

ORIGINAL ARTICLE

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Role of IL-13R α 2 in modulating IL-13-induced MUC5AC and ciliary changes in healthy and CRSwNP mucosa

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Abstract

Background: The IL-13 receptor α 2 (IL-13R α 2) is a receptor for IL-13 which has conflicting roles in mediating IL-13 responses in the lower airway, with little known about its impact on upper airway diseases. We sought to investigate the expression of IL-13 receptors, IL-13R α 1 and IL-13R α 2, in chronically inflamed nasal epithelium, and explore IL-13-induced signaling pathways in an in vitro model of human nasal epithelial cells (hNECs).

Methods: The protein and mRNA expression levels of IL-13 and its receptors in nasal biopsies of patients with nasal polyps (NP) and healthy controls were evaluated. We investigated goblet cell stimulation with mucus hypersecretion induced by IL-13 (10 ng/mL, 72 hours) treatment in hNECs using a pseudostratified epithelium in air-liquid interface (ALI) culture.

Results: There were significant increases in IL-13, IL-13R α 1, and IL-13R α 2 mRNA and protein levels in NP epithelium with healthy controls as baseline. MUC5AC mRNA positively correlated with IL-13R α 2 ($r = .5886$, $P = .002$) but not with IL-13R α 1 in primary hNECs. IL-13 treatment resulted in a significant increase in mRNA and protein levels of IL-13R α 2 only in hNECs. IL-13 treatment induced an activation of extracellular signal-regulated kinases (ERK)1/2 and an upregulation of C-JUN, where the IL-13-induced effects on hNECs could be attenuated by ERK1/2 inhibitor (50 μ mol/L) or dexamethasone (10^{-4} - 10^{-7} mol/L) treatment.

Conclusions: IL-13R α 2 has a potential role in IL-13-induced MUC5AC and ciliary changes through ERK1/2 signal pathway in the nasal epithelium. IL-13R α 2 may contribute to airway inflammation and aberrant remodeling which are the main pathological features of CRSwNP.

KEYWORDS

ERK1/2, IL-13, IL-13R α 2, MUC5AC, nasal mucosal inflammation

1 | INTRODUCTION

A normal human nasal epithelium is a pseudostratified layer composed of ciliated and nonciliated columnar cells, goblet cells, and basal cells. It protects the upper airway by increasing mucus production, activating mucociliary clearance (MCC), secreting inflammatory mediators, and recruiting immune cells.¹ In response to injury, nasal epithelium can undergo rapid remodeling, such as goblet cell hyperplasia and metaplasia, epithelial denudation, loss or dysfunction of ciliated cell, and thickening of the epithelial basement membrane.^{2,3} Chronic rhinosinusitis with nasal polyps (CRSwNP) is a chronic mucosal inflammatory condition of the nose and paranasal sinuses which is associated with MCC dysfunction, nasal epithelium barrier alteration, and epithelial remodeling, and is driven by a typical type 2 inflammatory response.^{4,5}

IL-13 is a key T helper 2 cells (Th2) cytokine that acts as an important mediator in chronic airway inflammation and remodeling.^{6,7} IL-13 can induce goblet cell hyperplasia, inducible nitric oxide synthases production, and fibrosis to accelerate inflammation and promote remodeling.^{6,8} The effects of IL-13 are mediated through a complex receptor system consisting of two cognate receptors IL-13R α 1 and IL-13R α 2. It is widely believed that IL-13R α 1 paired with IL-4R α serves as the functional IL-13 receptor.^{9,10} In contrast, IL-13R α 2 does not perform any signaling function due to the absence of functional signaling motifs.¹¹ In the human airways, studies mainly performed in the lower airways have shown that IL-13R α 2 has a negative regulatory role as a decoy receptor to diminish IL-13 responses.¹²⁻¹⁴ However, an increasing number of studies have shown that IL-13R α 2 may have some signaling functions. There are recent evidences that found IL-13R α 2 to be highly expressed in various solid tumors to mediate cancer metastasis by inducing downstream pathways ERK/AP-1, PI3K/AKT, or ERK/PNR.¹⁵⁻¹⁷ In human airways, S. Fichtner-Feigl found that IL-13R α 2 is involved in IL-13 signaling through the transcription factor AP-1 to induce the activation of TGF- β in lung fibrosis.¹⁸ Recently, a study has also shown that IL-13R α 2 contributes to development of experimental allergic asthma.¹⁹ The exact mechanism by which IL-13R α 2 regulates IL-13 responses is still under investigation, and whether IL-13R α 2 contributes to biological activities of IL-13 in the upper airway remains unknown.

In this study, we investigated the expression levels of IL-13 and its receptors in nasal epithelium from healthy controls and chronically inflamed nasal polyps (NP) tissue. Additionally, a well-established model of human nasal epithelial cells (hNECs) that form a pseudostratified layer in air-liquid interface (ALI) culture was used to investigate the effect of IL-13 on goblet cell differentiation, mucus production, and ciliary beating frequency (CBF). Furthermore, we sought to determine whether these effects are mediated through IL-13R α 2 and its modulation by dexamethasone or specific ERK1/2 inhibitor PD98059. These findings provide opportunities to exploit the IL-13-mediated pathways to identify novel therapeutic interventions for common nasal diseases such as CRSwNP.

2 | MATERIALS AND METHODS

2.1 | Study population

CRSwNP patients and healthy controls were collected from the Department of Otolaryngology, National University Health System (Singapore), and the Department of Otolaryngology, Qilu Hospital (China). Patients' characteristics are listed in Table 1. NP biopsies were obtained from functional endoscopic sinus surgery in 49 patients with bilateral NPs, who are not on any corticosteroid medications. Healthy control tissues were taken from the inferior turbinate of 27 nasal septum deviation patients undergoing septal plastic surgery.

2.2 | hNECs culture and treatment

hNECs were derived from primary human nasal epithelial stem/progenitor cells (hNESPCs) isolated from fresh healthy control specimens. Methods for culturing hNECs are described in previous papers.^{20,21} hNECs cells were treated with IL-13 (10 ng/mL, R&D System, Minneapolis, MN, USA) for 72 hours. In pretreatment experiments, cells were pretreated with the testing compounds separately: ¹ cells were ex-exposed in the PD98059, a specific ERK1/2 inhibitor (50 μ mol/L, 9900L, Cell Signaling Technology Inc., Beverly, MA, USA) for 1 hour, DMSO (0.1%) as the vehicle control; ² cells were pretreated with anti-human IL-13R α 1

TABLE 1 Patients' characteristics and histopathologic patterns for nasal specimens

	Healthy controls	NP Patients	P value ^a
Sample size (Number)	27	49	NA
Age, years[median (1st-3rd quartile)]	30 (18-41)	49 (31.5-53)	.001
Gender [N (%)]			
Male	22 (81.5)	37 (75.5)	.384
Female	5 (18.5)	12 (24.5)	
Asthma ^b [N (%)]	0 (0)	8 (16.3)	.035
Atopy ^c [N (%)]	5 (18.5)	9 (18.4)	.505
Smoker [N (%)]	5 (18.5)	9 (18.4)	.597
Hyperplasia ^d [N (%)]	2 (7.4)	35 (71.4)	<.0001
Eosinophilia ^e [N (%)]	1 (3.7)	23 (46.9)	<.0001
Neutrophilia ^e [N (%)]	5 (18.5)	16 (32.6)	.033

NA, not applicable.

^aThe level of significance was evaluated using the Fisher's exact test. A P value of less than .05 was considered statistically significant.

^bPhysician-diagnosed asthma with the treatment of β 2-agonist (n = 3) or β 2-agonist plus GC inhaler (n = 2).

^cFive control subjects had allergic rhinitis but they did not have any topical or systemic treatment within 3 months before the study. All healthy control subjects were free of upper respiratory tract infections.

^dEpithelium with more than 4 layers was defined as "epithelial hyperplasia."

^eThe results of cell counting were shown as mean percentages, which are described in detail in the Methods section. Infiltration of eosinophils/neutrophils in nasal tissues was categorized as eosinophilia or neutrophilia when the percentage of eosinophils or neutrophils exceeded 10%, respectively.^{21,38}

(20 µg/mL, AF-152, R&D systems, Minneapolis, MN, USA); IL-13R α 2 (2 µg/mL, AF-146, R&D systems, Minneapolis, MN, USA)-neutralizing antibodies or IgG isotype control for 1 hour; and 3° cells were pretreated with dexamethasone (10^{-4} - 10^{-7} mol/L) for 30 minutes.

2.3 | Immunohistochemistry (IHC) and immunofluorescent (IF)

Nasal tissue sections, cytospin samples, and transwell membranes were used to perform IHC and IF staining. Details of antibodies and staining procedures are described in the Supporting Information.

2.4 | RNA extraction and quantitative real-time PCR

TaqMan real-time PCR analysis was performed on the ABI ViiA 7 Real-Time PCR System (Applied Biosystems) and calculated using the comparative $2^{-\Delta\Delta CT}$ methods with the housekeeping gene: GAPDH or PGK1 as a reference. More details are described in the Supporting Information.

2.5 | Ciliary beat frequency (CBF) measurement

CBF was auto-analyzed using the Sisson-Ammons Video Analysis system (SAVA, Omaha, NE). Full details are described in the Supporting Information.

2.6 | Western blot analysis

The preparation of the cell lysates, SDS-PAGE, and Western blot analysis was performed as standard protocols. Equal loading was demonstrated by detecting total ERK, p38, JNK, or GAPDH, and the final assessment was evaluated as ratio of target protein to total protein or housekeeping gene (See the Supporting Information).

2.7 | Statistical analysis

All data were analyzed using SPSS software (version 19.0; SPSS, Chicago, Ill). Differences in categorical variables (eg, eosinophilia/neutrophilia and epithelial hyperplasia) between groups were compared using the Fisher's exact test. The Mann-Whitney 2-tailed U test was used to compare the expression of IL-13, IL-13R α 1, and IL-13R α 2 between healthy controls and that from CRSwNP patients in RNA and paraffin-embedded tissues. Wilcoxon signed rank test was used to analyze the effect of IL-13 treatment in the ALI cultures of normal nasal epithelial cells. Median values with interquartile range (25th and 75th percentiles) are indicated by error bar in each graph. In all analyses, values are considered significant if $P < .05$.

3 | RESULTS

3.1 | Study subjects characteristics

Patients' characteristics and IHC findings of the study subjects are summarized in Table 1. Tissue eosinophilia (46.9%, $P < .0001$) and

neutrophilia (32.6%, $P = .033$) were all more common in CRSwNP patients compared with healthy controls. Epithelial hyperplasia was found in 71.4% (35/49) of the biopsies from CRSwNP patients but only in 7.4% (2/27) of the controls ($P < .0001$). Although a total of 76 subjects took part in the study, sample sizes and subject used varied for each experimental system. Studies on nasal tissue were performed on mRNA and protein from 76 subjects (27 of healthy control subjects and 49 of CRSwNP subjects for tissue extraction RT-PCR; 12 of healthy control subjects and 13 of CRSwNP subjects for primary hNECs RT-PCR; 18 of healthy controls and 25 of CRSwNP subjects for IHC staining). Cell culture studies were performed on cells obtained from 9 specimens, and the patients' characteristics are shown in Table S2.

3.2 | IL-13 and its receptors' expression in nasal tissues and its relation to the MUC5AC expression

In nasal specimens, we found that IL-13 ($P < .001$, 8.25-fold) and IL-13R α 2 ($P = .0402$, 1.4-fold) mRNA increased significantly in NPs tissue extraction as compared to healthy controls (Figure 1A,C). However, there was no significant difference between these two groups on IL-13R α 1 expression (Figure 1B) and IL-4R α expression (Figure S1A). Despite the known increase of MUC5AC protein in CRSwNP, MUC5AC mRNA expression was not increased in NPs tissue extraction (Figure 1D). As such, we further detect the mRNA expression of these markers in the isolated primary hNECs obtained from the tissue specimen from NPs and healthy controls. The results showed that IL-13 ($P = .0303$, 13.24-fold), IL-13R α 1 ($P = .0036$, 5.7-fold), IL-13R α 2 ($P = .001$, 19.6-fold), MUC5AC ($P = .0043$, 5.9-fold), and IL-4R α ($P = .001$, 6.5-fold) were significantly increased in primary hNECs of NPs as compared to healthy controls (Figure 1A-D and Figure S1A). IHC staining showed that IL-13, IL-13R α 1, and IL-13R α 2 protein expressions were greater in NPs compared to healthy controls (Figure 1E-H). Moreover, we found that MUC5AC mRNA positively correlated with IL-13R α 2 ($r = .5886$, $P = .002$, Figure 1J) and IL-4R α mRNA ($r = .5005$, $P = .01$, Figure S1B) but not with IL-13R α 1 ($r = .2497$, $P = .2288$, Figure 1I) in primary hNECs. In the nasal tissue subgroup analysis, we tested the differences in the mRNA level of IL-13, IL13Rs, and IL-4R in NP patients with different characteristics. We found that IL-13 (0.008351, 0.001573-0.018668, $P = .021$) and IL-13R α 2 (0.000185, 0.0006-0.001728, $P = .07$) were increased in asthmatic NPs; IL-13R α 2 (0.000629, 0.000245-0.002058, $P = .035$) was increased in NPs from smokers; and IL-4R α (0.074325, 0.068534-0.095302, $P = .043$) was increased in neutrophilic NP (Table S1).

3.3 | IL-13 stimulates mucus overproduction and impairment of ciliary function

To validate the effect of IL-13 on mucociliary function in the nasal epithelium, we established a model of goblet cell hyperplasia induced by IL-13 on hNECs from healthy controls. Fully differentiated hNECs were treated with recombinant human IL-13 (10 ng/mL) for 72 hours. From IF staining, we were able to see that the positive area of goblet cell marker MUC5AC was significantly increased

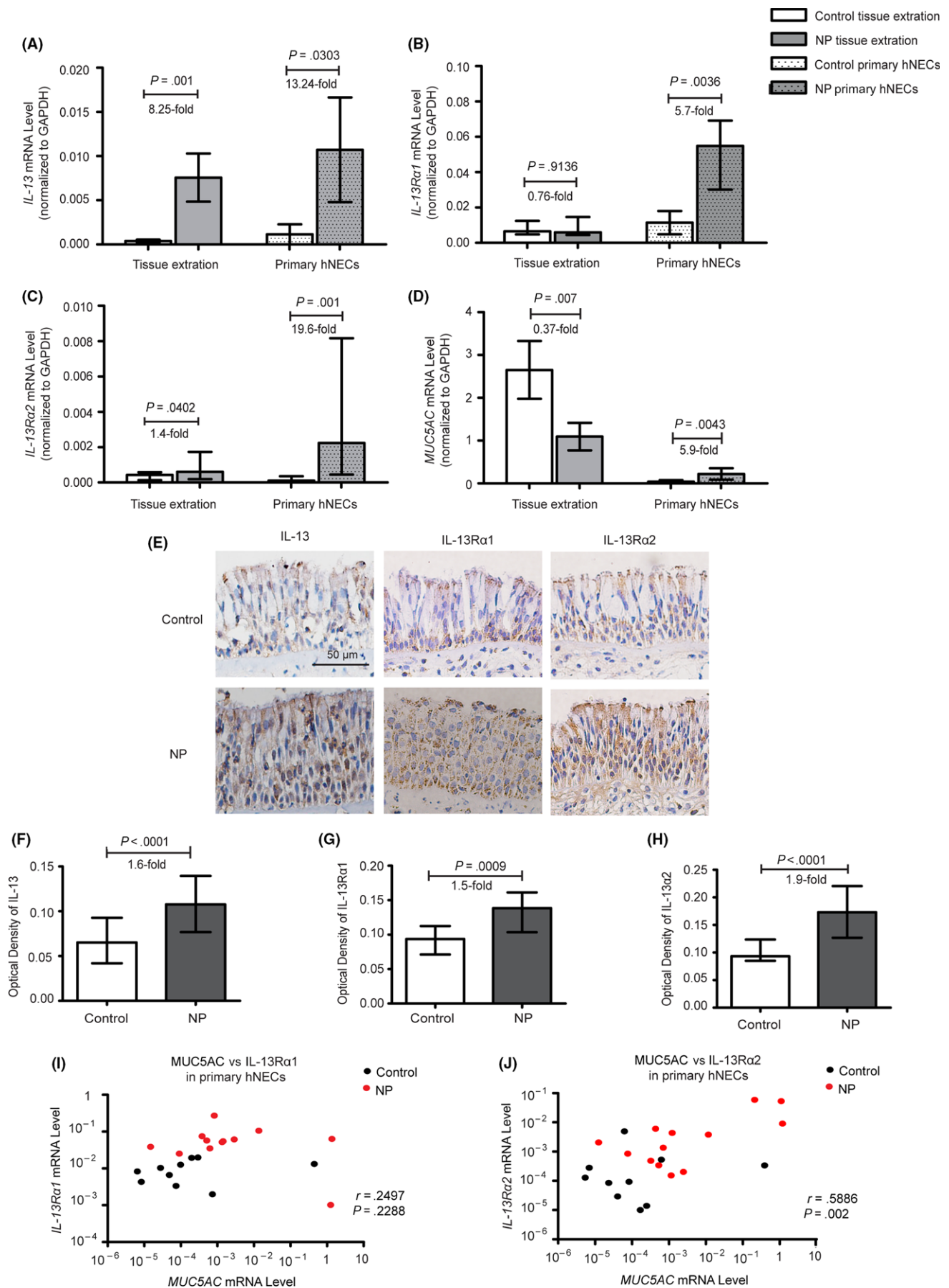


FIGURE 1 Expression levels of IL-13, IL-13R α 1, and IL-13R α 2 in nasal tissues. A-D, Quantitative real-time RT-PCR analysis of *IL-13*, *IL-13R α 1*, and *IL-13R α 2* mRNA expressions in tissues extractions and primary hNECs of CRSwNP patients ($n = 49$ and $n = 13$) with healthy control subjects as baseline ($n = 27$ and $n = 12$), using *GAPDH* as the internal control. Relative mRNA expression levels were calculated using $2^{-\Delta\Delta Ct}$ method; the fold change is the fold increases of from healthy control subjects. E-H, IHC staining of IL-13, IL-13R α 1 and IL-13R α 2 in CRSwNP patients and healthy controls ($\times 200$ amplification; scale bar $50 \mu\text{m}$); Semi-quantitative analysis of IL-13, IL-13R α 1, and IL-13R α 2 immunohistochemistry optical density in tissue sections of CRSwNP patients ($n = 25$) and healthy controls ($n = 18$) (three fields for each slide were measured). I-J, The correlations between mRNA level of IL-13R α 1 and IL-13R α 2 with *MUC5AC* (one-to-one) in primary hNECs were analyzed (x-axis scale and y-axis scale are logarithmic (base 10) transformed). Median values with 25th and 75th percentiles are indicated by error bar

($P = .0313$, 281-fold) with the treatment of IL-13 compared to untreated cells (Figure 2A-B). *MUC5AC* ($P = .0039$, 9.6-fold) mRNA expression was significantly increased after treatment with IL-13 (Figure 2C). The positive area of ciliated cell marker β IV-Tubulin and mRNA expression of the ciliogenesis marker *FOXJ1* had no significant changes (Figure S2). We then measured the CBF of the hNECs

to test the ciliary function in IL-13-treated and untreated conditions. A significant reduction in CBF ($P = .0313$, 0.6-fold) was observed in IL-13 treatment (Figure 2D).

We were not able to detect IL-13 (mRNA or protein) expression from both untreated and IL-13-treated hNECs, suggesting that hNECs were not a producer of IL-13 cytokine. IL-13 treatment could

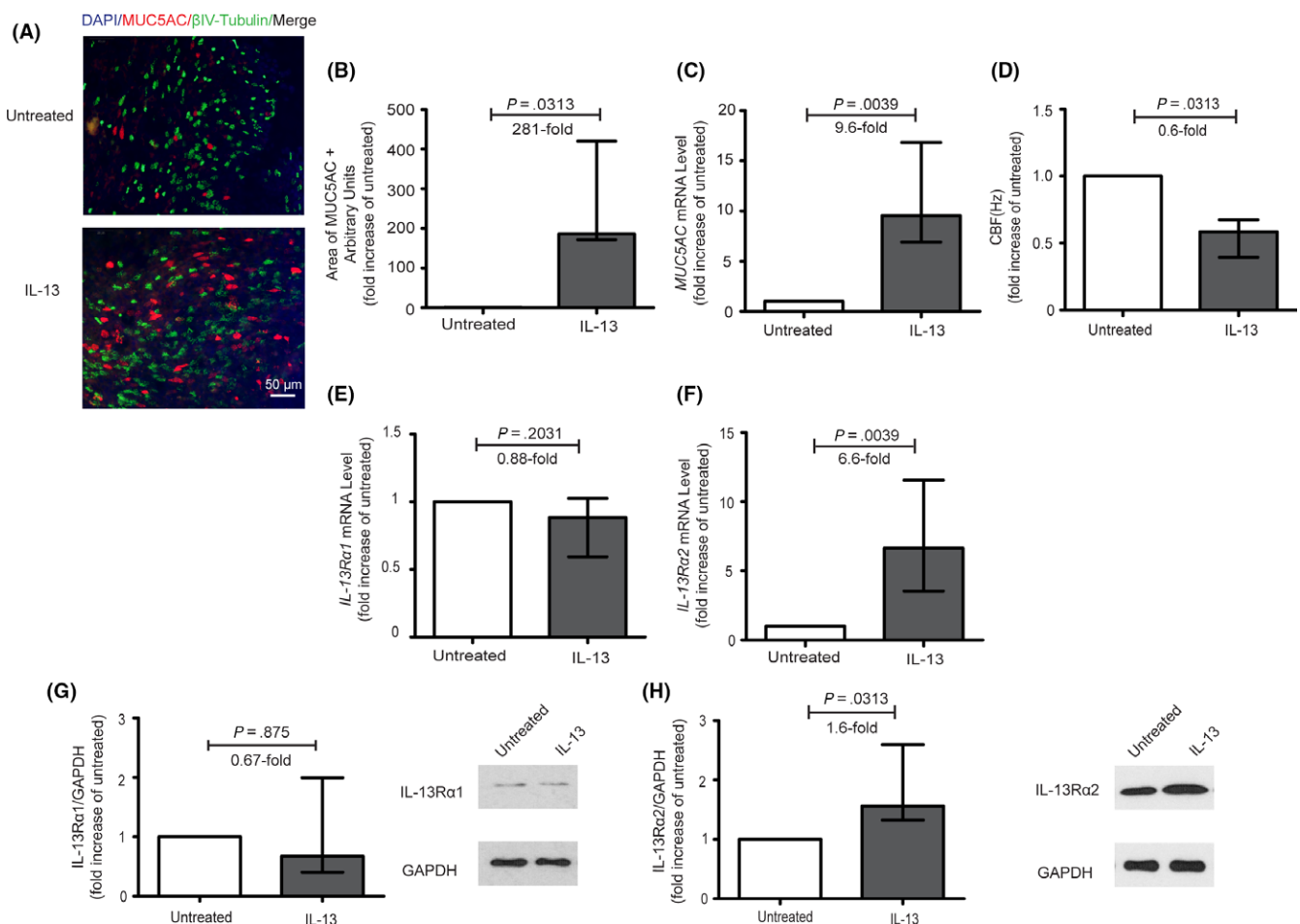


FIGURE 2 IL-13 stimulates mucus overproduction and IL-13R α 2 in vitro and impairs ciliary function on hNECs ALI culture. A, IF staining of MUC5AC (red) with β IV-Tubulin (green) on fully differentiated hNECs with or without the treatment of IL-13 (10 ng/mL, 72 h) in ALI cultures ($\times 200$ amplification; scale bar $50 \mu\text{m}$; cell nuclei are shown in blue, DAPI, 49-6-diamidino-2-phenylindole dihydrochloride). B, Semi-quantitative analysis of MUC5AC (in arbitrary unit) of IF staining (three fields per individual, $n = 6$, evaluated at $\times 200$ amplification by ImageJ). C, Quantitative real-time RT-PCR analysis of *MUC5AC* mRNA expressions on hNECs with or without the treatment of IL-13 (10 ng/mL, 72 h) in ALI cultures ($n = 9$), with *PGK1* as the internal control. D, CBF was measured on hNECs with or without the treatment of IL-13 (10 ng/mL, 72 h) in ALI cultures ($n = 6$, three measurements per individual). E-F, Quantitative real-time RT-PCR analysis of *IL-13R α 1* and *IL-13R α 2* mRNA expressions on hNECs with or without the treatment of IL-13 (10 ng/mL, 72 h) in ALI cultures ($n = 9$), with *PGK1* as the internal control. G-H, Western blot quantification of IL-13R α 1 ($n = 4$) and IL-13R α 2 ($n = 6$) protein levels on hNECs with or without the treatment of IL-13 (10 ng/mL, 72 h) in ALI cultures. The Western blot band intensities were measured with ImageJ. Protein levels were normalized to GAPDH. Representative blot images are shown. Median values with 25th and 75th percentiles are indicated by error bar

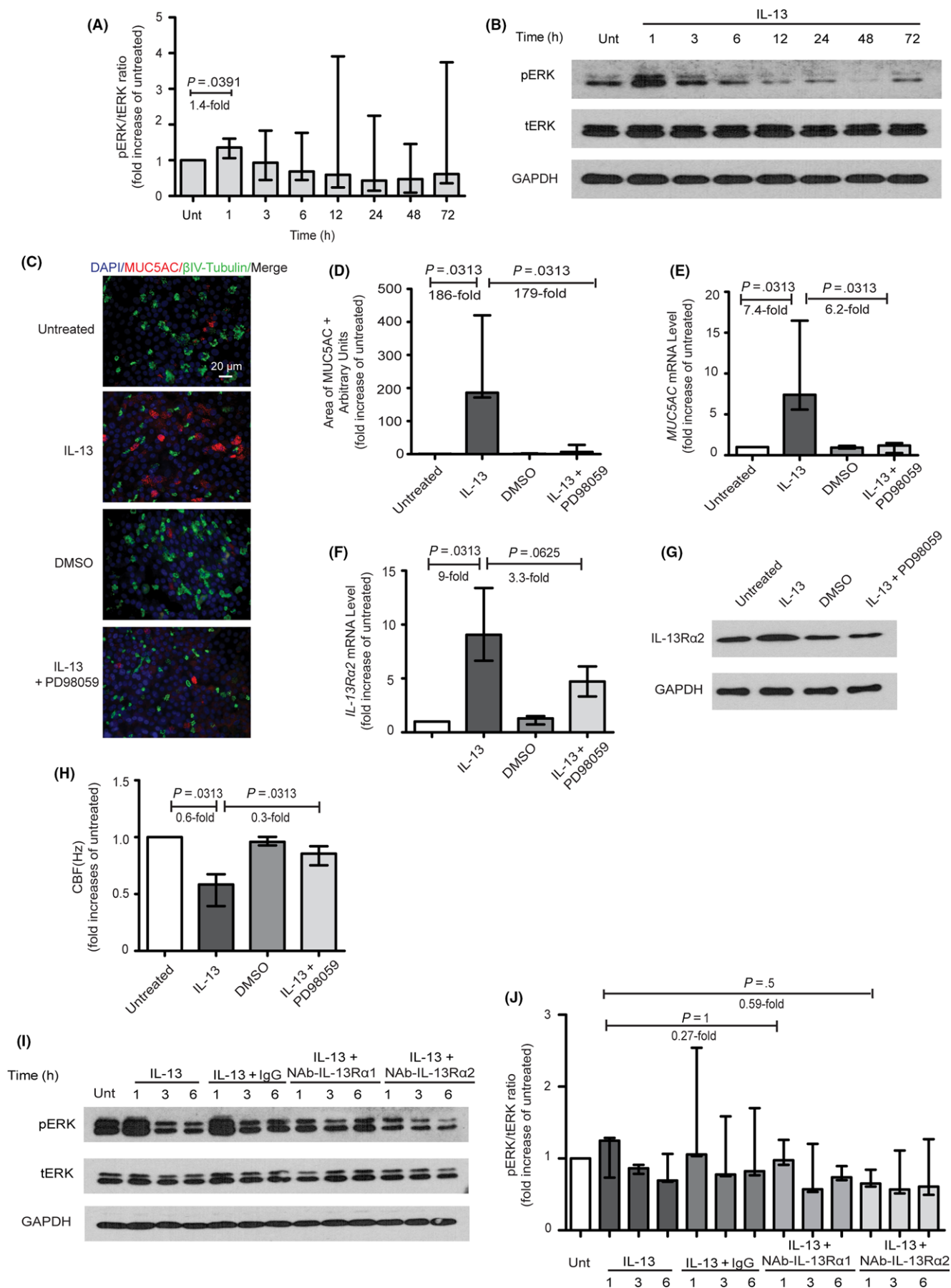


FIGURE 3 IL-13 induces ERK1/2 activation, and ERK1/2 inhibitor PD98059 attenuates IL-13-induced effect. A-B, Western blot analysis of ERK1/2 activation on hNECs with the treatment of IL-13. Cells were harvested at various times as indicated ($n = 8$). The Western blot band intensities were measured with ImageJ. Graph is presented as the ratio of phosphorylated-to-total ERK1/2. Representative blot images are shown. C, IF staining of MUC5AC (red) with β IV-Tubulin (green). hNECs in the presence of IL-13 (10 ng/mL) treated with PD98059 (50 μ mol/L) or vehicle DMSO (0.11%) for 72 h ($\times 400$ amplification; scale bar = 20 μ m; cell nuclei are shown in blue, DAPI, 49-6-diamidino-2-phenylindole dihydrochloride). D, Semi-quantitative analysis of MUC5AC-positive area in arbitrary unit (three fields per individual, $n = 6$, evaluated at $\times 200$ amplification by ImageJ). E-F, Quantitative real-time RT-PCR analysis of MUC5AC and IL-13R α 2 mRNA expressions on hNECs in the presence of IL-13 (10 ng/mL) treated with PD98059 (50 μ mol/L) or vehicle DMSO (0.11%) for 72 h ($n = 6$, PGK1 as the internal control). G, Western blot analysis of IL-13R α 2 protein levels on hNECs in the presence of IL-13 (10 ng/mL) treated with PD98059 (50 μ mol/L) or vehicle DMSO (0.11%) for 72 h. H, CBF were measured on hNECs in the presence of IL-13 (10 ng/mL) treated with PD98059 (50 μ mol/L) or vehicle DMSO (0.11%) for 72 h ($n = 6$, three measurements per individual). I-J, Western blot analysis of ERK1/2 activation on hNECs with the treatment of IL-13 (10 ng/mL) alone and in cultures pretreated with either goat IgG isotype control (20 μ g/mL), IL-13R α 1-neutralizing antibody (20 μ g/mL), or IL-13R α 2-neutralizing antibody (2 μ g/mL) one hour prior to IL-13 exposure. Median values with 25th and 75th percentiles are indicated by error bar

only induce the expression of IL-13R α 2 but not IL-13R α 1 of hNECs at both mRNA ($P = .0039$, 6.6-fold) (Figure 2E-F) and protein ($P = .0313$, 1.6-fold) levels (Figure 2G-H and Figure S3). IL-13 treatment had no effect on mRNA expression of IL-4R α (Figure S4).

3.4 | IL-13 induces ERK1/2 activation and its response to the ERK1/2 inhibitor PD98059 treatment

As mitogen-activated protein kinase (MAPK) plays an important role in the transcriptional and post-transcriptional regulations of inflammatory genes that mediates airway inflammation and remodeling,²²⁻²⁴ we further detect the activation of three main pathways of MAPK: ERK1/2, p38, and JNK. A Western blot analysis of hNECs showed that IL-13 treatment increased pERK1/2 expression at 1 hour after IL-13 treatment when compared to untreated cells (Figure 3A-B). No p38 and JNK pathway activations were observed (data not shown). To confirm the specificity of the signal, cells were pretreated with the ERK1/2-specific inhibitor, PD98059 (50 μ mol/L) or vehicle DMSO (0.11% dimethyl sulfoxide). The result showed that PD98059 inhibited the baseline ERK1/2 activation in the untreated group, as well as the ERK1/2 activation in the IL-13-treated groups (Figure S5A). This concentration of PD98059 did not affect cell viability (Figure S5B).

To study whether the activation of ERK1/2 is associated with the induction of IL-13 effect, PD98059 was added to ALI culture in the presence of IL-13 for 72 hours. Inhibition of the ERK1/2 pathway by PD98059 resulted in a decrease in MUC5AC ($P = .0313$, 179-fold)-positive cells (Figure 3C-D), which also significantly decreased MUC5AC ($P = .0313$, 6.2-fold) mRNA expression as compared to that induced by IL-13 (Figure 3E). Moreover, an inhibition of ERK1/2 by PD98059 resulted in a 3.3-fold decrease in IL-13R α 2 mRNA, where its protein expression was similarly reduced (Figure 3F-G) compared to IL-13 only treatment, with no changes observed in either IL-13R α 1 or IL-4R α (Figure S6). We also found that PD98059 could suppress an IL-13-induced reduction in the CBF (Figure 3H, $P = .0313$, 0.3-fold).

To assess whether this effect is specific to IL-13R α 2, cells were pretreated with IL-13R α 1- and IL-13R α 2-neutralizing antibodies prior to IL-13 treatment. The results showed that IL-13-induced phosphorylation of ERK1/2 level was to a large degree blocked by disruption of IL-13 binding to IL-13R α 2 and to a lesser extent, IL-13R α 1 (Figure 3I-J).

3.5 | Expressions of activator protein-1 (AP-1)-related genes

Studies on sinusitis and many other mucosal inflammatory conditions have shown that transcription factor activator protein-1 (AP-1), a downstream factor of the ERK1/2 pathway, was activated.^{25,26} Previously, we have suggested that the regulation of AP-1 gene network is involved in a mechanism wherein glucocorticoids decrease nasal polyp formation.²⁷ We thus measured the AP-1-related genes *C-JUN*, *C-FOS*, *EGR1*, *ARGE*, *COX-2*, and *IL-6* expressions in ALI cultures after IL-13 and PD98059 treatment. From the results in Figure 4A, we can observe that IL-13 could induce a 2.9-fold increase in *C-JUN* mRNA expression albeit not statistically significant compared to untreated hNECs. This upregulation of *C-JUN* expression could be reduced using PD98059 but has no effect on other genes (Figure S7). Double IF staining with IL-13R α 2 demonstrates that IL-13 could induce *C-JUN* activation, but deactivated using the ERK inhibitor PD98059 (Figure 4B).

3.6 | Effect of dexamethasone (DEX) on IL-13-induced effect

Glucocorticoids (GCs) have been widely used as the first-line drugs to control airway inflammation in CRS.^{28,29} We investigated the effect of dexamethasone (DEX) on IL-13-induced effects. Figure 5A demonstrates that DEX decreased MUC5AC-positive area when compared to the IL-13 treatment. It also suppressed MUC5AC mRNA expression with a mean 8.5-fold decrease in the highest concentration (10^{-4} mol/L) of DEX when compared to the IL-13 treatment (Figure 5B). This concentration did not affect the viability of the cells (Figure S8). The mRNA expression of IL-13R α 2 and *C-JUN* could be downregulated by DEX at a fold decrease of 2.8 and 1.5, respectively, when compared to the IL-13 treatment (Figure 5D-E). The DEX treatment, however, did not affect the IL-13R α 1 mRNA expression (Figure 5C). In addition, DEX was found to be able to suppress the CBF reduction in hNECs induced by IL-13 (Figure 5F), as well as decreased IL-13-induced phosphorylation of ERK1/2 level (Figure 5G-H) in the highest concentration (10^{-4} mol/L) of DEX.

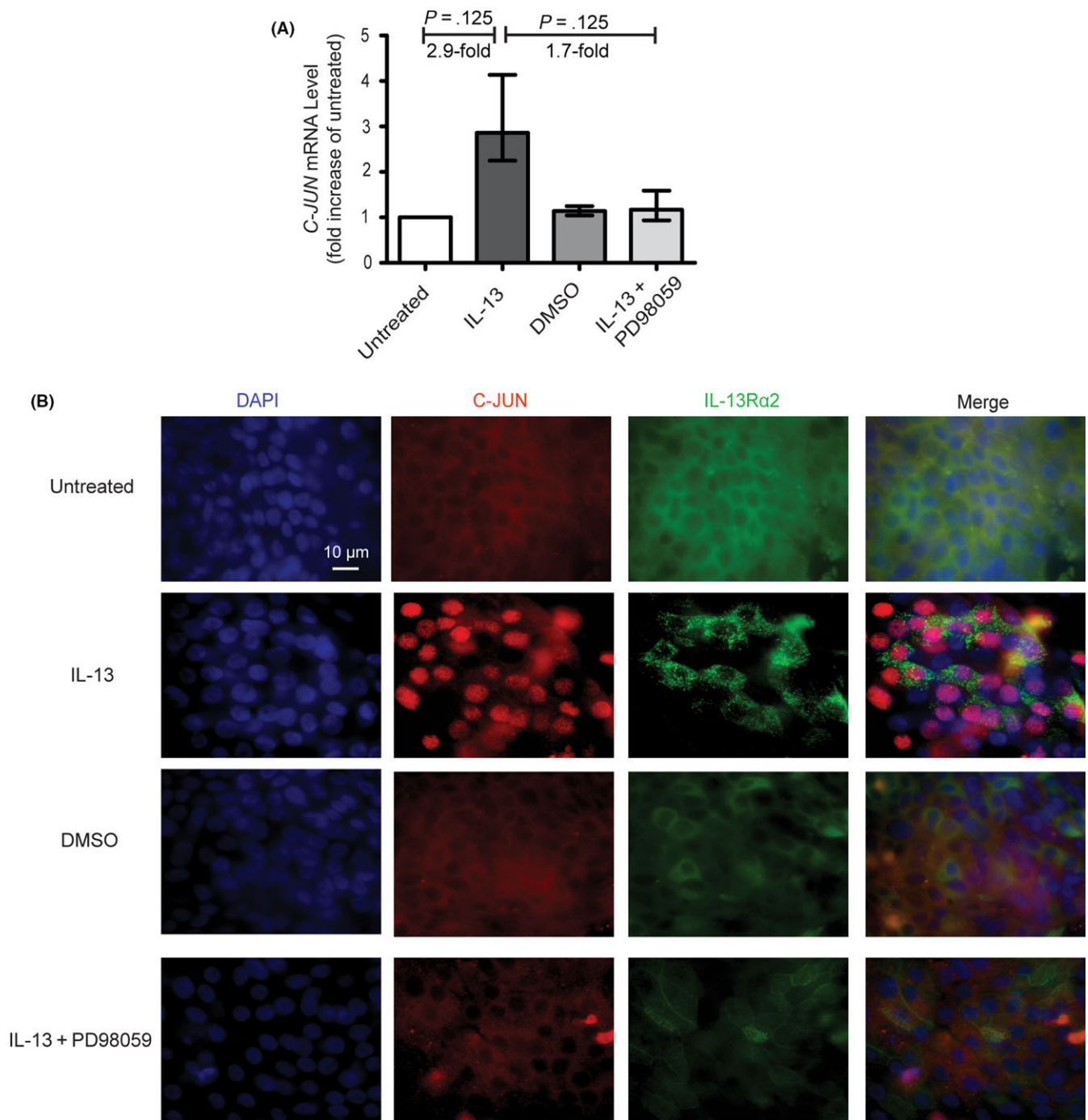


FIGURE 4 IL-13 induces activator protein-1 (AP-1)-related gene C-JUN activation which was deactivated after PD98059 treatment. A, Quantitative real-time RT-PCR analysis of *C-JUN* mRNA expressions on hNECs in the presence of IL-13 (10 ng/mL) treated with PD98059 (50 μ mol/L) or vehicle DMSO (0.11%) for 72 h ($n = 4$, *PGK1* as the internal control). B, IF staining of C-JUN (red) with IL-13R α 2 (green) on hNECs in the presence of IL-13 (10 ng/mL) treated with PD98059 (50 μ mol/L) or vehicle DMSO (0.11%) for 72 h ($\times 1000$ amplification; scale bar –10 μ m; cell nuclei are shown in blue, DAPI, 49-6-diamidino-2-phenylindole dihydrochloride). Median values with 25th and 75th percentiles are indicated by error bar

4 | DISCUSSION

In the present study, we have found that the increase in IL-13R α 2 with IL-13 treatment activates ERK1/2, which is associated with IL-13-induced MUC5AC upregulation and impaired ciliary function on normal nasal epithelial cells. IL-13-induced effects on hNECs could

be attenuated either by ERK1/2 inhibitor PD98059 or by dexamethasone treatment.

IL-13 is an important mediator in the development and progression of chronic airway inflammation and remodeling. The IL-13 signaling works through a complex cognate receptor system. It is generally recognized that IL-13R α 1 paired with IL-4R α is the

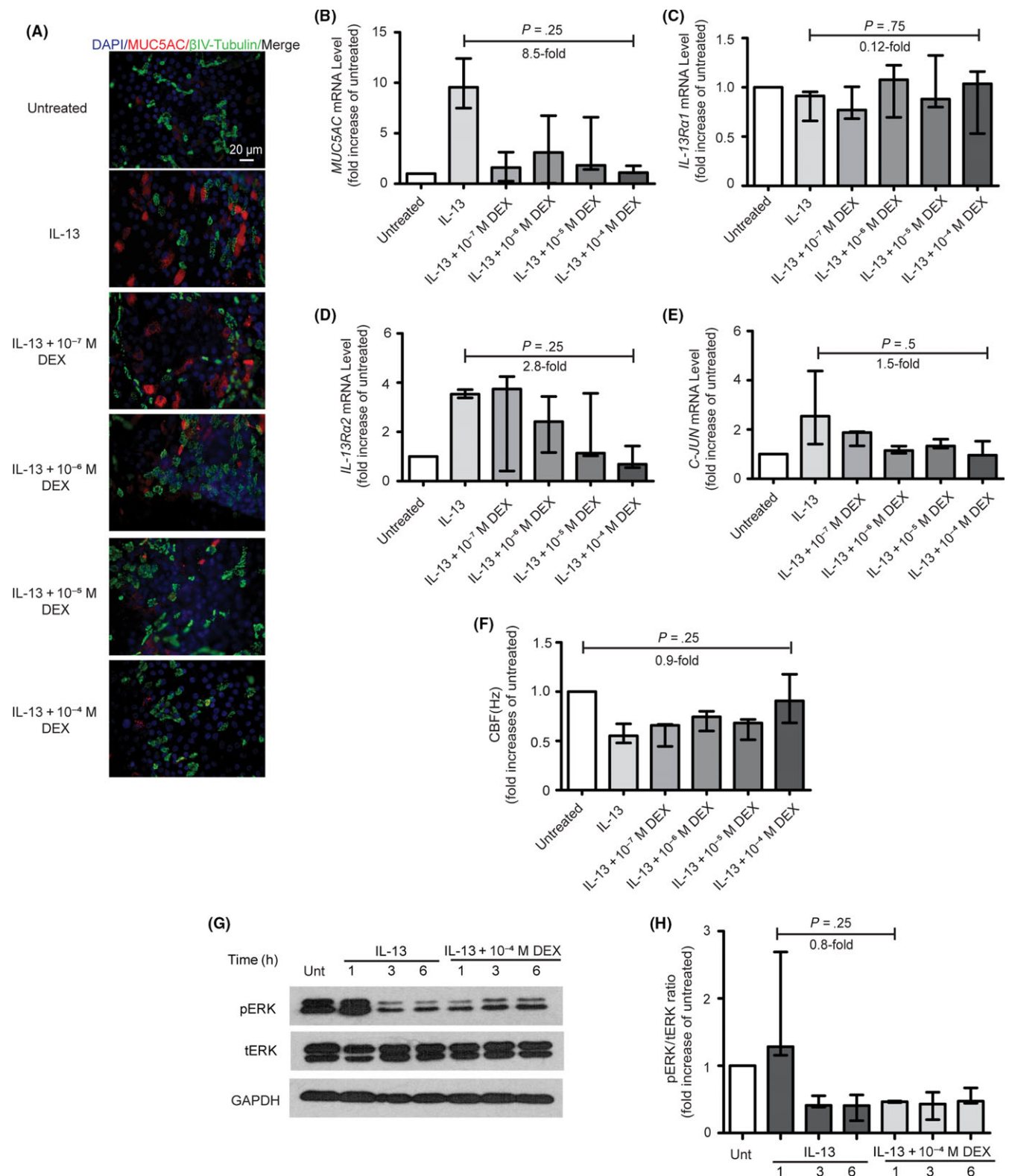


FIGURE 5 Dexamethasone (DEX) suppresses IL-13-induced effect. A, IF staining of MUC5AC (red) with β IV-Tubulin (green). hNECs in the presence of IL-13 (10 ng/mL) treated with DEX (10^{-4} - 10^{-7} mol/L) for 72 h ($\times 400$ amplification; scale bar = 20 μ m; cell nuclei are shown in blue, DAPI, 49-6-diamidino-2-phenylindole dihydrochloride). B-E, Quantitative real-time RT-PCR analysis of MUC5AC, IL-13R α 1, IL-13R α 2, and C-JUN mRNA expressions on hNECs in the presence of IL-13 (10 ng/mL) treated with DEX (10^{-4} - 10^{-7} mol/L) for 72 h ($n = 3$, PGK1 as the internal control). F, CBF were measured on hNECs in the presence of IL-13 (10 ng/mL) treated with DEX (10^{-4} - 10^{-7} mol/L) for 72 h ($n = 3$). G-H, Western blot analysis of ERK1/2 phosphorylation on hNECs in the presence of IL-13 treated with DEX (10^{-4} mol/L). Cells were harvested at various times as indicated. Median values with 25th and 75th percentiles are indicated by error bar

functional IL-13 receptor that activates the STAT6 signaling pathway to initiate downstream gene transcription.⁹ On the other hand, the roles of IL-13R α 2 are controversial in existing studies. IL-13R α 2 has long been considered a decoy receptor with no signaling activity due to its short cytoplasmic domain and the lack of JAK/STAT binding sequences.^{11,30} Studies have shown that as a decoy receptor, IL-13R α 2 may negatively modulate IL-13 signaling to inhibit IL-13 effect.^{12–14,31} However, emerging evidences suggest that IL-13R α 2 might actually be a functional receptor. It has been reported that IL-13R α 2 is involved in IL-13 signaling through transcription factor, AP-1 to induce the activation of TGF- β in lung fibrosis which is STAT-6 independent.¹⁸ Recently, it was observed that airway hyperresponsiveness (AHR) and inflammation were attenuated in IL-13R α 2-deficient mice compared to wild-type mice after a house dust mite challenge. After adding exogenous IL-13R α 2, it reconstituted AHR and inflammation to levels similar to those observed in the house dust mite challenged wild-type mice.¹⁹ Similarly, our results strongly suggest that IL-13R α 2 is involved in IL-13 signaling and contributes to mucus overproduction possibly associated with MUC5AC gene upregulation in the upper airway.

Although fibroblasts and airway epithelial cells are demonstrated to be able to produce IL-13R α 2, no previous study has quantified the IL-13R α 2 expression level in NPs compared with normal tissues. Our results showed that both mRNA and protein expression levels of IL-13, IL-13R α 1, and IL-13R α 2 increased in NPs epithelium compared with healthy control MUC5AC mRNA positively correlated with IL-13R α 2 and IL-4R α mRNA but not with IL-13R α 1 in primary hNECs. In the nasal tissue subgroup analysis, we only found that IL-13 and IL-13R α 2 were increased in asthmatic NP; while IL-13R α 2 was increased in NP of smokers. This suggested that a potential relationship between IL-13 α 2 induced airway inflammation and epithelial remodeling in asthmatics and smokers.

To better understand the roles of IL-13 and its receptors in the upper airway, we used primary hNECs derived from hNESPcCs of biopsy samples, which forms a pseudostratified layer in ALI cultures, as there is currently no human upper airway epithelial cell line with differentiation capacity available. The system comprises of homogeneous nasal epithelial cells with no effect from contaminating immune counterparts such as lymphocytes, neutrophils, and eosinophils that allow us to investigate the effect of IL-13 on pure hNECs. Although existing literatures have reported that airway epithelial cells are capable of producing IL-13,³² it was not found in our study, where both IL-13 mRNA and protein expression remained undetectable in untreated or IL-13-treated hNECs. This result suggests that IL-13 likely induces alterations in nasal epithelium from a paracrine source (inflammatory cells or Th2 lymphocytes) but not autocrine source (epithelial cells). We have confirmed that IL-13 stimulation resulted in upregulation of MUC5AC gene and protein expression and induced a reduction in CBF. The cell culture model differentiates into a multilayered nasal epithelial that closely resembles the arrangement in the nasal epithelia. The model captured the effects of IL-13 secreted onto the nasal epithelium cells obtained from human donors. Additionally, the air-liquid interface of the culture

system closely modeled the condition in the nasal epithelium, and therefore, the epithelial responses we observed in this model are highly comparable to the in vivo epithelium of the nasal mucosa.

The role of mitogen-activated protein kinase (MAPK) in mediating post-transcriptional regulation of the inflammatory genes, T cell differentiation, and apoptosis has been suggested previously.^{24,33} Additionally, ERK1/2, P38, and JNK, three subfamilies of MAPKs, have suggested roles in the pathogenesis of asthma with potential as novel targets for the treatment of asthma.^{23,34} In our study, we found that IL-13 activated ERK1/2 at 1 hour after IL-13 treatment but no p38 and JNK activation were observed during the 72-hour treatment. IL-13 is also found to be able to induce the transcription factors activator protein-1 (AP-1)-related gene *C-JUN* upregulation. As AP-1 is found to be activated in nasal polyposis and many other mucosal inflammatory conditions, the expression of IL-13R α 2 in response to IL-13 treatment may induce changes leading to nasal polypogenesis via AP-1 activation.²⁵ These observations are also consistent with findings from Lee et al., who linked ERK1/2 to rhinosinusitis through cellular functions such as inflammatory cytokine secretion, cell proliferation and differentiation, and mucus production.²² We validated this observation in our study by treating the epithelial cells with PD98059, a specific inhibitor of ERK1/2. Strikingly, this treatment resulted in an inhibition of IL-13-induced effects such as MUC5AC overproduction, CBF reduction, and C-JUN upregulation. Notably, the ERK1/2 inhibitor PD98059 could also suppress IL-13-induced IL-13R α 2 up-expression but did not affect IL-13R α 1 expression.

To further study whether this effect is specific to IL-13R α 2, we used IL-13R α 1- and IL-13R α 2-neutralizing antibodies to block the two receptors of IL-13. We found that IL-13-induced phosphorylation of ERK1/2 level was strongly reduced with the disruption of IL-13 binding to IL-13R α 2. In comparison, a weaker inhibition of ERK1/2 activation using anti-IL-13R α 1 was observed, suggesting possible ERK1/2 activation via IL-13R α 1 or may be attributed to the cross-reactivity of the neutralizing antibody to both receptors. Additionally, whether this lies in the cross-regulation through IL-13R α 1/STAT6 needs to be further determined. Nevertheless, these data suggested that IL-13-induced IL-13R α 2 expression may involve both transcriptional and/or post-transcriptional regulation via ERK1/2 signaling pathways. If ERK1/2 has direct effect on IL-13R α 2 gene transcription, it needs to be further characterized (Figure 6)

Glucocorticoids (GCs) have a broad anti-inflammatory effect in controlling airway inflammation and are commonly used in CRS patients.²⁹ In our study, we found that DEX suppressed IL-13-induced inflammation on hNECs by decreasing MUC5AC gene and protein production as well as restoring CBF. Furthermore, we found that DEX suppressed IL-13R α 2 induction but does not affect IL-13R α 1 expression. In addition, DEX was also found to affect the IL-13-induced phosphorylation of ERK and activation of C-JUN. These results indicate that IL-13R α 2/ERK is involved in DEX repression of MUC5AC, and ERK1/2 inhibitor has similar efficacy in anti-inflammatory activity to DEX. However, the anti-inflammatory actions of GCs are through binding to the glucocorticoid receptor (GR) to modulate gene expression.^{29,35} It is therefore crucial to investigate whether

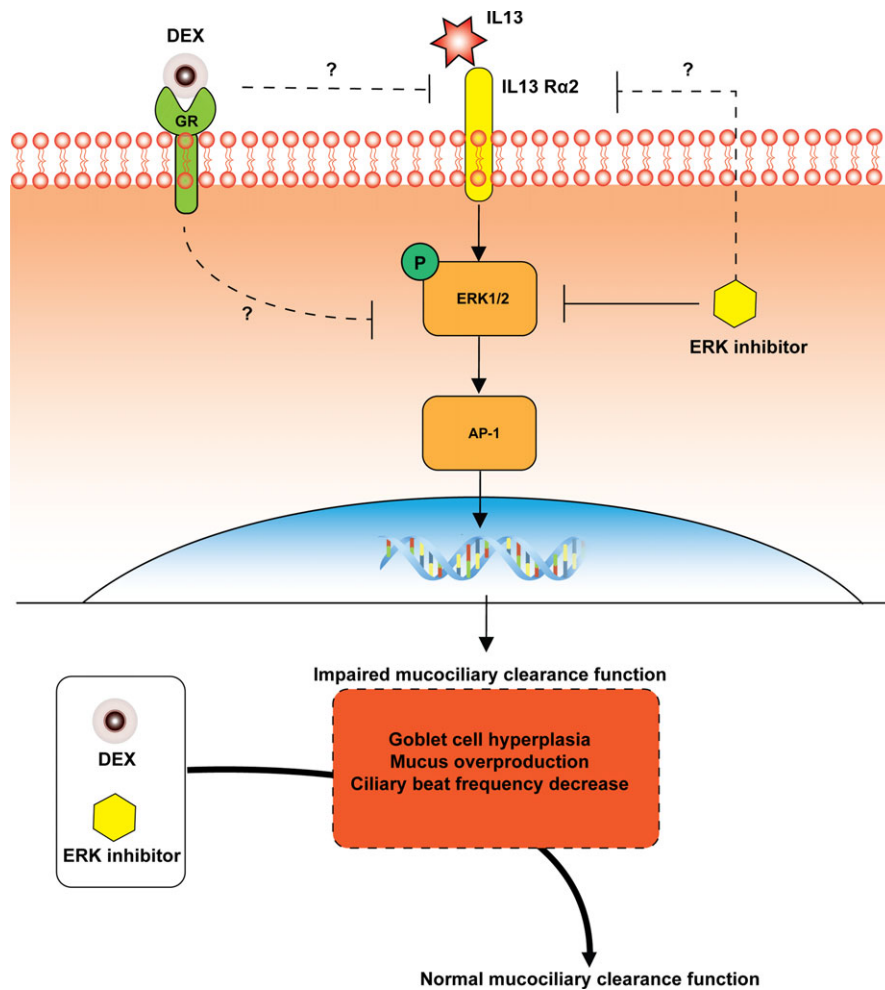


FIGURE 6 The proposed mechanism of IL-13 α 2-mediated in IL-13-induced mucus overproduction and ciliary dysfunction in CRSwNP patients. The increase in IL-13 α 2 with IL-13 treatment activates mitogen-activated protein ERK1/2 pathway and downstream AP-1-related gene *C-JUN*, which is associated with IL-13-induced goblet cell hyperplasia, mucus overproduction, and impaired ciliary function on normal nasal epithelial cells. IL-13-induced effects on hNECs could be attenuated either by ERK1/2 inhibitor or DEX treatment

GCs directly bind to GC-GR complex or indirectly inhibit the activities of other transcription factors such as AP-1 in future studies (Figure 6). Although GCs are the most effective drugs for the treatment of inflammatory upper airway diseases, the chance of failure in treating CRSwNP is high³⁶ due to cellular resistance to GCs and systemic side effects in long-term usage.²⁹ In view of this, novel therapeutic alternatives are urgently needed. Recently, some genomewide methylation studies identified that IL-13-mediated pathways such as ERK1/2, IFN- γ , and NF- κ B-induced methylation changes contribute to asthma pathogenesis.^{37,38}

IL-13 is an attractive target for intervention in allergic airway inflammation diseases. Our findings provide opportunities to exploit the IL-13-mediated pathways to identify novel therapeutic interventions that can provide functionally specific (eg, modulating mucus production and cilia function) alternatives to steroid treatment. These strategies could potentially target the genes and their products in these inflammatory pathways to control the development of the airway inflammation and remodeling, which may produce beneficial clinical effects, especially if applied topically to avoid systemic

side effects. The effect of IL-13 α 2 in airway inflammation is still not clear, and delineation of the roles of IL-13 α 2 will help to further design more effective IL-13-targeted therapies.

There are limitations in this study. One limitation is that cell culture studies were performed from relatively low number of specimens. However, given the primary ALI cell culture is time-consuming and with limited patient samples in obtaining healthy inferior turbinate, such limitation is inevitable in this study. Additionally, obtaining tissues from healthy inferior turbinate removes most confounding factors from other underlying diseases and establishes a stringent baseline for the in vitro experiments, thus strengthening the finding of the study despite the low number of specimens. Another limitation is that this is an in vitro system that does not encapsulate systemic changes or chronic effects of IL-13 in vivo. However, the simplicity of this in vitro system that closely mimics the nasal epithelium allows for clearer elucidation of the IL-13 pathway at the nasal epithelial level. Based on current results, we hypothesize that increased IL-13 α 2 has a potential role in elevating IL-13-induced mucus overproduction and ciliary dysfunction in the upper airway

epithelial cells, contributing to polypogenesis. To confirm and fully investigate IL-13R α 2's mechanism that contributes to polypogenesis, future experiments using IL-13R α 2 knockout or overexpression cell or animal models can be employed to observe their responses to IL-13 leading to subsequent polypogenesis and its associated features. Additionally, the knockout of IL-13R α 2 using antibodies or locked nucleic acid (LNA) RNAi strategies could be further applied to a recently established mouse nasal polyp model³⁹ to observe IL-13R α 2's role in accelerating polyp formation.

In conclusion, our data demonstrated that IL-13R α 2 has a potential role in IL-13-induced mucus overproduction and ciliary dysfunction through the activation of ERK1/2 signal pathway in the nasal epithelium. IL-13R α 2 may contribute to airway inflammation and aberrant remodeling which are the main pathological features of CRSwNP. More importantly, IL-13 receptors and ERK1/2 inhibitors are attractive targets for therapeutic intervention in inflammatory disease in the upper airway.

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CONFLICTS OF INTEREST

The authors declared that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

DYW conceived the study. JL performed most of the experiments and wrote the manuscript. YYL performed some of the experimental work and most of the data analysis. DAKA and KST contributed protocol advice and editing the manuscript. YY and KST reviewed histology and CBF. HHO and HBL contributed protocol advice and performed some the experiments. KTT, YKO, and LS contributed to subject recruitment and obtaining informed consent, as well as the collection and analysis of clinical samples and data. YLZ and FGY helped with editing the manuscript. All authors approved the manuscript.

ETHICAL APPROVAL

National Healthcare Group Domain-Specific Review Board of Singapore (Singapore) and from the Institutional Review Boards of Qilu Hospital, Shandong University (China) approved to conduct this study.

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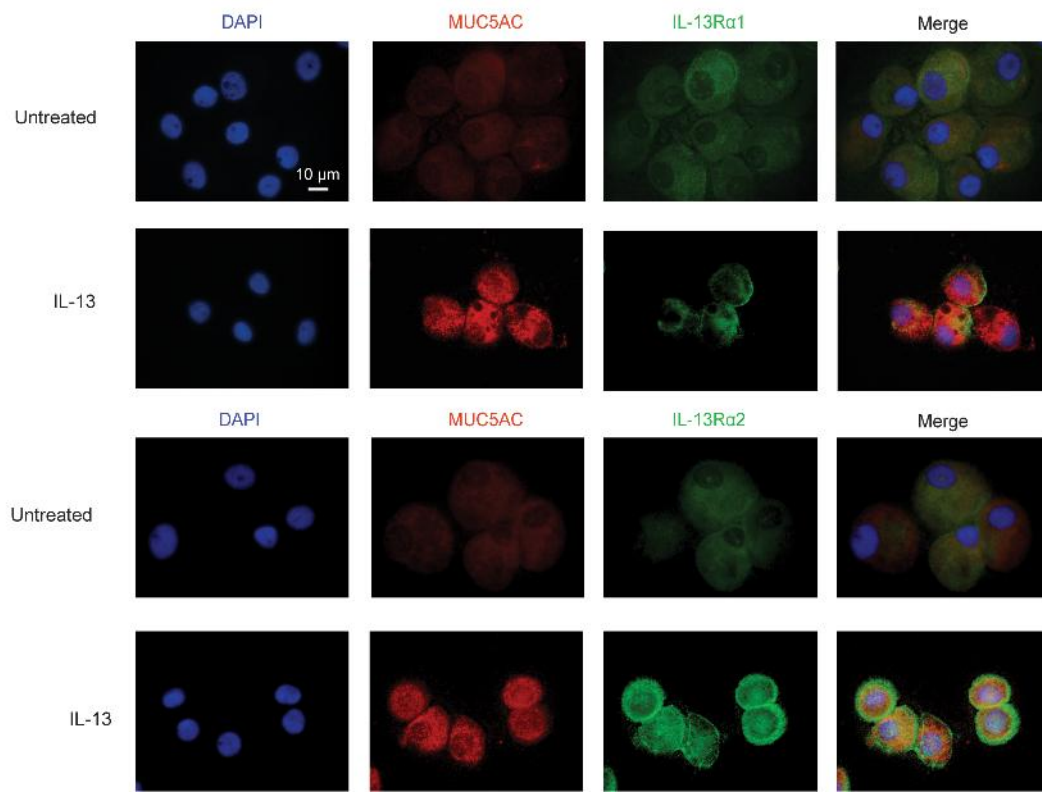
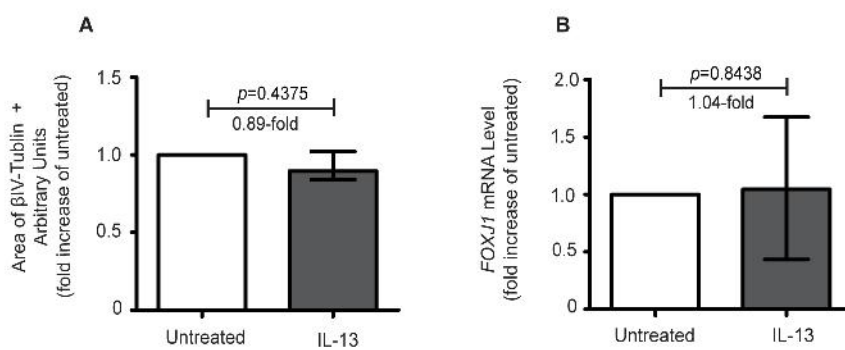
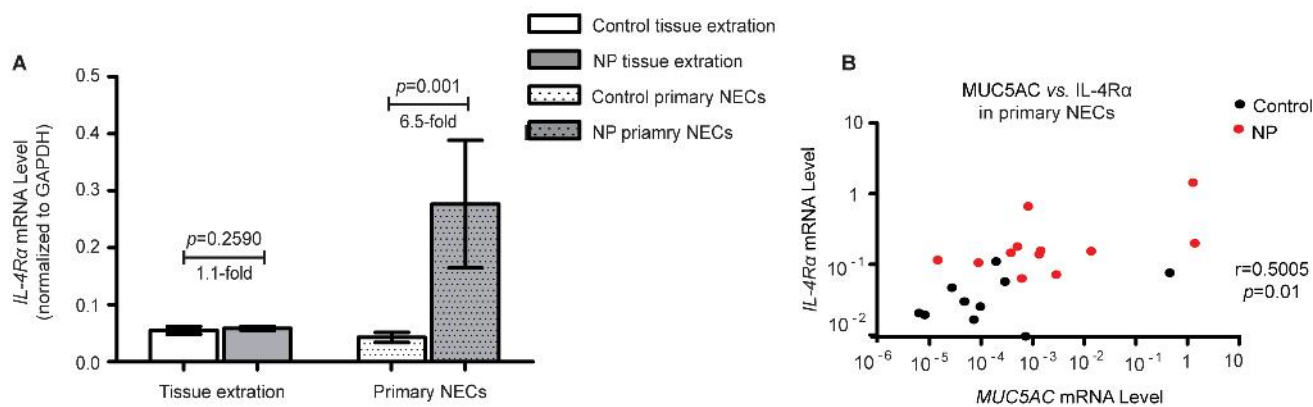
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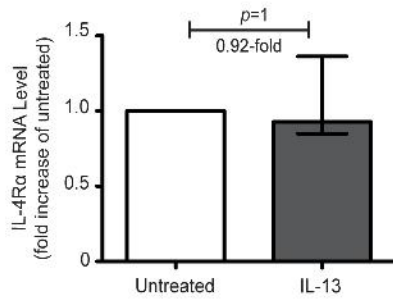
SUPPORTING INFORMATION

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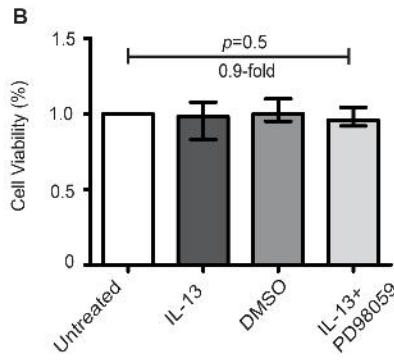
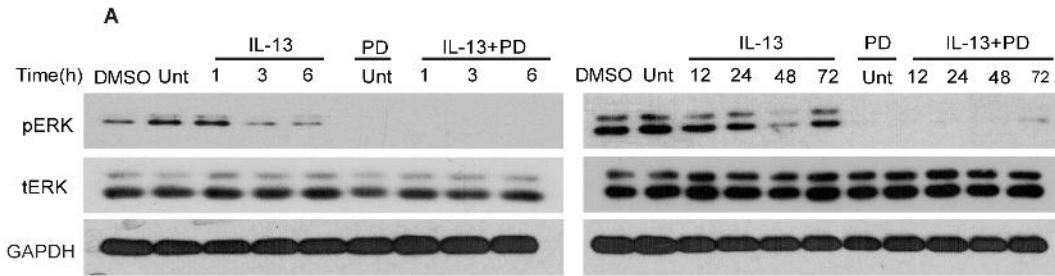
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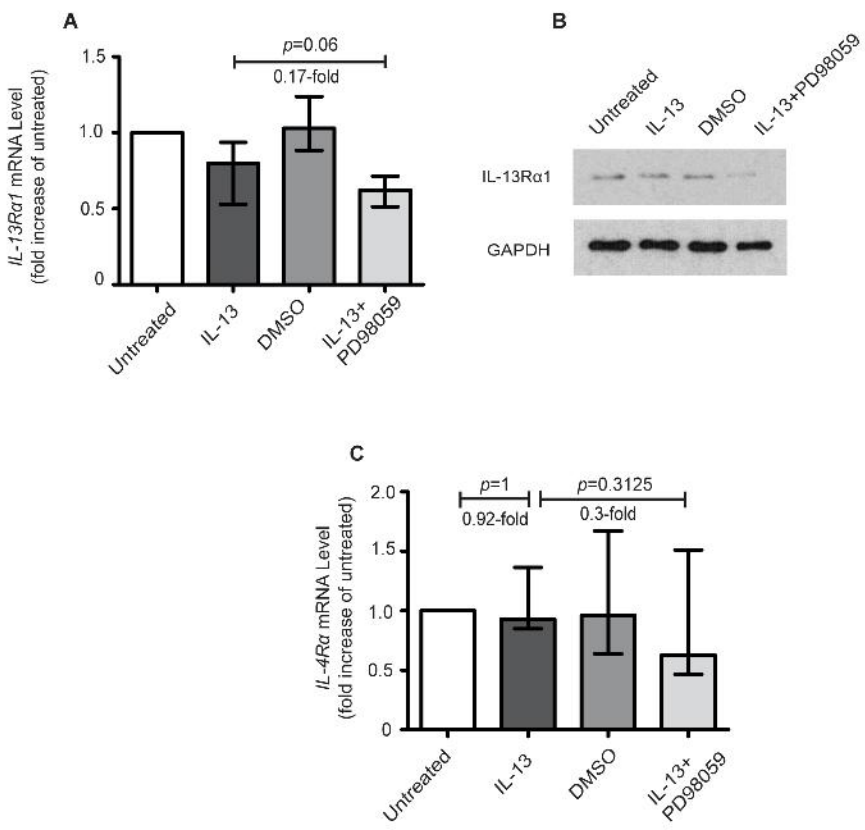
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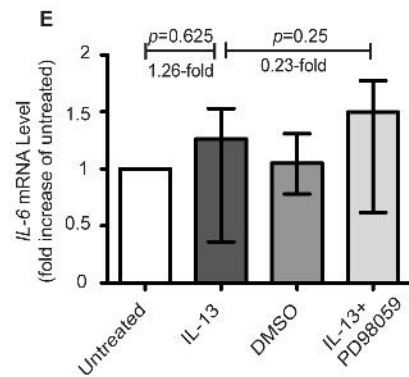
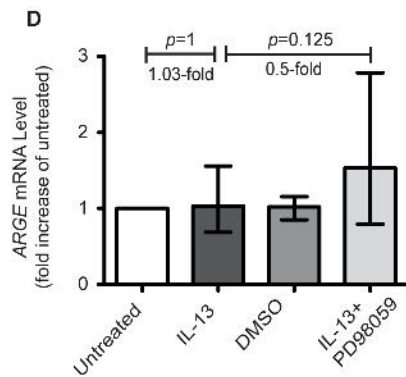
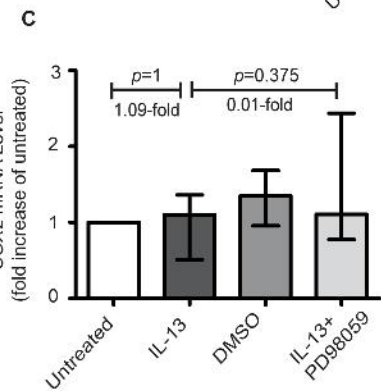
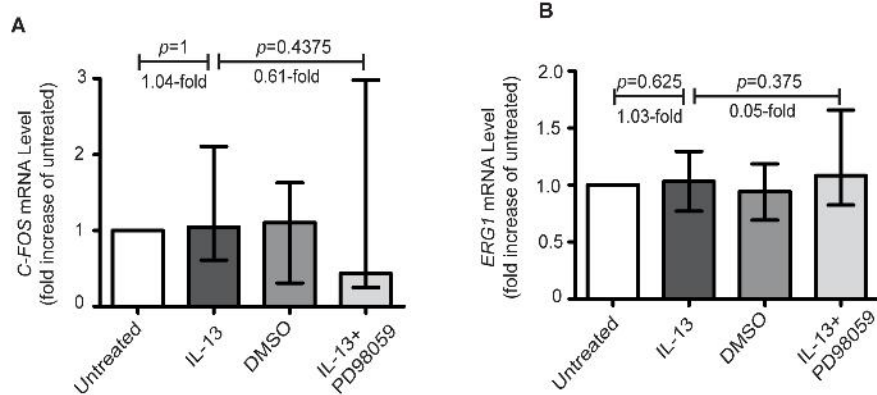
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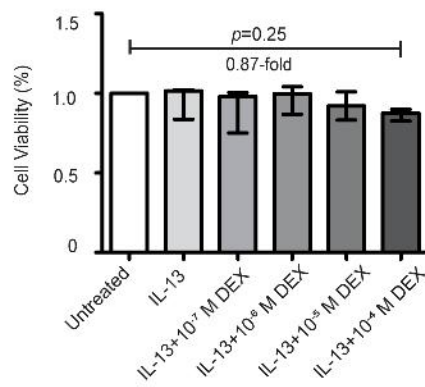
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Supporting information

Role of IL-13R α 2 in modulating IL-13 induced MUC5AC and ciliary changes in healthy and CRSwNP mucosa

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Supplemental methods

IHC and IF staining

Nasal tissue sections, cytospin samples and transwell membranes were used to perform IHC and IF staining.

Antibodies Mouse monoclonal against β IV-Tubulin and IL-13R α 2 (Abcam, Cambridge, MA), rabbit monoclonal against C-JUN and IL-13R α 1 (Abcam, Cambridge, MA), rabbit monoclonal against MUC5AC (Santa Cruz, CA, USA) were used at dilutions of 1:800, 1:500, 1:400, 1:200, 1:400 respectively.

IHC staining First, nasal biopsy tissues were fixed in formalin, embedded in paraffin and sectioned at 4 μ m with Leica microtome (Leica, Wetzlar, Germany). Then, the paraffin sections were deparaffinized, hydrated through graded alcohols. The sections were heated in a microwave oven for 10 minutes at 100 °C with pH6 retrieval buffer (Dako A/S) for antigen retrieval. Endogenous peroxidase activity was blocked with 3% H₂O₂ for 5 minutes at room temperature. Non-specific immune reactivity was blocked with 10% goat serum for 30 minutes at room temperature. Slides were stained with anti- IL-13R α 1 and anti-IL-13R α 2 primary antibody and incubated at 4 °C overnight. The slides were then incubated with DAKO EnVision+System-HRP (Dako A/S) for 30 minutes at room temperature. Diaminobenzidine was used as substrate for color development. All slides were counterstained with hematoxylin (Sigma Aldrich), dehydrated with serial concentration of ethanol, hydrated with xylene and mounted with mounting medium (Dako A/S).

IF staining Double staining was performed in IF staining by using two different host species antibodies. The combinations of the primary antibodies include: IL-13R α 1 with

MUC5AC, IL-13R α 2 with MUC5AC, β IV-Tubulin with MUC5AC and IL-13R α 2 with C-JUN.

- (1) Paraffin tissue sections were deparaffinization, rehydration and processed with pH6 retrieval buffer (Dako A/S) before blocking with 10% goat serum.
- (2) hNECs from ALI cultures with or without cytokine/test compounds treatments were spun onto microscopic slides at 500 rpm for 5 minutes using a cytospin. Cell smears were fixed in cold acetone for 10 minutes at -20°C and were permeabilized with 0.1% TritonX-100 for 10 minutes at room temperature before blocking with 10% goat serum.
- (3) Transwell membranes from ALI cultures with or without cytokine/test compounds treatments were fixed by formalin for 15 minutes. After washing by PBS, the cells were then permeabilized with 0.1% TritonX-100 for 10 minutes at room temperature before blocking with 10% goat serum.

Paraffin tissue sections, cytospin samples and transwell membranes were incubated with primary antibodies at 4°C overnight and then incubated with Alexa Fluor 488 or 594 conjugated secondary antibodies (goat-anti mouse or rabbit IgG (H+L), molecular probes, Carlsbad, CA) at 1:400 in the dark for 1 hour at room temperature and followed by mounting the slides with Antifade reagent with DAPI (molecular probes, Carlsbad, CA).

Evaluation of staining results

All IHC staining slides were examined and photographed with a light microscope. To ensure standardization in the staining results evaluation, at least two researchers independently assessed all specimens and the researchers were blinded to each other's findings.

For semi-quantitative analysis of the expression of IL-13R α 1 and IL-13R α 2, the measurement parameter was the optical density values of the positive cells showed brown articles or clumps in the cytoplasm. The positive staining area in the healthy controls and NPs were captured under $\times 400$ amplification and was assessed by using ImageJ (three measurements per slide).

For semi-quantitative analysis of the expression of MUC5AC, β IV-Tubulin positive area (in arbitrary unit) in transwell membranes from untreated/treated cells (three measurements per individual) were evaluated under $\times 200$ amplification by Image J using the following formula: MUC5AC or β IV-Tubulin positive area / Total area of $\times 200$ amplification $\times 100\%$

RNA extraction and real-time (RT) – PCR

RNA extraction Total RNA was extracted from solid tissues (Healthy control and NPs) or epithelial cells from ALI cultures by using the mirVanaTM miRNA isolation kit (Applied Biosystems, AM1561). Briefly, the procedures were described as follows:

(1) Total RNA was extracted from solid tissues (Healthy controls and NPs)

(i) tissues were removed from RNA later and submerged in Lysis/Binding buffer, followed by homogenizing until all visible clumps are dispersed, (ii) Homogenate was mixed well with 1/10 volume of miRNA Homogenate Additive, (iii) Acid-Phenol: Chloroform was added in homogenate and centrifuge at $1,000\times g$ to separate the mixture into a lower, organic phase, an interphase, and a upper aqueous phase, (iv) RNA remained in the aqueous phase while DNA and proteins were in the interphase and organic phase, (v) aqueous phase was mixed thoroughly with 100% ethanol and transferred into a Filter Cartridge-collection tube and RNA was binding to the filter, (vi)

after washing steps, the RNA was eluted through the filter. The purified total RNA was stored at -80 °C.

(2) Total RNA was extracted from epithelial cells of ALI cultures

Cells were first lysed in a denaturing Lysis/binding Buffer from the mirVana™ miRNA isolation kit (Applied Biosystems, AM1561), with the stem or progenitor cells and one transwell pooled from different time point. The lysate was frozen at -20 °C until processed further. Total RNA was extracted from cells, according to the manufacture protocol similar to the one applied on solid tissue. The purified total RNA was stored at -80 °C.

Maxima First Strand cDNA Synthesis Kits was used for reverse transcription PCR performed on all extracted RNA samples to obtain cDNA.

RT-PCR was performed on the ABI Vii 7 Real time PCR System (Applied Biosystems) using probes from TagMan assays (Applied Biosystem) with the following assay numbers. *FOXP1*, Hs00230964_m1; *MUC5AC*, Hs00873651_m1; *IL-13*, Hs00174379_m1; *IL-13Rα1*, Hs00609817_m1; *IL-13Rα2*, Hs00152924_m1; *IL-4Rα*, Hs00166237_m1; *C-FOS*, Hs00170630_m1; *C-JUN*, Hs00277190_s1; *ARG1*, Hs00155832_m1; *IL-6*, Hs00174131_m1; *EGR1*, Hs00152928_m1; *COX2*, Hs00153133_m1; *GAPDH*, Hs999999056_m1; *PGK1*, Hs99999906_m1 ; The reaction was performed in the following cycling conditions: 50 °C for 2 minutes and 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. *GAPDH* and *PGK1* were used as endogenous control.

All the values were calculated by using the comparative $2^{-\Delta\Delta CT}$ methods (1). Absolute value of $2^{-\Delta CT}$ was presented for comparing the gene expression level of *IL-13*, *IL-13R α 1* and *IL-13R α 2* in nasal tissues. A fold change of $2^{-\Delta CT}$ was presented for comparing the effect of IL-13 on hNECs after normalizing untreated cells as one.

CBF

CBF was autoanalyzed by using the Sisson- Ammons Video Analysis system (SAVA, Omaha, NE)(2). The sample IDs were coded confidentially and blinded to the two investigators who performed CBF and data analysis. Whole field analysis was performed by using software that automatically analyzed the entire captured video of all ciliated cells in a given field. For all experiments, the digital sampling rate was set at 100 frames per second (fps). The predominant frequency of a small group of cilia from each sample was viewed and recorded at a minimum of three separate fields every one minute for up to five minutes while they were maintained at a constant temperature (23 ± 0.5 °C).

Cell Viability Test

Cell viability was determined by the trypan blue dye exclusion test. At least 600 cells were counted in four different fields and the number of viable cells was calculated as a percentage of total cell population (3).

Western blotting

hNECs were exposed to IL-13 or pretreated with chemical compounds then plus IL-13 at various time points to study the effects of IL-13 on activation of MAPK, and expression

of IL-13R α 1, IL-13 α 2. hNECs from ALI cultures were stimulated with IL-13 (10 ng/mL). For ERK, p38 and JNK activation, the cells were harvested at various times (1, 3, 6, 12, 24, 48, 72 hours) in cell lysis buffer containing phosphatase inhibitor (Cell Signaling Technology). For the chemical compounds treatment, the cells were pretreated with PD98059 or neutralizing antibodies for 1 hour or DEX (10^{-4} Mol/L) for 30 minutes before stimulation with IL-13 or vehicle DMSO. Then the cells were harvested at 1, 3, 6, 12, 24, 48, 72 hours for PD98059 treatment. The cells were harvested at 1, 3, 6 hours for neutralizing antibodies and DEX treatments. Cell lysates were in cell lysis buffer and Incubated on ice for 20 minutes and centrifuged at 13.2k rpm for 20 minutes at 4 °C. Then cell lysates were transferred to a new eppendorf tube and measured the protein concentration using Bradford protein assay (BioRad). Protein samples (40 mg/sample) were run and separated with 10% SDS-PAGE and transferred to PVDF membranes. Membranes blocked with 5% milk solution in 1 $\times$ TBST at room temperature for 1 hour and incubated in the primary antibody solution against with Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) and p44/42 MAPK (ERK1/2) (1:1000; Cell Signaling Technology), Phospho-p38 MAPK (Thr180/Tyr182) and p38 MAPK (1:1000; Cell Signaling Technology), Phospho-SAPK/JNK (Thr183/Tyr185) and SAPK/JNK (1:1000; Cell Signaling Technology) and GAPDH (1:10000, ab9485, Abcam) at 4 °C overnight. The membranes were incubated with anti-rabbit HRP-conjugated secondary antibody in 5% milk solution 1 $\times$ TBST (1:4000, NA934, GE Healthcare, UK). For IL-13 receptors expression, protein samples (30 mg/sample) were run and incubated in the primary antibody solution against with IL-13R α 1 (1:500, ab79277, Abcam), IL-13R α 2 (1:200, ab55275, Abcam) and GAPDH (1:10000, ab9485, Abcam). The membranes were

incubated with anti-mouse HRP-conjugated secondary antibody in 5% milk solution 1×TBST (1:2000, ab98693, Abcam). The signal was detected with ECL reagent (BioRad). Semi-quantitative analysis was performed on the western blot bands. Intensities of the bands were measured by imageJ.

Supplemental Table legend

Table S1. Sub analysis of mRNA level of IL-13, IL-13R α 1, IL-13R α 2 and IL-4R α in asthmatic, atopic patients, smoke patients, eosinophilic group, neutrophilic group and hyperplastic group in healthy control subjects and NPs.

	asthmatic IT	non-asthmatic IT	<i>p</i> value
mRNA	n=0	n=22	
[Median(IQR)]			
IL-13	0	0.000030(0-0.000428)	N/A
IL-13R α 1	0	0.006375(0.004581-0.013411)	N/A
IL-13R α 2	0	0.000433(0.000129-0.000552)	N/A
IL-4R α	0	0.046423(0.030608-0.069549)	N/A
	atopic IT	non-atopic IT	<i>p</i> value
mRNA	n=5	n=17	
[Median(IQR)]			
IL-13	0.000099(0.000011-0.001833)	0.000023(0-0.000542)	0.543
IL-13R α 1	0.006511(0.005657-0.019931)	0.005941(0.003147-0.011387)	0.359
IL-13R α 2	0.000252(0.000109-0.000488)	0.000457(0.000129-0.000655)	0.446
IL-4R α	0.0576446(0.042415-0.083638)	0.0415(0.028413-0.060631)	0.218
	smoke IT	non-smoke IT	<i>p</i> value
mRNA	n=5	n=21	
[Median(IQR)]			
IL-13	0(0-0.000624)	0.000099(0.000007-0.019994)	0.138
IL-13R α 1	0.00594(0.000733-0.019994)	0.006511(0.004928-0.011971)	0.85
IL-13R α 2	0.000433(0.000251-0.000694)	0.000369(0.000123-0.000534)	0.486
IL-4R α	0.035494(0.030223-0.121932)	0.049336(0.03143-0.068664)	0.753
	eosinophilic IT	non-eosinophilic IT	<i>p</i> value
mRNA	n=1	n=21	
[Median(IQR)]			

IL-13	0.042(0.018-0.094)	0.058(0.029-0.181)	0.182
IL-13R α 1	0.012(0.008-0.016)	0.014(0.009-0.031)	0.545
IL-13R α 2			0.364
IL-4R α	0.003(0.002-0.007)	0.004(0.001-0.045)	0.182
	neutrophilic IT	non-neutrophilic IT	p value
mRNA	n=5	n=16	
[Median(IQR)]			
IL-13	0.000414(0.000041-0.008947)	0.001434(0.000817-0.007227)	0.032
IL-13R α 1	0.005817(0.003127-0.011346)	0.005359(0.004038-0.009496)	0.842
IL-13R α 2	0.000502(0.000154-0.002455)	0.000365(0.000174-0.000901)	0.354
IL-4R α	0.067805(0.048521-0.08238)	0.051271(0.041485-0.061294)	0.011
	hyperlplastic IT	non-hyperplastic IT	p value
mRNA	n=2	n=21	
[Median(IQR)]			
IL-13	0.000105(0-0.00021)	0.000089(0-0.000257)	0.711
IL-13R α 1	0.501811(0.003769-0.999854)	0.00594(0.004781-0.011305)	0.711
IL-13R α 2	0.000146(0.000111-0.000181)	0.000433(0.000152-0.000577)	0.237
IL-4R α	0.044155(0.03041-0.057901)	0.04971(0.040246-0.069371)	0.711
	asthmatic NP	non-asthmatic NP	p value
mRNA	n=8	n=38	
[Median (IQR)]			
IL-13	0.008351(0.001573-0.018668)	0.001313(0.000076-0.006254)	0.021
IL-13R α 1	0.005236(0.002675-0.013824)	0.005817(0.004491-0.013421)	0.579
IL-13R α 2	0.001537(0.000507-0.00262)	0.000537(0.000174-0.001131)	0.07
IL-4R α	0.054491(0.023676-0.076294)	0.0555(0.041546-0.073911)	0.522
	atopic NP	non-atopic NP	p value
mRNA	n=9	n=25	
[Median (IQR)]			
IL-13	0.003314(0.000789-0.015720)	0.001335(0.004866-0.015543)	0.316

IL-13R α 1	0.007355(0.004866-0.015543)	0.005603(0.002132-0.015994)	0.316
IL-13R α 2	0.001451(0.000318-0.003547)	0.000607(0.000365-0.002344)	0.591
IL-4R α	0.055995(0.049656-0.070893)	0.052987(0.032954-0.079214)	0.645
	NP smokers	NP non-smokers	<i>p</i> value
mRNA	n=9	n=36	
[Median (IQR)]			
IL-13	0.001841(0.000218-0.009627)	0.000833(0.000051-0.002612)	0.211
IL-13R α 1	0.006016(0.004408-0.014908)	0.005461(0.003239-0.018747)	0.856
IL-13R α 2	0.000629(0.000245-0.002058)	0.000274(0.00016-0.000456)	0.035
IL-4R α	0.057726(0.042204-0.076768)	0.05123(0.041297-0.061386)	0.267
	eosinophilic NP	non-eosinophilic NP	<i>p</i> value
mRNA	n=23	n=9	
[Median (IQR)]			
IL-13	0.001499(0.000361-0.007744)	0.00136(0.000072-0.004775)	0.621
IL-13R α 1	0.005461(0.002724-0.011961)	0.005638(0.004821-0.008532)	0.711
IL-13R α 2	0.00045(0.000144-0.001451)	0.000874(0.000229-0.003547)	0.433
IL-4R α	0.064909(0.044052-0.076211)	0.053687(0.039238-0.059773)	0.341
	neutrophilic NP	non-neutrophilic NP	<i>p</i> value
mRNA	n=16	n=14	
[Median (IQR)]			
IL-13	0.000513(0.000126-0.002774)	0.000029(0-0.000129)	0.154
IL-13R α 1	0.006511(0.004712-0.008968)	0.005794(0.002678-0.011516)	0.728
IL-13R α 2	0.000235(0.000123-0.000524)	0.00049(0.000177-0.000716)	0.728
IL-4R α	0.074325(0.068534-0.095302)	0.045069(0.032118-0.055776)	0.043
	hyperplastic NP	non-hyperplastic NP	<i>p</i> value
mRNA	n=35	n=13	
[Median (IQR)]			
IL-13	0.00137(0.00008-0.008896)	0.00083(0.000135-0.006272)	0.844
IL-13R α 1	0.007355(0.005166-0.015917)	0.005199(0.00248-0.007019)	0.123

IL-13R α 2	0.000799(0.000274-0.001899)	0.000371(0.000136-0.001219)	0.19
IL-4R α	0.057863(0.045997-0.076211)	0.047109(0.039444-0.070097)	0.302

N/A, not available.

Relative mRNA expression levels were calculated using $2^{-\Delta\Delta Ct}$ method with *GAPDH* as the internal control

Data showed median values with IQR (interquartile range, 25th and 75th percentiles).

The *p* value was calculated by Mann-Whitney two-tailed test. A *p* value of less than 0.05 was considered statistically significant.

Table S2. Patients' characteristics for ALI cultures

ID	Age	Gender	Allergic	Asthma	Smoker	GC-treatment
Patient 1	52	M	N	N	N	N
Patient 2	35	M	N	N	N	N
Patient 3	27	M	N	N	N	N
Patient 4	19	M	N	N	N	N
Patient 5	30	M	N	N	Y	N
Patient 6	38	M	N	N	N	N
Patient 7	57	F	N	N	N	N
Patient 8	36	F	N	N	N	N
Patient 9	39	M	N	N	N	N

Supplemental Figure legend

Figure S1. (A) Quantitative real-time RT-PCR analysis of *IL-4R α* mRNA expressions in tissues extractions and primary hNECs of CRSwNP patients (n=49 and n=13) and healthy control subjects (n=27 and n=12), *GAPDH* as the internal control. Relative mRNA expression levels were calculated using $2^{\Delta(-\Delta C_t)}$ method; **(B)** The correlations of mRNA level of *IL-4R α* and *MUC5AC* (one-to-one) were analyzed (X and Y-axis scale are logarithmic (base 10) transformed). Median values with 25th and 75th percentiles are indicated by error bar.

Figure S2. (A) Semi-quantitative analysis of β IV-Tubulin positive area (in arbitrary unit) in transwell inserts from untreated/treated cells (three fields per individual, n=6, evaluated at $\times 200$ amplification by Image J). **(B)** Quantitative real-time RT-PCR analysis of *FOXJ1* mRNA expressions on hNECs with or without treatment of IL-13 (10 ng/mL, 72 hours) in ALI cultures (n=6), *PGK1* as the internal control). Median values with 25th and 75th percentiles are indicated by error bar;

Figure S3. Cytospin IF staining of IL-13R α 1 (green) or IL-13R α 2 (green) with MUC5AC (red) on hNECs with or without treatment of IL-13 (10 ng/mL, 72 hours) in ALI cultures ($\times 1000$ amplification; scale bar - 10 μ m; cell nuclei are shown in blue, DAPI, 49-6-Diamidino-2-phenylindole dihydrochloride).

Figure S4. Quantitative real-time RT-PCR analysis of *IL-4R α* mRNA expressions on hNECs from healthy control subjects (n=5) with or without treatment of IL-13 (10 ng/mL, 72 hours) in ALI cultures. *PGK1* is the internal control. Relative mRNA expression levels were calculated using $2^{\Delta(-\Delta C_t)}$ method; median values with 25th and 75th percentiles are indicated by error bar.

Figure S5. (A) Western blot analysis of ERK1/2 activation on hNECs with pretreatment of PD98059 (50 μ Mol/L) or vehicle DMSO (0.11% dimethyl sulfoxide) to confirm the specificity of the ERK1/2 signals. (B) PD98059 (50 μ Mol/L) demonstrated no significant influence on the viability of ALI cells as compared to untreated cells (n=3). Median values with 25th and 75th percentiles are indicated by error bar..

Figure S6. (A and C) Quantitative real-time RT-PCR analysis of *IL-13R α 1* (n=6) and *IL-4R α* (n=5) mRNA expressions on hNECs in the present of IL-13 (10 ng/mL) treated with PD98059 (50 μ Mol/L) or Vehicle DMSO (0.11%) for 72 hours (PGK1 as the internal control). (B) Western blot analysis of IL-13R α 1 protein levels on hNECs in the present of IL-13 (10 ng/mL) treated with PD98059 (50 μ Mol/L) or Vehicle DMSO (0.11%) for 72 hours. Median values with 25th and 75th percentiles are indicated by error bar.

Figure S7. (A-E) Quantitative real-time RT-PCR analysis of *C-FOS*, *ERG1*, *COX2*, *ARGE* and *IL-6* mRNA expressions on hNECs in the present of IL-13 (10 ng/mL) treated with PD98059 (50 μ Mol/L) or Vehicle DMSO (0.11%) for 72 hours (n=4, *PGK1* as the internal control). Median values with 25th and 75th percentiles are indicated by error bar.

Figure S8. Dexamethasone (10^{-4} mol/L) demonstrated no significant influence on the viability of ALI cells as compared to untreated cells (n=3). Median values with 25th and 75th percentiles are indicated by error bar.

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