IL-13 modulates Interferon-mediated ACE2 expression in Upper Airway Epithelial Cells

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Capsule Summary: This study presents evidence of IL-13-mediated *ACE2* gene reduction in the human upper respiratory epithelium, in addition to demonstrating ability to significantly repress IFN-mediated *ACE2* upregulation.

Key words: sinonasal airway epithelium, chronic rhinosinusitis, cell culture, SARS-CoV-2, *ACE2*, IFNα

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To the Editor:

SARS-CoV-2 primarily causes respiratory illness and presents with a range of severities (COVID-19). While the majority of cases are thought to be asymptomatic or mild, some individuals develop severe disease with pneumonia and acute respiratory distress syndrome (ARDS), and there is a sizeable fatality rate in high-risk groups [1]. Some of the risk factors identified for COVID-19 disease include older age, diabetes, obesity, cardiovascular disease, and an immunocompromised state [1]. While the US Centers for Disease Control and the World Health Organization mention asthma as a COVID-19 risk factor, the ARIA-EAACI statement suggests a more complex relationship without clear evidence that asthma is a risk factor for severe COVID-19. Furthermore, it is unestablished whether individuals with other chronic respiratory illnesses, such as chronic rhinosinusitis (CRS), are at elevated risk for COVID-19 disease.

Increased expression of the receptor for SARS-CoV-2, angiotensin-converting enzyme (*ACE2*), is a theoretical risk factor for infection supported by *in vitro* data [2]. Secretion of interferon (IFN) upon viral infection results in the induction of a large family of interferon-stimulated genes (ISGs), many of which have antiviral activity. Ziegler et al. demonstrated that *ACE2* is an ISG that is upregulated upon treatment with IFNα in undifferentiated human airway cell cultures [3]. Subsequently, it was reported that *ACE2* expression is downregulated by the type 2 cytokine IL-13 [4], [5]. A further study reported that brush airway samples from cohorts of asthmatics displayed reduced *ACE2* expression [6].

The lack of published evidence to support chronic type 2 airway inflammatory diseases, such as asthma and chronic rhinosinusitis with nasal polyps (CRSwNP), as risk factors for COVID-19 raises the intriguing possibility that reduced *ACE2* expression in the upper

respiratory airway may be protective against SARS-CoV-2 infection. To investigate this possibility, we compared *ACE2* expression in total RNA from sinonasal tissue of CRSwNP and control subjects. In a series of experiments, we analyzed the effect of the type 2 cytokine IL-13 on *ACE2* expression in differentiated epithelial cell cultures and investigated the modulating effects of IL-13 and IFNα.

ACE2 is primarily expressed in the goblet/secretory and ciliated cells of the nasal epithelium [7]. For SARS-CoV-2, ACE2 expression is suggested to be the limiting factor for viral entry [7]. We found that the expression of *ACE2* mRNA was significantly lower in sinonasal tissue of CRSwNP compared to control subjects (18 vs. 14 subjects, respectively, Figure 1A). We also observed significantly lower *TMPRSS2* expression in whole tissue (Figure 1A). *TMPRSS2*, which has been shown to be important for priming of the viral spike (S) protein [8], has a broad tissue distribution profile but is present in only a subset of $ACE2^{+}$ cells [7].

IL-13 is a pleiotropic cytokine shown to be important in the pathogenesis of CRS and other type-2 related diseases [9]. To understand the expression of *ACE2* and *TMPRSS2* in an IL-13-enriched cellular environment, we treated fully differentiated human sinonasal epithelial cultures (hSNEC) with increasing concentrations of IL-13 (1, 10, and 50 ng/ml). Within 24 hours, *ACE2* expression was significantly downregulated with increasing doses (10ng/ml and 50ng/ml) of IL-13-treated cells (Figure 1B). However, *TMPRSS2* expression trended higher, with significantly greater expression after 50 ng/ml IL-13 treatment (Figure 1B). This observation is in agreement with recent publications showing similar opposing effects of IL-13 on *ACE2* and *TMPRSS2* expression *in vitro* [4][5]. Interestingly, our data further shows a dose-dependent effect of IL-13 on *ACE2* expression, and it would be of great interest to understand whether that leads to variable vulnerability to viral attachment and entry. We then performed a two-week time

course analysis, which suggested that prolonged exposure to IL-13 maintains repression of *ACE2* expression (Figure 1C). *TMPRSS2* expression continued to be significantly upregulated for the two-week duration (Supplementary Figure 1). Finally, our data conclusively showed that *ACE2* expression was significantly reduced in hSNECs treated with IL-13 for 24 hours, in five independent experiments on different patient tissues (Figure 1D). Furthermore, multiple independent studies on different patient tissues after one- and two-week IL-13 treatment also indicated a continued decreased level of *ACE2* expression (Supplementary Figure 2). Our *in vitro* data corresponds well with *ACE2* expression in tissue samples obtained from CRSwNP and control subjects, suggesting that a chronic type 2 inflammatory microenvironment with high IL-13 may act to limit ACE2 on the epithelial cell surface available for SARS-CoV-2 binding.

Given the report by Ziegler et al. [3] that ACE2 is an ISG, we explored whether IFN treatment can reverse IL-13-mediated repression of *ACE2* in hSNECs. First, we titrated the IFNα concentration and observed that 120×10^2 Units/ml induced the greatest *ACE2* expression after 12 hours of apical treatment of hSNECs (Supplementary Figure 3). IFNα stimulation of untreated and IL-13 pre-treated (7 days) hSNECs showed significantly reduced *ACE2* expression in the IL-13 pretreated cells (Figure 2A). Therefore, IFNα was unable to reverse IL-13-mediated repression of *ACE2* expression. We furthermore observed that IFNα did not counter the IL-13 mediated repression of other ISGs, including *IFITM2*, *IFITM3*, and *GBP5* (Supplementary Figure 4). Finally, we investigated whether IL-13 can reverse *ACE2* expression induced by IFNα-pretreatment in hSNECs. While 72 hour IFNα treatment significantly increased *ACE2* expression, subsequent addition of IL-13 for 24 hours resulted in significantly reduced *ACE2* expression (Figure 2B). Similar results were observed in two independent experiments on tissue from different patients, suggesting that IL-13 suppresses IFNα-induced *ACE2* expression.

Taken together, our results suggest that the suppression of *ACE2* expression by IL-13 is dominant to IFN upregulation in the upper airway epithelium.

In summary, we provide evidence here that IL-13, a well characterized cytokine important in the pathogenesis of many type 2 inflammatory diseases, reduces *ACE2* expression in the upper airway epithelium. Furthermore, our data demonstrate not only that IFNα cannot overcome IL-13-mediated repression of ACE2, but that IL-13 suppresses the up-regulation of *ACE2* induced by IFNα. While more studies are needed to understand the effect of IL-13 at molecular level, these initial results could have important implications for strategies to prevent and treat COVID-19 disease.

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REFERENCES

[1] W. J. Guan *et al.*, "Clinical Characteristics of Coronavirus Disease 2019 in China," *N. Engl. J. Med.*, 2020, doi: 10.1056/NEJMoa2002032.

[2] H. P. Jia *et al.*, "ACE2 Receptor Expression and Severe Acute Respiratory Syndrome Coronavirus Infection Depend on Differentiation of Human Airway Epithelia," *J. Virol.*, 2005, doi: 10.1128/jvi.79.23.14614-14621.2005.

[3] C. Ziegler *et al.*, "SARS-CoV-2 Receptor ACE2 is an Interferon-Stimulated Gene in Human Airway Epithelial Cells and Is Enriched in Specific Cell Subsets Across Tissues," *SSRN Electron. J.*, 2020, doi: 10.2139/ssrn.3555145.

[4] S. P. Sajuthi *et al.*, "Type 2 and interferon inflammation strongly regulate SARS-CoV-2 related gene expression in the airway epithelium," *bioRxiv*, 2020, doi: 10.1101/2020.04.09.034454.

[5] H. Kimura *et al.*, "Type 2 Inflammation Modulates ACE2 and TMPRSS2 in Airway Epithelial Cells," *J. Allergy Clin. Immunol.*, 2020, doi: https://doi.org/10.1016/j.jaci.2020.05.004.

[6] D. J. Jackson *et al.*, "Association of Respiratory Allergy, Asthma and Expression of the SARS-CoV-2 Receptor, ACE2," *J. Allergy Clin. Immunol.*, 2020, doi: 10.1016/j.jaci.2020.04.009.

[7] W. Sungnak *et al.*, "SARS-CoV-2 entry factors are highly expressed in nasal epithelial cells together with innate immune genes," *Nat. Med.*, 2020, doi: 10.1038/s41591-020-0868-6.

[8] M. Hoffmann *et al.*, "SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor," *Cell*, 2020, doi: 10.1016/j.cell.2020.02.052.

[9] C. Bachert, L. Zhang, and P. Gevaert, "Current and future treatment options for adult chronic rhinosinusitis: Focus on nasal polyposis," *Journal of Allergy and Clinical Immunology*. 2015, doi: 10.1016/j.jaci.2015.10.010.

MATERIALS & METHODS

Human Subjects

Forty-five adult subjects undergoing endonasal surgical procedures were enrolled in this study to allow collection of epithelial cells from brushings (CytoSoft Cytology Brush, Medical Packaging Corperation, Camarillo, CA) or sinonasal surgical tissue samples. The Johns Hopkins Institutional Review process approved the research protocol and all subjects signed informed consent statement. The CRSwNP patients were characterized by continuous symptoms of rhinosinusitis for at least 12 weeks as defined by the American Academy of Otolaryngology–Head and Neck Surgery (AAO-HNS) Chronic Rhinosinusitis Task Force and the EPOS guidelines. The computed tomography (CT) of the sinuses revealed isolated or diffuse sinus mucosal thickening or air-fluid levels, and nasal polyps visible on diagnostic endoscopy. Participating subjects had no history of tobacco use, cystic fibrosis, ciliary dyskinesia, systemic inflammatory or autoimmune disease, or immunodeficiency. Control subjects had no signs of CRS and were undergoing endoscopic sinonasal procedure for cerebrospinal fluid leak repair or orbital decompression.

Sinonasal tissue and brushing processing

Human sinus tissue was digested with 0.05 mg/mL Liberase TL (Roche) and 0.5 mg/ml DNaseI (Sigma) for 45 min at 37°C and 5% CO_2. The digested tissue was filtered through a 70-µm cell strainer (BD Biosciences). Liberase was inactivated with the addition of RPMI 1640 (Corning) media with 10% fetal bovine serum (FBS; Sigma). After centrifugation, the pellet was treated with ACK Lysis Buffer to lyse red blood cells. The lysis was stopped with RPMI (10% FBS) media and the cells were re-filtered with a 40-µm cell strainer (BD Biosciences) followed by

centrifugation. The pellet was resuspended in media and counted by trypan blue exclusion method. Sinonasal tissue was then grown in human collagen (Type IV, Sigma, St. Louis, MO) - coated culture dishes and expanded to confluence in expansion media (Pneumacult-Ex Plus, Stemcell Technologies, Vancouver, Canada). Upon confluency, the submerged cells were washed with PBS followed by trypsinization for 5-10 minutes. The detached cells were washed with PBS and seeded for ALI culture as described below. The sinonasal cells from brushings was obtained by gentle hand vortexing in PBS followed by centrifugation and lysis of RBCs with ACK buffer. The rest of the culturing process was similar to sinus tissue culture described above.

Air-Liquid Interface (ALI) Culture

Epithelial cells were seeded onto collagen-IV-coated (Sigma-Aldrich, St. Louis, MO) transwell plates (Corning, Corning, NY) and expanded to confluence in expansion media (Pneumacult-Ex Plus, Stemcell Technologies, Vancouver, Canada). After confluency, the apical media was aspirated and the basolateral media was replaced with ALI media (Pneumacult-ALI, Stemcell Technologies, Vancouver, Canada). The cells are considered fully differentiated after 21 days of ALI. After at least three weeks of differentiation, the ALI cells are treated with recombinant human IL-13 (BioLegend; Cat. No. 571102) and/or IFNα (Bio-Rad; Prod. Code PHP107Z) according to the experimental conditions described in the manuscript.

RNA extraction and real-time PCR

RNA was extracted from frozen human sinonasal tissue or ALI cultured cells using TRIzol (Invitrogen). Complementary DNA (cDNA) was generated by reverse transcribing 0.15 to 1 µg of RNA using SuperScript III and random primers (Invitrogen kit). cDNA was diluted five- to

ten-fold using RNase/DNase-free double-distilled water before polymerase chain reaction (PCR). Real-time PCR was performed using appropriate TaqMan Fast Advanced master mix and TaqMan primer probes (Thermo Fisher Scientific): *ACTB* (Hs99999903_m1), *ACE2* (Hs1085333_m1), *TMPRSS2* (Hs01122322_m1), *IFITM2* (Hs00829485_sH), *IFITM3* (Hs03057129_s1), and *GBP5* (Hs00369472_m1).

FIGURE LEGENDS

Figure 1. ACE2 gene expression is reduced in sinonasal epithelium of CRSwNP subjects and IL-13 can recapitulate the effect in an in vitro model. (A) Whole tissue mRNA expression of ACE2 and TMPRSS2 genes from a total of 14 control and 18 CRSwNP subjects were analyzed by real-time PCR. (B) The effect of three different doses—1, 10, and 50 ng/ml—of recombinant human IL-13 on ACE2 and TMPRSS2 gene expression in differentiated ALI culture cells. The culture cells were stimulated for 24 hours before RNA extraction and real-time PCRs were performed. Each condition was tested in triplicates. (C) Time-course of the effect of recombinant human IL-13 (50 ng/ml) on ACE2 gene expression in differentiated ALI culture cells. The samples were collected on days 1, 2, 5, 7, 9, 12, and 14 post-stimulation. Each condition was tested in triplicates. (D) ACE2 gene expression in IL-13 stimulated differentiated ALI culture cells. The cells were stimulated with 50 ng/ml recombinant human IL-13 for 24 hours before RNA extraction and real-time PCR. The results represent five independent experiments with three replicates in each experiment. (A), (B), and (C) The results are represented as $1/2^{\Delta CT}$, where ΔCT was calculated by normalizing target gene CT value to β-actin housekeeping gene. (D) Fold change was calculated by normalizing the $1/2^{\Delta CT}$ value of IL-13 stimulated cells to the value of media wells. (A) Statistics was calculated using Mann-Whitney nonparametric t-test with significance listed. (B) and (C) Statistics was calculated using 2-way ANOVA and applying Tukey's multiple comparison test with significance at $p < 0.05$. (D) Statistics was calculated using One sample t and Wilcoxon test.

Figure 2. IL-13 can reduce the expression of IFNα-mediated ACE2 expression in differentiated upper airway epithelium. (A) ACE2 gene expression in IL-13 treated differentiated ALI culture

cells followed by stimulation with IFNα. The cells were treated basolaterally with 50 ng/ml of recombinant human IL-13 for 7 days before apical stimulation with 120×10^2 Units/ml of IFNα for 12 hours. RNA extraction and real-time PCR was performed subsequently. (B) ACE2 gene expression in IFNα-stimulated differentiated ALI culture cells followed by treatment with IL-13. The cells were stimulated basolaterally with 120×10^2 Units/ml of IFNα for 48 hours before basolateral addition of 120×10^2 Units/ml of IFNα and 50 ng/ml recombinant human IL-13 for 24 hours. Results in (A) and (B) are representative of two independent experiment. The results are represented as $1/2^{\Delta CT}$, where ΔCT was calculated by normalizing target gene CT value to β-actin housekeeping gene. Statistics was calculated using 2-way ANOVA and applying Tukey's multiple comparison test with significance at $p < 0.05$.

Supplementary Figure 1. Time-course of the effect of recombinant human IL-13 (50 ng/ml) on TMPRSS2 gene expression in differentiated ALI culture cells. The samples were collected on days 1, 2, 5, 7, 9, 12, and 14 post-stimulation. Each condition was tested in triplicates. The results are represented as $1/2^{\Delta CT}$, where ΔCT was calculated by normalizing target gene CT value to β-actin housekeeping gene. Statistics was calculated using 2-way ANOVA and applying Tukey's multiple comparison test with significance at $p < 0.05$.

Supplementary Figure 2. ACE2 gene expression in IL-13 (50 ng/ml) stimulated differentiated ALI culture cells before RNA extraction and real-time PCR. The results represent four (1-week stimulation) or three (2-week stimulation) independent experiments on different patient tissue with three replicates in each experiment. (Exception: One independent experiment for 1-week

stimulation only had a single well for each condition) Fold change was calculated by normalizing the $1/2^{\Delta CT}$ value of IL-13 stimulated cells to the value of media wells.

Supplementary Figure 3. ACE2 gene expression after stimulation with different doses of IFNα. Fully differentiated ALI culture cells were stimulated apically for 12 hours with 6×10^2, 24×10^2, or 120×10^2 Units/ml of IFNα. Apical inoculum was removed and the ALI cells were washed with PBS before collection for RNA analysis.

Supplementary Figure 4. Gene expression of interferon stimulated genes (ISGs) IFITM2, IFITM3, and GBP5 in IL-13 treated differentiated ALI culture cells followed by stimulation with IFNα. The cells were treated basolaterally with 50 ng/ml of recombinant human IL-13 for 7 days before apical stimulation with 120×10^2 Units/ml of IFNα for 12 hours. RNA extraction and real-time PCR was performed subsequently. The results are represented as $1/2^{\Delta CT}$, where ΔCT was calculated by normalizing target gene CT value to β-actin housekeeping gene. Statistics was calculated using 2-way ANOVA and applying Tukey's multiple comparison test with significance at $p < 0.05$.