

# Induction of IL-25 Expression in Human Nasal Polyp Epithelium by Influenza Virus Infection is Abated by Interferon-Alpha Pretreatment

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**Background:** Epithelial cytokines including IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) are recently established as drivers of type 2 chronic inflammatory diseases such as chronic rhinosinusitis with nasal polyps (CRSwNP). Here, we further confirmed the increased expression of IL-25 in CRSwNP and investigated potential contributors of IL-25 in CRSwNP epithelium.

**Methods:** Sixty CRSwNP, 25 CRSsNP and 15 healthy control tissues were examined for IL-25 expression and for the accompanying type 2 inflammatory cytokines. We then tested different respiratory virus infections on human nasal epithelial cells (hNECs) for their ability to trigger IL-25 expression. In addition, we subjected hNECs generated from CRSwNP tissues to pretreatment with recombinant interferon-alpha (IFN- $\alpha$ ) prior to viral infection to evaluate IFN effects on IL-25 induction.

**Results:** We confirmed that significantly enhanced levels of IL-25 were observed in CRSwNP tissues, and that IL-25 expression correlated with type 2 inflammatory cytokine expression. In vitro, we observed significantly elevated IL-25 in hNECs infected with influenza A virus as early as 24 hours post-infection (hpi), regardless of tissue origin, and IL-25 correlated positively with viral load. While other respiratory viruses exhibited increasing trends of IL-25, these were not significant at the time-points tested. IFN- $\alpha$  treatment of CRSwNP epithelium was found to exert bimodal effects, ie IFN- $\alpha$  treatment alone induced moderate IL-25 expression, whereas IFN- $\alpha$  pretreatment of hNECs before influenza infection significantly diminished IL-25 induction by active influenza virus infection.

**Conclusion:** We have authenticated the observation of elevated IL-25 in CRSwNP, which is correlated with type 2 inflammatory cytokines. Notably, we identified influenza virus infection as a potential contributor of IL-25 in both control and CRSwNP epithelium during active infection. This IL-25 induction can be abated by IFN- $\alpha$  pretreatment which ameliorated active influenza infection.

**Trial Registration:** [Chictr.org.cn](http://www.chictr.org.cn) ChiCTR-BON-16010179, Registered 18 December 2016, <http://www.chictr.org.cn/showproj.aspx?proj=17331>. The authors agree on the sharing of deidentified participant data where it pertains to request directly related to the data in this article when contacted (Haiyu Hong; honghy@mail.sysu.edu.cn).

**Keywords:** chronic rhinosinusitis with nasal polyps, interferon-alpha, interleukin 25, respiratory viruses, influenza virus, type 2 inflammation

## Introduction

Chronic rhinosinusitis (CRS) is one of the most common diseases in the upper respiratory tract, with an estimated prevalence of about 10% globally.<sup>1-3</sup> CRS is classified as CRS with or without nasal polyps (CRSwNP or CRSsNP), based on

clinical examination of visible NP in the nasal meatus.<sup>3</sup> CRSwNP is an inflammatory condition of the paranasal sinuses that lasts at least 12 weeks in duration, and the risk of CRSwNP exacerbation is increased by external pathogen infections, including bacterial, viral, and fungal infections.<sup>3</sup> Although emerging evidence implicates numerous cytokines and chemokines in the interactions of host and environmental factors during occurrence and exacerbation of CRSwNP, factors that contribute to and perpetuate type 2 inflammation in existing CRSwNP are not fully elucidated.<sup>4</sup>

The nasal epithelium plays crucial roles in the host defenses by forming a physical barrier that mechanically clears foreign materials via its mucociliary functions.<sup>5</sup> In addition, human nasal epithelial cells (hNECs) are also an important source of cytokines and chemokines, including the recently discovered trio of epithelial-derived cytokines (thymic stromal lymphopoietin or TSLP, IL-25, and IL-33) that are highly associated with type 2 inflammation. Among them, IL-25 (also known as IL-17E) is a member of the IL-17 cytokine family, and plays a variety of roles in different inflammatory disorders such as asthma, atopic dermatitis, pulmonary fibrosis, and CRSwNP.<sup>6,7</sup> It was shown that intraperitoneal or intranasal administration of IL-25 protein results in the accumulation of eosinophils or production of type 2 inflammatory cytokines in the bronchoalveolar lavage fluid and the lung tissues.<sup>8</sup> Conversely, blocking IL-25 decreases type 2 inflammatory cytokine production in a murine asthma model.<sup>9</sup> These studies suggest that that IL-25 may be an important biomarker in the diagnosis and management of CRSwNP. However, the potential triggers in CRSwNP epithelium that can cause the expression of IL-25 are poorly understood.

Respiratory viruses are well-established triggers of exacerbation of chronic airway inflammatory diseases.<sup>10</sup> In addition, as the primary target cells for respiratory viruses,<sup>10,11</sup> the hNECs also play roles in mediating antiviral innate and adaptive immunity that orchestrate both the type 1 and type 2 inflammatory responses at the local mucosa site.<sup>12</sup> Interestingly, while viral infection typically triggers type 1 inflammatory responses geared towards viral clearance, they have also been shown to trigger the expression of type 2 inflammatory cytokines.<sup>10,13,14</sup> Therefore, it is interesting to interrogate their roles in inducing IL-25 in CRSwNP epithelium which may contribute to the increased accumulation of local IL-25 at the diseased site.

Recently, there is increasing evidence to support the association between respiratory viruses and CRSwNP, especially in Asian cohorts.<sup>15–17</sup> Hence, our study aims to reaffirm the importance of IL-25 in CRSwNP pathogenesis; as well as to implicate respiratory viruses as potential triggers of IL-25 expression in local CRSwNP epithelium. We hypothesize that IL-25 expression is a part of the immune response repertoire in the nasal epithelium during virus infection due to its role in epithelial injury and barrier damage. This in turn contributes to the accumulation of IL-25 and its associated type 2 inflammatory cytokines in the local CRSwNP environment. At the same time, given that anti-infective agents are proposed as a means of managing chronic airway inflammatory diseases,<sup>16,18</sup> we therefore investigated the antiviral effect of IFN- $\alpha$  and whether it can modulate IL-25 expression in hNECs infected with respiratory viruses.

## Materials and Methods

This study was approved by the Ethical Committees of the First and Fifth Affiliated Hospitals of Sun Yat-sen University (clinical trial registration no. ChiCTR-BON-16010179 and NCT02110654), and National University of Singapore (National Healthcare Group Domain-Specific Board of Singapore, DSRB Reference no. D/11/228, and Institutional Review Board of the National University of Singapore, IRB code 13–509) in accordance with the Declaration of Helsinki. Written informed consent was obtained from each subject.

## Patients and Tissue Samples

60 CRSwNP patients and 25 CRSsNP patients were recruited from the First and Fifth Affiliated Hospitals of Sun Yat-sen University (Guangzhou and Zhuhai, China). Diagnosis of CRSwNP and CRSsNP was carried on the basis of on disease history, nasal endoscopy, and computed tomography scan of the paranasal cavities, in accordance with the current European position paper on rhinosinusitis and nasal polyps.<sup>2,3</sup> The atopic status was evaluated by skin prick test (SPT) and serum IgE levels (Phadia, Uppsala, Sweden) specific to common inhaled allergens (eg pollens, house dust mites, pets, molds, and cockroaches). For healthy controls, 15 subjects with traumatic optic neuropathy were enrolled, and uncinat process tissues were sampled during endoscopic optic nerve decompression. Peripheral blood samples were collected before surgery.

## Immunohistochemical Staining

Sino-nasal tissues were embedded in paraffin and cut into 5- $\mu$ m thick sections. Hematoxylin-eosin (HE) staining and immunochemistry using peroxidase-labeled streptavidin-biotin technique were performed for histological analyses, following published protocol.<sup>7</sup>

## Real-Time Quantitative PCR

The mRNA expression profiles of target genes were determined using real-time quantitative PCR (RT-qPCR) as previously described.<sup>19</sup> Briefly, total RNA was extracted from the tissue or cell samples using mirVana miRNA isolation kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Reverse transcription was performed to synthesize cDNA from 2  $\mu$ g of total RNA using oligo(dT) 18 primer and M-MLV reverse transcriptase (Takara, Shiga, Japan). Expression of mRNA was determined by qPCR using the ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Taq™ (Takara). The mean threshold cycle (Ct) values were normalized to  $\beta$ -actin and PGK1 housekeeping genes for clinical specimens and hNEC samples, respectively. The relative mRNA levels of target genes were analyzed using the formula  $2^{-\Delta\Delta C_t}$ . Experiments were performed in triplicate for each datapoint. The primer sequences used in the study for IL-25, IL-17RB, IL-4, IL-5, IL-13, IFN- $\gamma$ , IL-17A, intercellular adhesion molecule 1 (ICAM-1), interferon regulatory factor 7 (IRF7), retinoic acid-inducible gene I (RIG-I), Toll-like receptors (TLR) TLR3 and TLR7,  $\beta$ -actin, and PGK1 are listed in [Table S1](#).

## Luminex-Based Multiplex Assay

Cytokines and chemokines from polyp and uncinated process tissues were measured using Luminex-based multiplex assay. Briefly, 30 mg of each sample was homogenized in 0.5 mL lysis buffer supplemented with protease inhibitor (Millipore, Burlington, MA, USA) for 1 minute on ice and centrifuged for 20 minutes at 4000 rpm at 4°C. Supernatants were collected, and total protein concentrations in the supernatants were determined by standard BCA assay normalized to a concentration of 50  $\mu$ g total protein per sample. All samples were analyzed for IL-25 (IL-17E), IL-4, IL-5, IL-13, IFN- $\gamma$ , and IL-17A using Luminex-based Multiplex kits (Millipore), following the manufacturer's protocol. IL-25 levels from supernatants (apical and basal) of influenza virus-infected hNECs

were detected using the human IL-17E/IL-25 XL Magnetic Luminex performance assay (R&D Systems, Minneapolis, MN, USA) according to manufacturer's protocol.

## Virus Infection and Antiviral Pretreatment with Recombinant IFN- $\alpha$ Protein in hNECs Model

Infections with influenza virus, rhinovirus and respiratory syncytial virus (RSV) were performed as previously described.<sup>11,20,21</sup> Briefly, in vitro differentiated hNECs cultured in Transwells were infected at multiplicity of infection (MOI) of 0.1 (influenza A/Aichi/2/1968/H3N2 strain), 2.5 (rhinovirus RV16 strain), and 3.0 (RSV A2 strain), and incubated at 33°C (rhinovirus), and 35°C (influenza and RSV) for 1 hour, respectively. The inocula were then removed, and infected hNECs were incubated for 24 or 48 hours. Pretreatment with recombinant human IFN- $\alpha$  2A protein expressed in HEK293 cells (Sigma-Aldrich, St Louis, MO, USA) was performed 48 hours prior to viral infection. The cells were first subjected to pretreatment with IFN- $\alpha$ 2A at four different concentrations (5, 25, 50, 100 ng/mL) for 48 hours to determine the optimal/effective concentration, before selecting 5 and 25 ng/mL for the main pretreatment experiments.

## PrestoBlue™ Cell Viability Assay

PrestoBlue™ cell viability assay was conducted to check if any toxicity effect was induced by IFN- $\alpha$ 2A treatment. Cell viability assay using PrestoBlue™ reagent (Life Technologies) was performed following the manufacturer's protocol, as previously described.<sup>22</sup> A "no cell" control (1 $\times$  working solution without incubation with cells) was used as the baseline fluorescence value. The fluorescence reading from the mock treatment group was used as a negative control for normalization.

## Virus Plaque Assay

Viral plaque assay was performed as previously described.<sup>20</sup> Plaque-forming unit (PFU) values were calculated as follows: Number of plaques  $\times$  Dilution factor = Number of PFU per 100  $\mu$ L. The final data were presented as PFU per mL.

## Cytospin and Viral Staining

Cytospin and viral staining were performed as previously described.<sup>20</sup> Briefly, at each time-point, single-cell

suspensions ( $1-2 \times 10^5$  cells) were dissociated from Transwells using  $0.5 \times$  Trypsin/EDTA solution (Gibco, Carlsbad, CA, USA) at  $37^\circ\text{C}$ . Dissociated cells were fixed in 4% paraformaldehyde at room temperature for 10 minutes, followed by 2 rounds of washing with  $1 \times$  DPBS, and centrifuged at 3000 rpm for 5 minutes. Cytospin preparations ( $2 \times 10^4$  cells per slide) were prepared at 500 rpm for 5 minutes with mild acceleration with Shandon Cytospin 3 Cyto centrifuge (Thermo Fisher Scientific, Waltham, MA, USA). Mouse anti-influenza A nucleoprotein antibody (Abcam, Cambridge, MA, USA) was used at 1:200 dilution for immunofluorescence (IF) staining to visualize viral replication in host cells, while rabbit anti-IL-25 antibody (Abcam) was used at 1:250 dilution to analyze IL-25 protein expression. Alexa Fluor 488 (anti-rabbit) and Alexa Fluor 594 (anti-mouse)-labeled IgG (H1L) secondary antibodies (Life Technologies) were both used at 1:500 dilutions for IF staining. ProLong AntiFade mounting medium with DAPI (Life Technologies) was used for mounting of the stained slides.

## Statistical Analyses

Clinical data were expressed as median and interquartile range (IQRs) and analyzed using the Kruskal–Wallis *H*-test and nonparametric Mann–Whitney *U*-test, unless otherwise stated. For in vitro infection assays, the data were expressed as the means and standard errors of the mean (SEM). One-way analysis of variance (ANOVA) and paired or unpaired Student's *t*-test were used for the statistical analyses. For in vitro pretreatment assays, two-way ANOVA was employed to assess the differences between treatment and infection groups. A *p*-value of less than 0.05 was considered statistically significant.

## Results

### Increased IL-25 in CRSwNP Promotes Type 2 Inflammation in NP Tissues

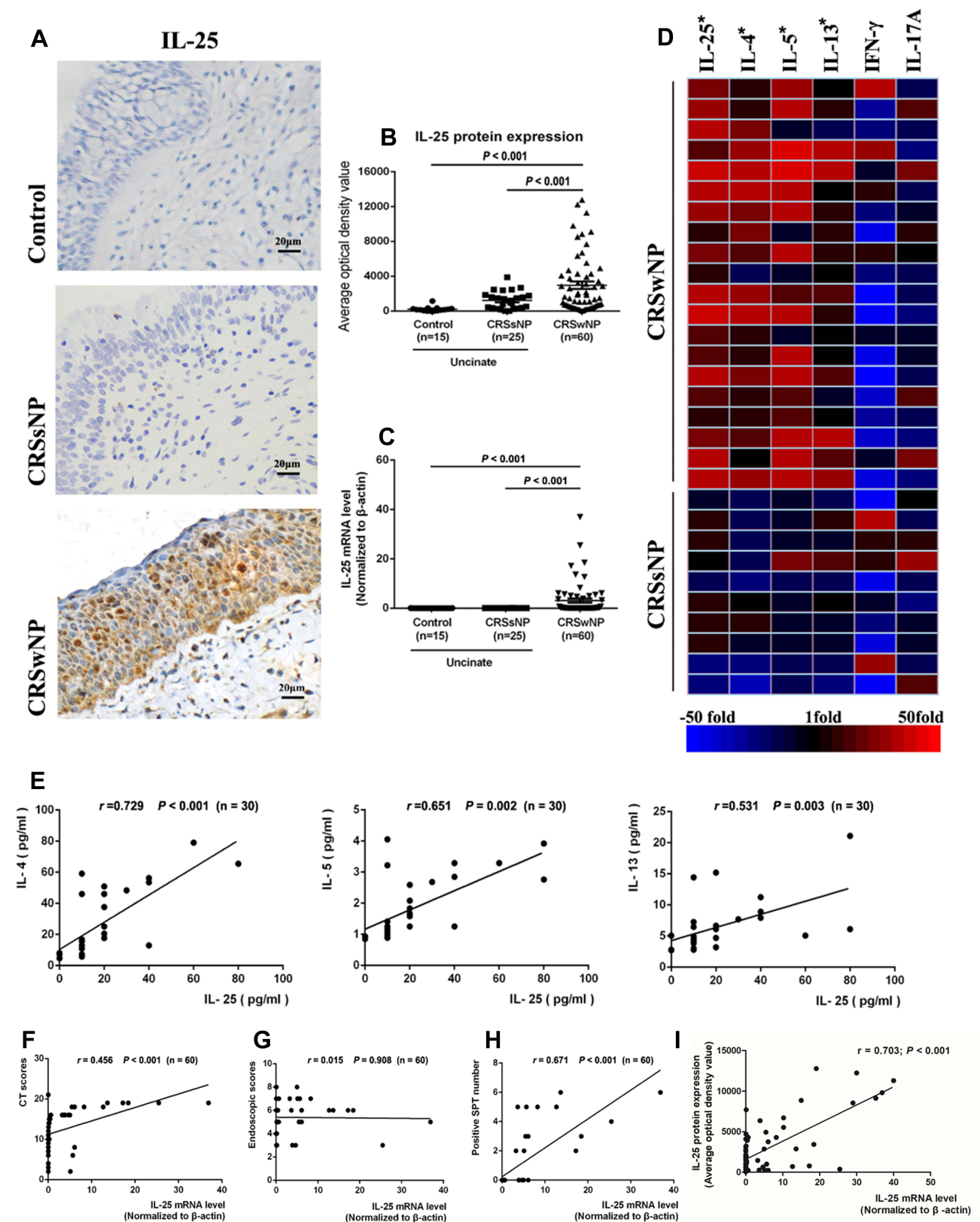
Sixty CRSwNP patients, 25 CRSsNP patients, and 15 healthy control subjects were enrolled in this study. The demographic data of all subjects are shown in [Table S2](#). Our results revealed that both protein and mRNA levels of IL-25 were significantly elevated in CRSwNP patients when compared to CRSsNP patients and healthy controls ( $p < 0.05$ , [Figure 1A–C](#)). In addition, LUMINEX cytokine analysis showed that the protein levels of type 2 inflammatory cytokines (IL-4, IL-5, and IL-13) were only

significantly increased in CRSwNP patients, affirming that IL-25 was more strongly associated with type 2 inflammation and was significantly more prevalent in CRSwNP ( $p < 0.05$ , [Figure 1D](#)). The findings corroborated our previous study which confirmed the role of polyp IL-25 in inducing type 2 inflammatory cytokines.<sup>23</sup> Moreover, we also further showed that IL-25 protein expression was positively correlated with expression of type 2 inflammatory cytokines, CT scores and sensitization to allergens, but not endoscopic scores ( $p < 0.05$ , [Figure 1E–H](#)), indicating a strong correlation between IL-25, CRSwNP and type 2 inflammation, particularly allergy-driven ones. In addition, we further demonstrated that the mRNA and protein levels of IL-25 were well correlated with each other, indicating that the mRNA levels are truly indicative of the IL-25 protein expression ([Figure 1I](#)).

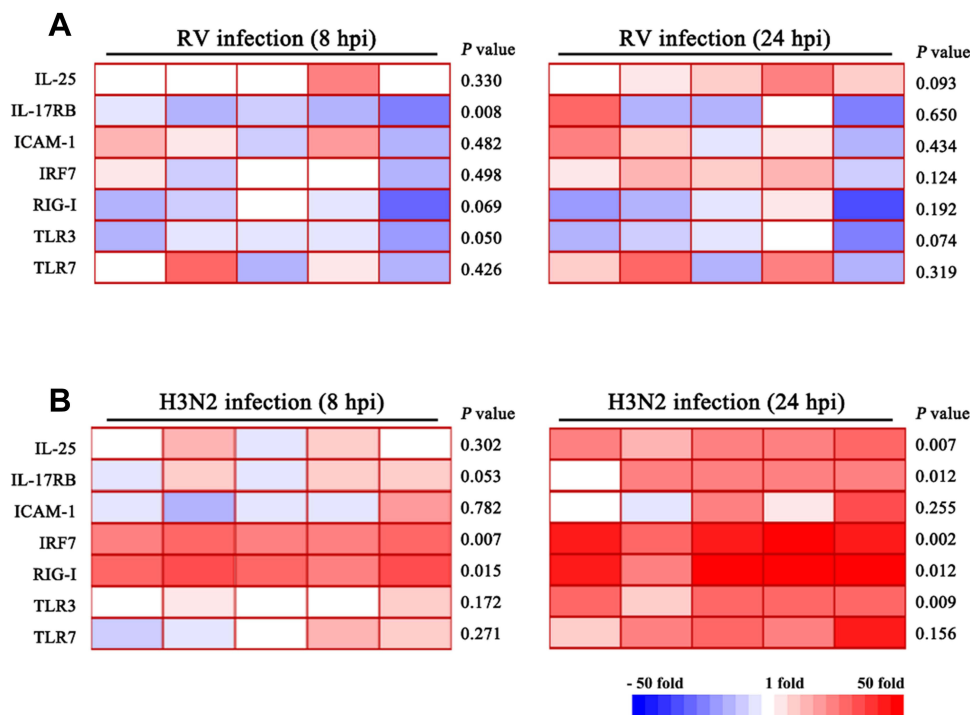
### Influenza Virus Infection of hNECs in vitro Induce IL-25 Expression

It has been established that respiratory virus infection of an inflamed airway can exacerbate symptoms of chronic airway inflammatory diseases.<sup>24</sup> Therefore, we investigated common respiratory viral infections (influenza, rhinovirus, RSV) of in vitro differentiated hNECs derived from control or CRSwNP tissues to investigate their ability to induce IL-25 ([Table S3](#)). Interestingly, among the respiratory viruses tested at the time-points in this study, H3N2 influenza virus stimulated significantly increased expression of IL-25 in hNECs derived from control and CRSwNP samples alike, when compared to rhinovirus (RV16) and RSV (A2) which exhibited weaker, non-significant increase of IL-25 ([Figure 2A and B](#); [Figure S1](#)). As shown in [Figure 3A](#), we observed significant fold changes of IL-25 expression at 24 hours and 48 hours post-infection (hpi) with influenza ( $p < 0.05$ ), which also positively correlated with IL-17RB ( $r = 0.578$ ,  $p < 0.05$ , [Figure 3B](#)). Additionally, we also observed the same magnitude of significant increase in secreted IL-25 protein following influenza infection of hNECs in both apical and basal supernatants ([Figure 3C](#)). This corroborated with the tissue data, and confirmed strong IL-25 correlation at mRNA and protein levels. Therefore, for the subsequent investigation, we used IL-25 mRNA levels as a surrogate for IL-25 protein expression levels.

To confirm that the induction of IL-25 was associated with virus infection and its host response, we also evaluated the correlation of IL-25 and IL-17RB transcripts and



**Figure 1** Increased IL-25 and type 2 inflammatory profiles in CRSwNP tissues (A and B) The expression of IL-25 in CRSwNP, CRSsNP and healthy control tissues (400 $\times$  magnification) and its quantification via optical density of IL-25 positive staining. (C) IL-25 mRNA levels in CRSwNP, CRSsNP and control tissues. (D) Heatmap of selected cytokines as determined by Luminex-based multiplex assay, showing up- or down-regulation in CRSwNP and CRSsNP samples, compared to controls. (E) Correlation of levels of IL-25 and type 2 inflammatory cytokines IL-4, IL-5, and IL-13 in CRSwNP patients. (F–H) Correlation of IL-25 mRNA expression with CT score, endoscopic score, and number of sensitizations to common environmental allergens (indicated by SPT) in CRSwNP patients. (I) Correlation of IL-25 protein expression and mRNA levels from CRSwNP tissues. Data are expressed as median values (IQRs). Comparison of median values was performed using Kruskal–Wallis test and Mann–Whitney U-test.  $p < 0.05$  is considered statistically significant.



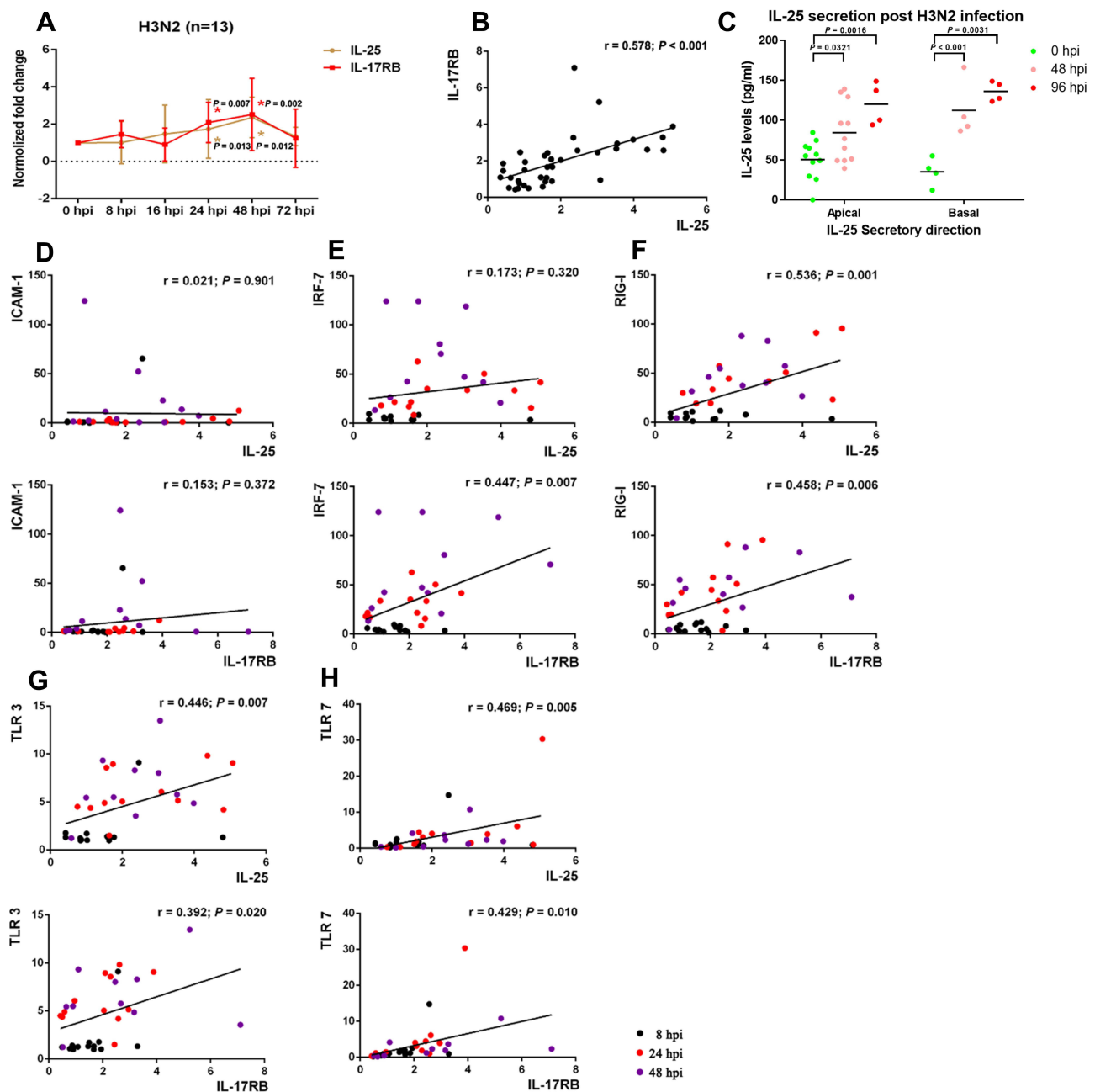
**Figure 2** Heatmap of expression profiles of IL-25 and related innate immune factors in response to influenza and rhinovirus infection at 8 and 24 hpi. Cytokine mRNA level changes in **(A)** rhinovirus infection and **(B)** influenza infection. Greater significant changes in cytokine levels were observed in influenza compared to rhinovirus infection at early stages of infection of hNECs. Changes in expression of cytokines at various time-points of virus infections were determined by RT-qPCR (n=5). Red color denotes up-regulation, while blue color denotes down-regulation of the corresponding cytokine mRNA. Comparison of means was performed by Student's *t*-test between infected and uninfected hNECs.  $p < 0.05$  is considered statistically significant.

the five most sensitive host response markers<sup>16,20,25</sup> at time-points of 8, 24, and 48 hpi after influenza infection. While ICAM1 and IRF7 were negatively correlated with IL-25 and IL-17RB (Figure 3D and E), the expression of viral sensors RIG-I, TLR3 and TLR7 was positively correlated with IL-25 and IL-17RB ( $p < 0.05$ , Figure 3F–H), signifying that the expression profiles of IL-25 and IL-17RB were associated with the induction of antiviral response in hNECs, particularly with influenza which induced strong antiviral responses as early as 24 hpi (Figure 2).

### Induced Expression of IL-25 Correlates with Influenza Virus Replication in hNECs Derived from CRSwNP Tissues

In order to evaluate the relationship between IL-25 expression with replication dynamics of H3N2 influenza virus in NP epithelium, recombinant protein of IFN- $\alpha$ , an FDA-approved antiviral drug for hepatitis C (INTRON<sup>®</sup>A), was used to inhibit virus replication in infected hNECs derived from CRSwNP donors.<sup>16,26</sup> The effective concentration of human recombinant IFN- $\alpha$

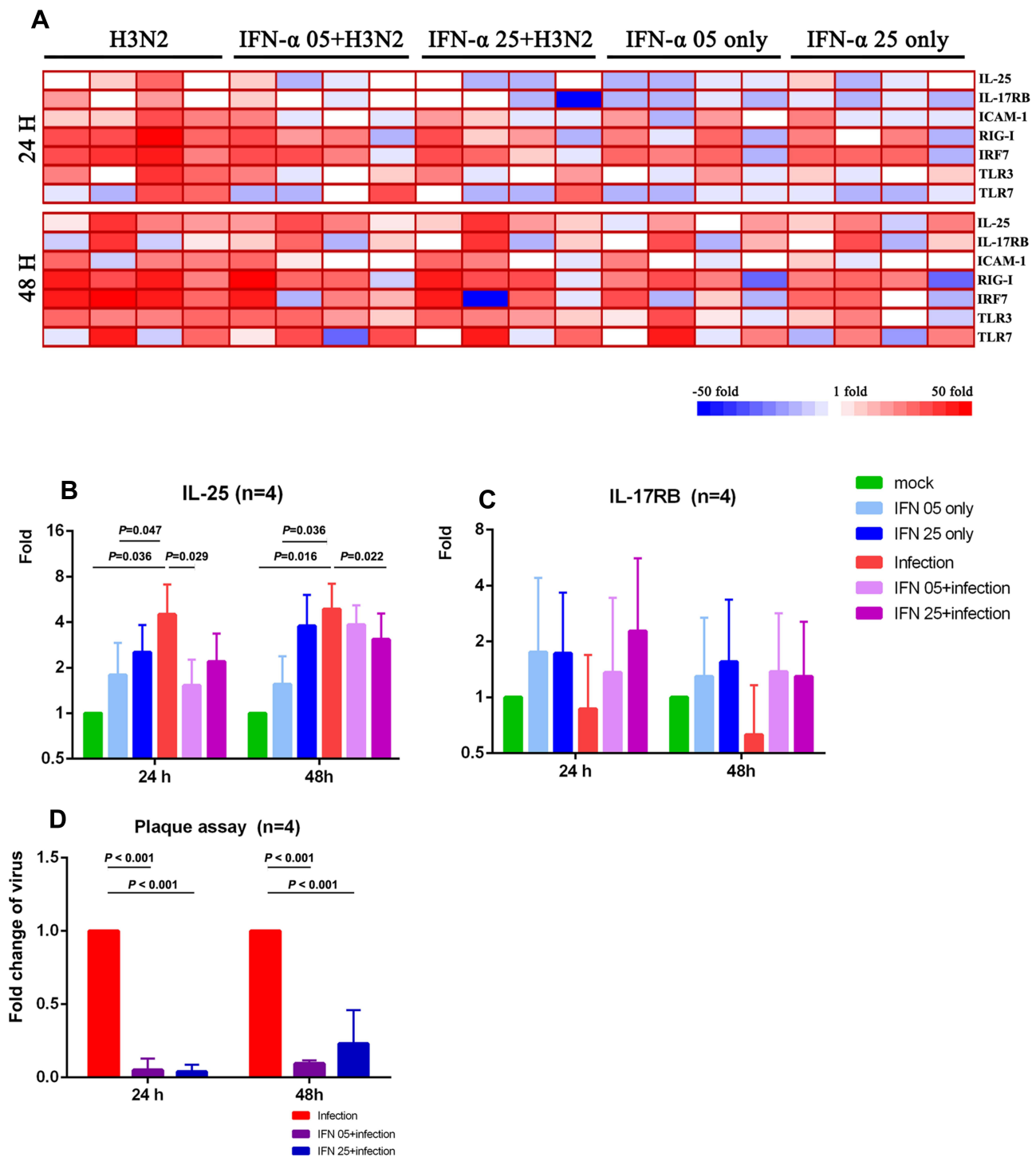
protein and pretreatment duration were screened during pilot experiments, and low doses of 5 and 25 ng/mL were employed for the inhibition of virus replication throughout the antiviral study, as shown by fold changes in virus titers (Figure S2). The viability of the hNECs following low-dose IFN- $\alpha$  treatment was reduced which was expected with IFN treatment, but this decreased viability was not significantly different than that of the infected cells (Figure S3). The heatmap of mRNA levels of pathogen sensors is shown in Figure 4A – we observed significant reduction of IL-25 expression at 24 and 48 hpi (Figure 4B), but not IL-17RB (Figure 4C) in CRSwNP hNECs pretreated with IFN- $\alpha$ . The results revealed that IL-25 was consistently elevated following influenza infection, but not IL-17RB which showed a different trend as in previous results (Figure 3A). The discrepancies suggested that IL-17RB expression may be more susceptible to batch variation, and its expression may change more dynamically and may not be entirely dependent on influenza infection. IFN- $\alpha$  treatment alone increased IL-25 expression, which was relatively lower than influenza-induced IL-25 expression. Interestingly,



**Figure 3** Significantly increased expression of IL-25 and IL-17RB upon influenza infection of hNECs. **(A)** The mRNA levels of IL-25 and IL-17RB at different time-points post-infection (n=13). **(B)** Correlation of IL-25 and IL-17RB mRNA expression following H3N2 influenza infection of hNECs. Comparison of means was performed by Student's *t*-test between infected and uninfected hNECs. **(C)** Apical and basal secretions of IL-25 protein in supernatants of influenza virus-infected hNECs as detected by Luminex assay at different time-points post-infection. Comparison of means was performed by one-way ANOVA with Dunnett's multiple comparison post-hoc test. **(D–H)** Correlation of mRNA levels of IL-25 and IL-17RB with the five most reactive markers as determined by RT-qPCR.  $p < 0.05$  is considered statistically significant.

the increased IL-25 by influenza infection was abated by the pretreatment of CRSwNP hNECs with IFN- $\alpha$ . This suggests bimodal effects of IFN- $\alpha$ , where its treatment alone moderately enhanced IL-25, whereas IFN- $\alpha$  pretreatment of hNECs instead suppressed the IL-25 expression induced by influenza. The bimodal effects may also explain the different levels of inhibition between 5 and 25

ng/mL of IFN- $\alpha$  at different time-points, in which the higher concentration treatment initially led to increased IL-25 (24 hpi), followed by stronger modulation of influenza-induced IL-25 at the later time-point (48 hpi). Congruent with this, we showed that the reduction of IL-25 was accompanied by efficient inhibition of influenza virus replication by a low dose of IFN- $\alpha$



**Figure 4** Expression of IL-25, IL-17RB, and host factors following inhibition of viral replication by pretreatment with IFN- $\alpha$  (A) Changes of mRNA expression of IL-25, IL-17RB and innate antiviral factors at 24 and 48 hpi with IFN- $\alpha$  pretreatment, together with controls. Fold change of (B) IL-25 and (C) IL-17RB mRNAs shown individually following influenza infection, and pretreatment with IFN- $\alpha$ , together with controls (n=4). Comparison of means was performed by two-way ANOVA. (D) Fold changes in viral replication titers (by viral plaque assay) following pretreatment with IFN- $\alpha$  that accompanied the reduction in IL-25 expression. Comparison of means was performed by Student's t-test between infected and uninfected hNECs.  $p < 0.05$  is considered statistically significant.

(Figure 4D), suggesting a virus-dependent induction of IL-25 in differentiated CRSwNP epithelium. The induced expression of IL-25 by influenza infection in hNECs was

further illustrated in Figure S4 (24 hpi) – indicated by the co-localization of IL-25 and influenza viral-nucleoprotein on cytospin slides.



## Discussion

The epithelial-derived cytokine IL-25, acting through the receptor IL-17RB, has been implicated in promoting type 2 inflammatory responses in airway inflammation in concert with IL-33 and TSLP.<sup>12,27,28</sup> We have recently found that IL-25, part of the epithelial “alarmins”, was highly produced locally in CRSwNP tissues,<sup>23</sup> which is further verified in this study. However, the potential sources of such local IL-25 accumulation in CRSwNP patients remain relatively uncharacterized, especially given that IL-25 levels can differ between patients.<sup>29</sup> Respiratory viral infections are increasingly associated with chronic allergic and inflammatory diseases since their presence often exacerbates the type 2 inflammation in inflamed airways – despite their conventional activation of type 1 inflammation for viral clearance.<sup>16,18,24,30,31</sup> Therefore, to investigate the ability of viral infections to induce expression of IL-25 that is strongly associated with type 2 inflammation, we infected in vitro differentiated hNECs from control and CRSwNP donors, and quantitatively characterized the induction of IL-25. Interestingly, we demonstrated that among the viruses tested, influenza virus contributed the most to IL-25 expression in control and CRSwNP epithelium alike. The increased IL-25 correlated with the antiviral response genes induced by viral infections, with influenza infection inducing the strongest antiviral responses, as well as the highest IL-25 expression.

Respiratory virus infections are known to aggravate chronic allergic diseases via epithelial cytokines IL-25, IL-33 and TSLP, but most studies focus on the induction of type 2 inflammation in asthmatic patients, with emphasis on the lower airway.<sup>32,33</sup> To date, there are limited reports that document detailed analysis of the epithelial cytokines during respiratory virus infections with regards to upper airway diseases such as CRSwNP. In our study, we demonstrated that respiratory virus infections, especially influenza, can contribute towards IL-25 accumulation in upper airway epithelium, which may help predispose or perpetuate type 2 inflammation in CRSwNP pathogenesis. This finding is interesting considering that influenza infection is a less common exacerbator of type 2 inflammation, due to the relatively strong type 1 inflammation it induces to counteract type 2 responses.<sup>20,21</sup> Nevertheless, influenza virus may induce severe exacerbation under specific circumstances. Therefore, we selected influenza virus for further studies

due to its ability to induce the highest IL-25 expression in the model of differentiated hNECs derived from CRSwNP tissues.

CRSwNP is a complex disease but the consensus is that it is generally classified as a type 2 inflammatory disease with eosinophilic mucosal inflammation, with type 2 inflammatory cytokines such as IL-5 and IL-13 being treatment targets.<sup>34–36</sup> As the first line of mechanical and immunological defense against external stimuli, the airway epithelium is an important local source of pro-inflammatory cytokines, including the type 2 inflammation promoting IL-25, IL-33, and TSLP.<sup>37,38</sup> Therefore, the modulation of these cytokines as a strategy for management of chronic allergic airway diseases such as asthma and CRSwNP has been intensively researched.<sup>7,18,32,33,39</sup> In recent years, it was shown that proper management of viral infections may lead to better outcomes during exacerbations of chronic allergic and inflammatory airway diseases.<sup>10,16,18</sup> These studies highlighted that viral infections are a constant trigger of CRSwNP exacerbations. In view of the ever-present threat of emerging viruses, as exemplified by the recently emerging SARS-CoV-2 virus, it is hence desirable to identify interventions that can appropriately alleviate the key triggers of exacerbation in CRSwNP management.<sup>16</sup>

Studies of airway epithelium in asthmatic patients have pointed out deficiencies or dysregulations in the expression of antiviral molecules and type 2 inflammatory cytokines<sup>32,40–42</sup> highlighting the importance of antiviral responses under type 2 inflammatory conditions in the airways. IL-25 has been accepted as the primary mediator of airway inflammation in asthmatic patients by promoting type 2 inflammation. Several studies explored the role of IL-25 in promoting type 2 inflammatory response in CRSwNP patients.<sup>7,43</sup> Hence, we conducted this study which identified influenza virus as a potential factor that can contribute IL-25 expression and accumulation in CRSwNP epithelium. Interestingly, when influenza virus replication was inhibited by pretreatment with recombinant IFN- $\alpha$  protein, a reduction of IL-25 expression in the CRSwNP hNECs was observed. This indicates that IFN- $\alpha$  does not additively induce IL-25 with influenza infection; but instead decreases the viral load and modulates overall IL-25 expression in infected hNECs via reduction of active infection. This bimodal effect of IFN- $\alpha$  has hitherto not been reported, and thus offers insights into IFN's role in modulating IL-25 in the presence or absence of active infection. This finding also suggests that the reduction of

IL-25 using broad-spectrum antivirals may constitute a viable strategy in the management of CRSwNP exacerbations. This is further supported by the randomized controlled clinical trial by Gao et al<sup>26</sup> who reported that the usage of IFN- $\alpha$  nasal spray may prevent infections caused by influenza A and B viruses, parainfluenza viruses, and adenovirus, without severe adverse effects. Thus, nasal spray containing IFNs may be useful for preventing viral infections that can contribute to the accumulation of IL-25 in CRSwNP.

One limitation of this study is that the *in vitro* hNEC model was unable to show a direct link to enhanced type 2 inflammation due to the lack of immune cells in the system. While co-culture of the CRSwNP hNECs with immune cells is possible, the experimental system needs to be established with hNECs and immune cells from the same donor to prevent allogenic activation. Given that influenza virus stimulated the highest local epithelial IL-25 production, further experiments are warranted on IFN- $\alpha$  to account for its downstream effectors and interactions with cells such as group 2 innate lymphoid cells (ILC2s). It will therefore be interesting to include the co-culture of ILC2s to further explore the effects of influenza and other viral infections (rhinovirus, RSV) on the activation of type 2 inflammation. In addition, while we were able to correlate active viral infection and antiviral responses with IL-25 induction in CRSwNP epithelium, we are unable to further discern the mechanisms directly leading to the IL-25 induction during active influenza infection. Therefore, future studies should focus on studying the factors (eg TLR7, RIG-I) associated with IL-25 induction and evaluating their effects on IL-25 expression levels (eg by knockdown experiments of these factors).

## Conclusions

In conclusion, our data, together with other studies, have demonstrated that respiratory viruses (especially influenza virus) can directly induce the expression of IL-25 in active virus-infected nasal epithelial cells, which may contribute to the accumulation of IL-25 in CRSwNP epithelium (based on the working hypothesis summarized in [Figure S5](#)). Using influenza infection, we have also provided several lines of evidence that active viral replication is positively correlated with the expression of IL-25, and that pretreatment with low doses of IFN- $\alpha$  abates influenza-dependent IL-25 production in CRSwNP epithelium. We also reported for the first time, potential bimodal functions of IFN- $\alpha$  which modulates IL-25 expression with active influenza infection, but by itself may

moderately increase IL-25 expression (though not to the extent of active infection). Also noteworthy was the strong correlation between mRNA and protein levels of IL-25 in our study, indicating that IL-25 mRNA from epithelial tissue can serve as a marker for IL-25 secretion. Our findings may aid in designing novel strategies for the optimal prevention and management of chronic inflammatory upper airway diseases such as CRSwNP via the broad-spectrum amelioration of its viral triggers.

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## Author Contributions

HH, DYW, VC, and YY conceived and designed the study; HH, YO, JL, KST, and FC carried out all *in vivo* and *in vitro* experiments; FC, YKO, MT, and HH collected and processed the clinical nasal mucosa samples. KST, HHO, YO, RS and VC performed the *in vitro* viral infection experiments. HH, YY, KST, and FC did the statistical analyses. All authors contributed to data interpretation. HH, KST, VC and DYW wrote the original and/or revised draft of the manuscript. All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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## Disclosure

The authors declare no conflicts of interest in this work.

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