SR, Barrow KA, White MP, Rich LM, Naushab M, Debley JS. Stability of gene expression by primary bronchial epithelial cells over increasing passage number. *BMC Pulm Med* 2018;**18**:91.
27. Delemarre T, Bochner BS, Simon HU, Bachert C. Rethinking neutrophils and eosinophils in chronic rhinosinusitis. *J Allergy Clin Immunol* 2021;**148**:327–35.
28. Kato A. Immunopathology of chronic rhinosinusitis. *Allergol Int* 2015;**64**:121–30.
29. Carsuzaa F, Béquignon É, Bainaud M, Jégou JF, Dufour X, Lecron JC, et al. Oncostatin M counteracts the fibrotic effects of TGF-β1 and IL-4 on nasal-polyp-derived fibroblasts: a control of fibrosis in chronic rhinosinusitis with nasal polyps? *Int J Mol Sci* 2022;**23**:6308.
30. Van Bruaene N, Pérez-Novo CA, Basinski TM, Van Zele T, Holtappels G, De Ruyck N, et al. T-cell regulation in chronic paranasal sinus disease. *J Allergy Clin Immunol* 2008;**121**:1435–41. 41.e1-3.
31. Huang ZQ, Liu J, Ong HH, Yuan T, Zhou XM, Wang J, et al. Interleukin-13 alters tight junction proteins expression thereby compromising barrier function and dampens rhinovirus induced immune responses in nasal epithelium. *Front Cell Dev Biol* 2020;**8**:572749.
32. Wise SK, Laury AM, Katz EH, Den Beste KA, Parkos CA, Nusrat A. Interleukin-4 and interleukin-13 compromise the sinonasal epithelial barrier and perturb intercellular junction protein expression. *Int Forum Allergy Rhinol* 2014;**4**:361–70.
33. Saatian B, Rezaee F, Desando S, Emo J, Chapman T, Knowlden S, et al. Interleukin-4 and interleukin-13 cause barrier dysfunction in human airway epithelial cells. *Tissue Barriers* 2013;**1**:e24333.
34. Chalastras T, Athanassiadou P, Patsouris E, Eleftheriadou A, Kandiloros D, Papaxoinis K, et al. Differential rates of proliferation and apoptosis in nasal polyps correspond to alterations in DNA spatial distribution and nuclear polarization as observed by confocal microscopy. *Eur Arch Otorhinolaryngol* 2010;**267**:1075–80.
35. Zhao L, Li YY, Li CW, Chao SS, Liu J, Nam HN, et al. Increase of poorly proliferated p63(+)/Ki67(+) basal cells forming multiple layers in the aberrant remodeled epithelium in nasal polyps. *Allergy* 2017;**72**:975–84.
36. Cohen L, E X, Tarsi J, Ramkumar T, Horiuchi TK, Cochran R, et al. Epithelial cell proliferation contributes to airway remodeling in severe asthma. *Am J Respir Crit Care Med* 2007;**176**:138–45.
37. Zhou L, Leonard A, Pavel AB, Malik K, Raja A, Glickman J, et al. Age-specific changes in the molecular phenotype of patients with moderate-to-severe atopic dermatitis. *J Allergy Clin Immunol* 2019;**144**:144–56.
38. Jensen JM, Fölster-Holst R, Baranowsky A, Schunck M, Winoto-Morbach S, Neumann C, et al. Impaired sphingomyelinase activity and epidermal differentiation in atopic dermatitis. *J Invest Dermatol* 2004;**122**:1423–31.
39. Noda S, Suárez-Fariñas M, Ungar B, Kim SJ, de Guzman Strong C, Xu H, et al. The Asian atopic dermatitis phenotype combines features of atopic dermatitis and psoriasis with increased TH17 polarization. *J Allergy Clin Immunol* 2015;**136**: 1254–64.
40. Bachert C, Han JK, Desrosiers M, Hellings PW, Amin N, Lee SE, et al. Efficacy and safety of dupilumab in patients with severe chronic rhinosinusitis with nasal polyps (LIBERTY NP SINUS-24 and LIBERTY NP SINUS-52): results from two multicentre, randomised, double-blind, placebo-controlled, parallel-group phase 3 trials. *Lancet* 2019;**394**:1638–50.
41. Bachert C, Sousa AR, Lund VJ, Scadding GK, Gevaert P, Nasser S, et al. Reduced need for surgery in severe nasal polyposis with mepolizumab: randomized trial. *J Allergy Clin Immunol* 2017;**140**:1024–31. e14.
42. Gevaert P, Omachi TA, Corren J, Mullol J, Han J, Lee SE, et al. Efficacy and safety of omalizumab in nasal polyposis: 2 randomized phase 3 trials. *J Allergy Clin Immunol* 2020;**146**:595–605.
43. Diver S, Khalfaoui L, Emson C, Wenzel SE, Menzies-Gow A, Wechsler ME, et al. Effect of tezepelumab on airway inflammatory cells, remodelling, and hyperresponsiveness in patients with moderate-to-severe uncontrolled asthma (CASCADE): a double-blind, randomised, placebo-controlled, phase 2 trial. *Lancet Respir Med* 2021;**9**:1299–312.