

Proton pump inhibitors decrease eotaxin-3/CCL26 expression in patients with chronic rhinosinusitis with nasal polyps: Possible role of the nongastric H,K-ATPase

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Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is often characterized by tissue eosinophilia that is associated with poor prognosis. Recent findings that proton pump inhibitors (PPIs) directly modulate the expression of eotaxin-3, an eosinophil chemoattractant, in patients with eosinophilic diseases suggest therapeutic potential for PPIs in those with CRSwNP.

Objective: We assessed the effect of type 2 mediators, particularly IL-13 and eotaxin-3, on tissue eosinophilia and

disease severity in patients with chronic rhinosinusitis (CRS). Further investigation focused on PPI suppression of eotaxin-3 expression *in vivo* and *in vitro*, with exploration of underlying mechanisms.

Methods: Type 2 mediator levels in nasal tissues and secretions were measured by using a multiplex immunoassay. Eotaxin-3 and other chemokines expressed in IL-13-stimulated human sinonasal epithelial cells (HNECs) and BEAS-2B cells with or without PPIs were assessed by using ELISA, Western blotting, real-time PCR, and intracellular pH imaging.

Results: Nasal tissues and secretions from patients with CRSwNP had increased IL-13, eotaxin-2, and eotaxin-3 levels, and these were positively correlated with tissue eosinophil cationic protein levels and radiographic scores in patients with CRS ($P < .05$). IL-13 stimulation of HNECs and BEAS-2B cells dominantly induced eotaxin-3 expression, which was significantly inhibited by PPIs ($P < .05$). Patients with CRS taking PPIs also showed lower *in vivo* eotaxin-3 levels compared with those without PPIs ($P < .05$). Using intracellular pH imaging and altering extracellular K^+ , we found that IL-13 enhanced H^+ , K^+ -exchange, which was blocked by PPIs and the mechanistically unrelated H,K-ATPase inhibitor, SCH-28080. Furthermore, knockdown of *ATP12A* (gene for the nongastric H,K-ATPase) significantly attenuated IL-13-induced eotaxin-3 expression in HNECs. PPIs also had effects on accelerating IL-13-induced eotaxin-3 mRNA decay.

Conclusion: Our results demonstrated that PPIs reduce IL-13-induced eotaxin-3 expression by airway epithelial cells. Furthermore, mechanistic studies suggest that the nongastric H,K-ATPase is necessary for IL-13-mediated epithelial responses, and its inhibitors, including PPIs, might be of therapeutic value in patients with CRSwNP by reducing epithelial production of eotaxin-3. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Chronic rhinosinusitis, eotaxin-1/CCL11, eotaxin-2/CCL24, eotaxin-3/CCL26, omeprazole, proton pump inhibitors, IL-13, epithelial cells, $H(+)-K(+)$ -exchanging ATPase, eosinophils

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Chronic rhinosinusitis (CRS) is characterized by local inflammation of the sinonasal mucosa, with symptoms persisting for at least 12 weeks.¹ It is further classified into 2 clinical phenotypes: chronic rhinosinusitis with nasal polyps (CRSwNP) and CRS

Abbreviations used

CRS:	Chronic rhinosinusitis
CRSwNP:	Chronic rhinosinusitis with nasal polyps
ECP:	Eosinophil cationic protein
EoE:	Eosinophilic esophagitis
GERD:	Gastroesophageal reflux disease
gH,K-ATPase:	Gastric H,K-ATPase
HBEC:	Human bronchial epithelial cell
HNEC:	Human sinonasal epithelial cell
IT:	Inferior turbinate
[K ⁺] _e :	Extracellular K ⁺ concentration
ngH,K-ATPase:	Nongastric H,K-ATPase
NP:	Nasal polyp
pH _i :	Intracellular pH
PPI:	Proton pump inhibitor
siRNA:	Small interfering RNA
STAT6:	Signal transducer and activator of transcription 6
UT:	Uncinate tissue

without nasal polyps.¹⁻³ In Western populations, CRSwNP is frequently associated with type 2 inflammation and tissue eosinophilia.⁴ Because tissue eosinophilia has been implicated in increased postsurgical recurrence rates^{5,6} and decreased improvement in quality-of-life outcomes,⁷ strategies for blocking eosinophil recruitment could improve treatment for CRSwNP.

Eosinophil recruitment is generally regulated by type 2 cytokines (eg, IL-4, IL-5, and IL-13) and CC chemokines (eg, eotaxin-1/CCL11, eotaxin-2/CCL24, eotaxin-3/CCL26, and monocyte chemoattractant protein 4/CCL13).⁸⁻¹⁰ Among the cytokines, IL-13 appears to drive epithelial responses, including barrier dysfunction, mucus overproduction, and production of chemokines in type 2 inflammatory airway diseases.¹¹⁻¹⁴ The induced eotaxins are ligands for CCR3, which is highly expressed on eosinophils.^{9,15-19} Of the eotaxins expressed by human subjects, recent studies increasingly emphasize a critical role for eotaxin-3 in patients with eosinophilic diseases, showing greater and more sustained eosinophil recruitment in asthma^{8,20} and strong associations with susceptibility to eosinophilic esophagitis (EoE).²¹

In patients with CRSwNP, increased levels of type 2 mediators and type 2 cytokine-producing cells, such as T_H2 cells and group 2 innate lymphoid cells, are found in nasal mucosa,²²⁻²⁵ supporting a crucial role of tissue eosinophilia in CRSwNP pathogenesis. However, whether eotaxin-3 is associated with eosinophilic responses in patients with CRSwNP is yet to be established.

Although novel mAbs, such as mepolizumab (anti-IL-5) and dupilumab (anti-IL-4 receptor α), have shown promise for CRSwNP treatment,^{26,27} cost, parenteral administration, and lack of clinical approval for a CRSwNP indication will foreseeably limit access to these agents.²⁸ Interestingly, recent studies have shown that proton pump inhibitors (PPIs), which are used traditionally for treating gastroesophageal reflux disease (GERD), suppress IL-13-induced eotaxin-3 production in esophageal squamous cells²⁹⁻³¹ and have clinically relevant antieosinophil effects in patients with EoE, even in those without coexisting GERD.^{31,32} Because PPIs are not used for treating CRSwNP, mechanistic evidence that PPIs can also directly suppress IL-13 responses in the upper airway might open new avenues for treating this common chronic inflammatory condition.

Given the biological parallels between EoE and CRSwNP,³³ we characterized the relationship of type 2 mediators, particularly IL-13 and eotaxin-3, with tissue eosinophilia and disease severity in patients with CRSwNP. We further evaluated the relative production of eotaxins by IL-13-stimulated airway cells *in vitro* and explored the efficacy of PPIs on inhibiting eotaxin-3 expression *in vivo* and *in vitro*. Finally, we investigated potential mechanisms through which PPIs suppressed IL-13-induced eotaxin-3 expression in airway epithelial cells.

METHODS**Subjects and sample collection**

Healthy control subjects and patients with CRS^{2,34} were recruited from the Otolaryngology and Allergy-Immunology Clinics at Northwestern Medicine. Computed tomographic scans were graded according to the methods defined by Okushi et al,³⁵ and the history of taking PPIs listed in preoperative anesthesia records on the day of sinus surgery was obtained. Subjects' characteristics are included in Table E1 in this article's Online Repository at www.jacionline.org. All subjects provided informed consent. The Institutional Review Board of Northwestern University-Feinberg School of Medicine approved this study. Tissue specimens, including uncinate tissue (UT) and nasal polyps (NPs); nasal lavage fluid; and epithelial scrapings from the inferior turbinate (IT) and NPs were obtained from subjects and prepared, as previously described.^{36,37} Further details are provided in the **Methods** section in this article's Online Repository at www.jacionline.org.

Measurement of cytokines, eotaxins, and eosinophil cationic protein levels in specimens

We measured IL-4, IL-13, eotaxin-1, eotaxin-2, and eotaxin-3 levels by using the Milliplex Map Kit (EMD Millipore, Billerica, Mass) with a Luminex 200 instrument (Life Technologies, Gaithersburg, Md). We measured eosinophil cationic protein (ECP) levels using the Mesacup ECP Test (MBL International, Woburn, Mass). Tissue concentrations of these mediators were normalized to the total protein concentration measured by using the Bicinchoninic Acid Protein Assay (Thermo Fisher Scientific, Waltham, Mass).

Cell culture

BEAS-2B, a human bronchial epithelial cell (HBEC) line transformed with a hybrid adenovirus 12-simian virus 40, was obtained from ATCC (Manassas, Va). Primary human sinonasal epithelial cells (HNECs) were collected by means of epithelial scraping of IT and NPs and cultured. For cytokine (PeproTech, Rocky Hill, NJ) stimulation, submerged cultured cells were treated with 1 to 100 ng/mL IL-13, 10 ng/mL IFN- γ , 100 ng/mL TNF, or 50 ng/mL IL-17 for 6 or 48 hours. Cells were pretreated for 2 hours with acid-activated omeprazole (0.1-50 μ M/L) or other PPIs (lansoprazole, rabeprazole, pantoprazole, and esomeprazole [1-50 μ M/L]) before stimulation with 5 ng/mL IL-13 to study the effects of PPIs (Sigma-Aldrich, St Louis, Mo) on cytokine-induced chemokines. Additionally, SCH-28080 (1-50 μ M/L, Sigma-Aldrich) was used with the same protocol. In experiments altering extracellular K⁺ concentration ([K⁺]_e), modified Ringer solution that contained different contents of K⁺ (0-11.2 mmol/L KCl, see Table E2 in this article's Online Repository at www.jacionline.org) was used as culture medium. For mRNA stability assessment, actinomycin D (3 μ g/mL, Sigma-Aldrich) was used, and eotaxin-3 mRNA was measured by using real-time PCR. Supernatants, whole-cell lysates, and total RNAs were harvested for further analysis. Further detailed methods are described in the **Methods** section in this article's Online Repository.

ELISA

Eotaxin-1, eotaxin-2, and eotaxin-3 protein concentrations in supernatants were determined with the appropriate ELISA kits, as detailed in the **Methods** section in this article's Online Repository.

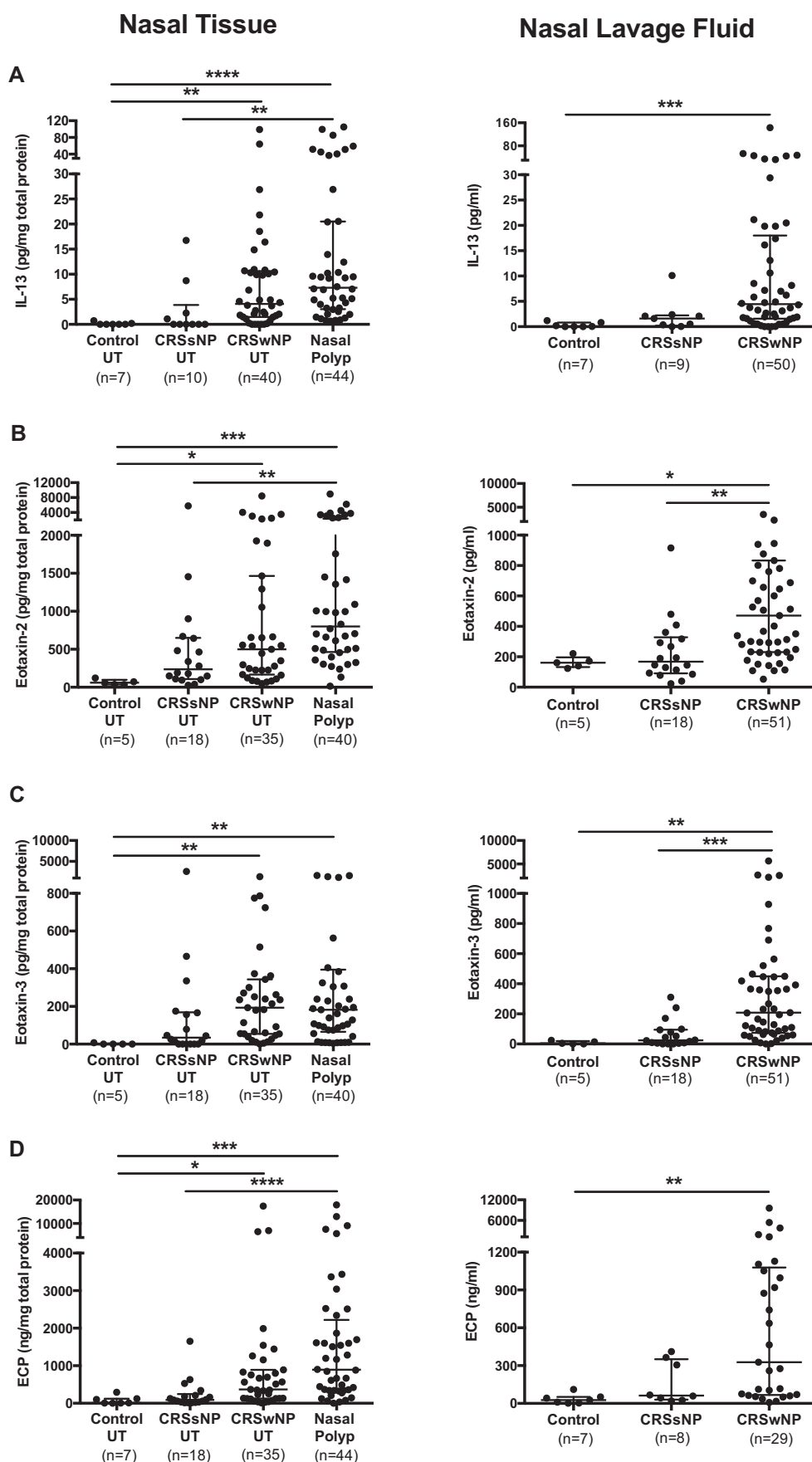


FIG 1. Increased levels of type 2 inflammatory mediators in nasal tissues and secretions of patients with CRSwNP. Protein levels of IL-13 (**A**), eotaxin-2 (**B**), eotaxin-3 (**C**), and ECP (**D**) were measured in UT, NPs, and nasal lavage fluid. Dot plots illustrate individual data points, and solid lines represent medians with interquartile ranges. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

TABLE I. Correlations between type 2 inflammatory mediators and tissue eosinophilia or radiographic severity

Type 2 inflammatory mediators	ECP in UT (total subjects*)		CT scores (patients with CRSwNP†)	
	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
In UT				
IL-13	0.84	<.0001	0.49	.002
Eotaxin-1	0.19	.31	0.34	.04
Eotaxin-2	0.70	<.0001	0.60	.0002
Eotaxin-3	0.54	.002	0.34	.049
ECP	—	—	0.58	.0003
In nasal lavage fluid				
IL-13	0.55	.001	0.11	.41
Eotaxin-1	0.12	.52	0.09	.49
Eotaxin-2	0.51	.003	0.15	.29
Eotaxin-3	0.49	.004	0.14	.31
ECP	0.26	.30	0.05	.81

Text in boldface indicates statistically significant correlations for that mediator and tissue eosinophilia or CT scores ($P < .05$).

CT, Computed tomography.

*N = 32 for correlations between ECP in UT with measures in UT and nasal lavage fluid.

†N = 34 and 55 for correlations between CT scores with measures in UT and nasal lavage fluid, respectively; UT tissue was not always available in instances of revision surgery.

Real-time PCR and Western blotting

mRNA levels of eotaxin-1, eotaxin-2, eotaxin-3, *CXCL1*, *CXCL10*, *ATP12A*, and *ATP4A* in total RNAs isolated from cells were measured by using quantitative real-time PCR. Western blots were performed to assess total signal transducer and activator of transcription 6 (STAT6), phosphorylated STAT6, and ATP12A protein in whole-cell lysates.³⁸ Further details are described in the [Methods](#) section in this article's Online Repository.

Intracellular pH imaging

The pH-sensitive pHrodo Green AM intracellular pH (pH_i) indicator (Life Technologies) that increases its fluorescence with decreasing pH_i was used.³⁹ Cells cultured in glass-bottom microwell dishes (MatTek, Ashland, Mass) were pretreated with omeprazole or vehicle before 6 hours of IL-13 stimulation. Then cells were incubated with dye (5 μ M/L) with live cell imaging solution (Life Technologies) at 37°C for 30 minutes per the manufacturer's instructions. Spinning disk confocal microscopy for live cell imaging was performed with Andor XDi Revolution (Andor Technologies, Belfast, United Kingdom). Fluorescence intensity was measured in 150 cells by using Image J software (National Institutes of Health, Bethesda, Md). For kinetic experiments, fluorescence intensity of cells cultured in 96-well plates with omeprazole, SCH-28080, or matched vehicle was measured at various times before and after IL-13 stimulation for up to 1 hour by using the SpectraMax Gemini EM Microplate Spectrofluorometer (Molecular Devices, Sunnyvale, Calif) at 485/538 nm (excitation/emission).

Small interfering RNA transfection

At 30% to 50% confluence, HNECs were transfected with 25 pmol ON-TARGETplus *ATP12A* small interfering RNA (siRNA) or nontargeting negative control siRNA (Dharmacon; GE Healthcare Life Sciences, Fairfield, Conn) in Lipofectamine RNAiMAX reagent (Life Technologies) per the manufacturer's instructions. At 96 hours after transfection, cells were treated with omeprazole or vehicle, followed by IL-13 stimulation for 6 hours. Knockdown efficiency was confirmed by using real-time PCR and Western blotting.

Statistical analyses

All data are reported as means $\pm$ SEMs, unless otherwise noted. A P value of less than .05 was considered significant. Further details are described in the [Methods](#) section in this article's Online Repository.

RESULTS

Levels of type 2 inflammatory mediators and their relationship with tissue eosinophilia and radiographic severity

We first assessed whether type 2 mediator *in vivo* levels were increased in patients with CRSwNP. Consistent with our recent study,²⁵ IL-13 levels, but not IL-4 levels (data not shown), were significantly increased in UT and NPs from patients with CRSwNP compared with control UT, with similar profiles in nasal lavage fluid (Fig 1, A). Among the eotaxins, eotaxin-2 (Fig 1, B) and eotaxin-3 (Fig 1, C) levels were significantly increased in tissues (UT and NPs) and lavage fluid of patients with CRSwNP compared with those of control subjects. Eotaxin-1 levels were significantly increased in NPs only compared with control UT (median, 61.0 vs 12.9 pg/mg total protein, respectively; $P < .05$). ECP levels were significantly increased in nasal tissues and secretions of patients with CRSwNP compared with those in control subjects (Fig 1, D).

We next evaluated the correlations between tissue eosinophilia, as determined based on ECP, and levels of type 2 mediators. ECP levels were significantly correlated with eotaxin-2, eotaxin-3, and IL-13 levels in UT and lavage fluid among all subjects (Table I). We further correlated radiographic severity³⁵ with these mediators in patients with CRSwNP and found that levels of all eotaxins, IL-13, and ECP in UT were significantly correlated with computed tomographic scores (Table I). Tissue and lavage eotaxin-2 and eotaxin-3 levels were also moderately correlated with UT IL-13 levels (see Table E3 in this article's Online Repository at www.jacionline.org). However, correlations carried out on type 2 mediators measured in NPs were uncorrelated with local eosinophilia and radiographic severity (see Table E4 in this article's Online Repository at www.jacionline.org).

Eotaxin-3 was the dominant eotaxin induced by IL-13 in airway epithelial cells

Given our *in vivo* findings, we evaluated the effect of IL-13 on production of the eotaxins in airway epithelial cells, including HNECs and BEAS-2B cells *in vitro*. We found that IL-13 significantly increased protein levels of all eotaxins in BEAS-2B cells (Fig 2, A) and HNECs (Fig 2, B). Notably, eotaxin-3 protein (Fig 2, A and B) and mRNA (see Fig E1, A and B, in this article's Online Repository at www.jacionline.org) expression was profoundly and concentration-dependently induced by IL-13 in both cell types. Considering that eotaxin-3 was most profoundly induced *in vitro* and was highly expressed and positively correlated with surrogate markers of tissue eosinophilia *in vivo*, we focused further experiments by using eotaxin-3 as our target mediator for stimulation with IL-13.

Omeprazole inhibited IL-13-induced eotaxin-3 production in airway epithelial cells

Next, we investigated whether recent findings that suggest omeprazole could inhibit IL-13-induced eotaxin-3 in esophageal squamous cells²⁹ could be replicated in airway epithelial cells. We found IL-13-induced eotaxin-3 protein secretion was significantly inhibited in BEAS-2B cells and HNECs treated with omeprazole at concentrations as low as 5 and 1 μ M/L,

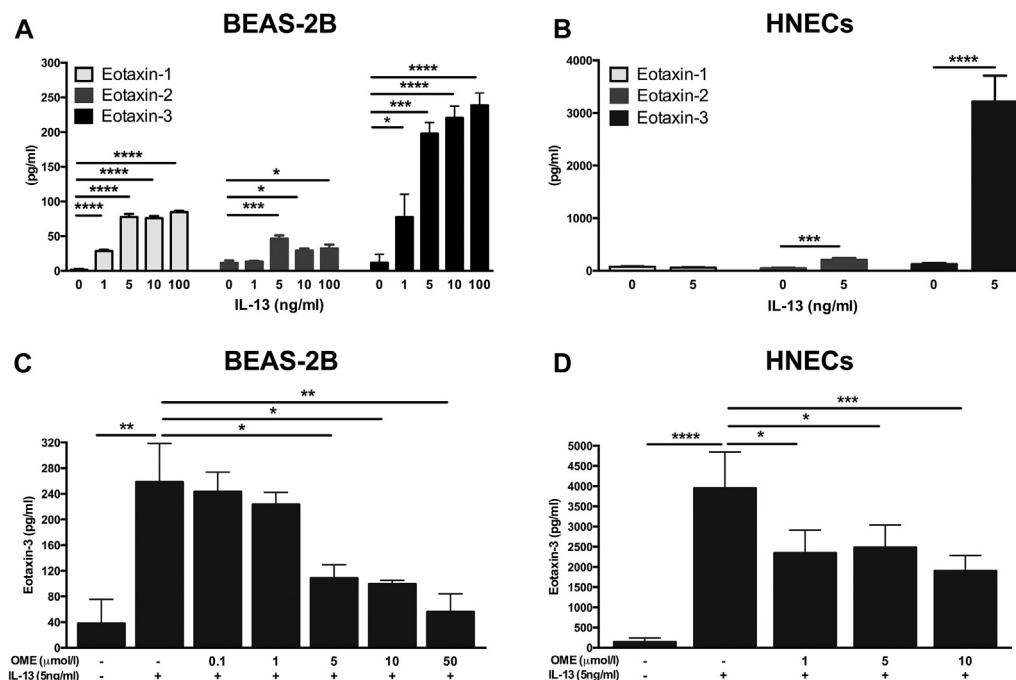


FIG 2. IL-13-induced eotaxins protein secretion and inhibitory effects of omeprazole (OME) in airway epithelial cells. **A** and **B**, BEAS-2B cells (Fig 2, **A**) and HNECs (Fig 2, **B**) were stimulated for 48 hours with IL-13. **C** and **D**, BEAS-2B cells (Fig 2, **C**) and HNECs (Fig 2, **D**) were pretreated with omeprazole for 2 hours and stimulated for 48 hours with IL-13. Eotaxins (Fig 2, **A** and **B**) and eotaxin-3 (Fig 2, **C** and **D**) levels in supernatants were measured by using ELISA. Data represent means $\pm$ SEMs of 3 (Fig 2, **A** and **C**), 15 (Fig 2, **B**), or 9 (Fig 2, **D**) independent experiments. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

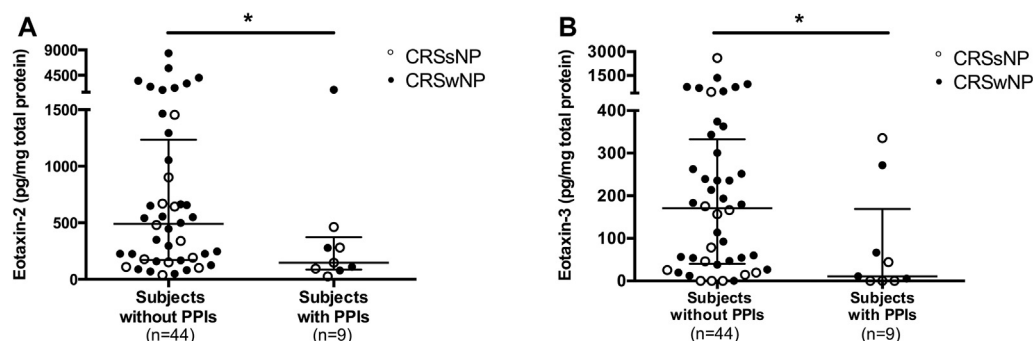


FIG 3. Eotaxin-2 and eotaxin-3 levels were decreased in patients with CRS taking PPIs at the time of sinus surgery. Protein levels of eotaxin-2 (**A**) and eotaxin-3 (**B**) in UT of patients with CRS taking PPIs and those without PPIs were measured by using multiplex immunoassay. Dot plots illustrate individual data points, and solid lines represent medians with interquartile ranges. * $P < .05$.

respectively (Fig 2, **C** and **D**). A similar pattern was observed in mRNA expression (see Fig E1, **C** and **D**).

To ensure that the observed effect was specific to IL-13–induced eotaxin-3 and not a result of general inhibition of gene expression, we measured mRNA expression of other chemokines (*CXCL10*, eotaxin-1, and *CXCL1*) in response to IFN- γ , TNF, and IL-17, respectively, with or without omeprazole pretreatment. These chemokines were significantly induced by their respective cytokines, as previously described,^{16,40,41} but their expression was not inhibited by omeprazole or other tested PPIs in BEAS-2B cells (see Fig E2 in this article's Online Repository at www.jacionline.org).

Association of PPI use and *in vivo* eotaxins levels in patients with CRS

Because we found the inhibitory effect of omeprazole on IL-13–induced eotaxin-3 expression in airway epithelial cells, we sought to determine whether *in vitro* findings might have *in vivo* effects. On medical record review, 9 (17%) of our patients with CRS were identified as taking PPIs, including omeprazole ($n = 5$), esomeprazole ($n = 1$), lansoprazole ($n = 2$), and rabeprazole ($n = 1$), at the time of sinus surgery. Interestingly, subjects taking PPIs had significantly lower eotaxin-2 and eotaxin-3 levels in UT compared with subjects without PPIs (Fig 3). Similar trends were observed in tissue

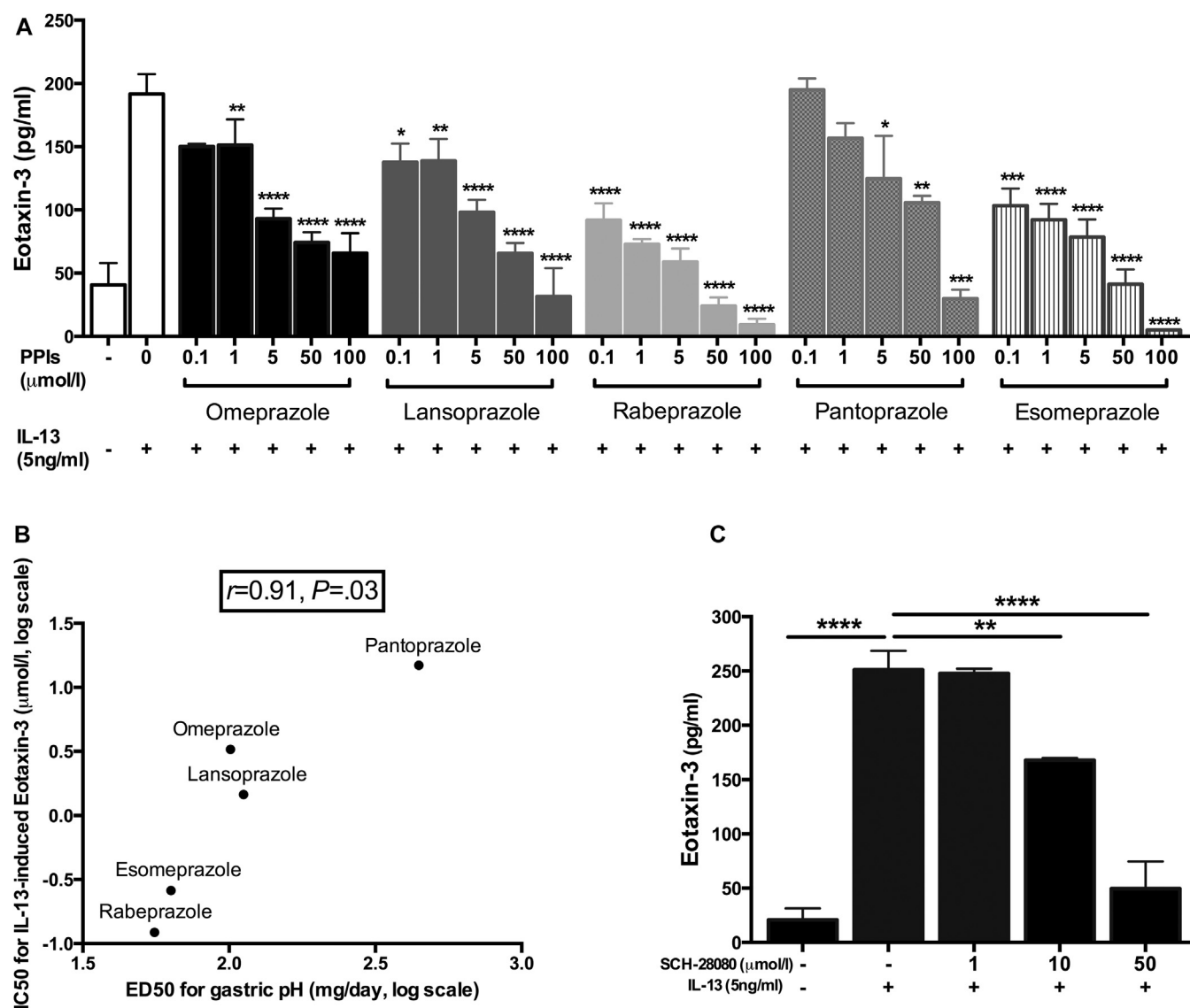


FIG 4. H,K-ATPase inhibitors decreased IL-13-induced eotaxin-3 protein secretion. **A**, BEAS-2B cells were pretreated for 2 hours with PPIs, followed by IL-13 stimulation for 48 hours. Eotaxin-3 levels in supernatants were measured by using ELISA. **B**, Correlations between the measured inhibitory concentration of 50% of PPIs for IL-13-induced eotaxin-3 with published median effective dose of PPIs for gastric pH.⁴² **C**, SCH-28080 was used with the same protocol as in Fig 4, A. Data represent means $\pm$ SEMs of 3 independent experiments. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$ compared with vehicle-treated/IL-13-stimulated cells (Fig 4, A and C).

eotaxin-1 and ECP levels, although these did not achieve statistical significance (data not shown).

Other PPIs and SCH-28080 inhibited IL-13-induced eotaxin-3 expression

Like omeprazole, other PPIs, including lansoprazole, rabeprazole, pantoprazole, and esomeprazole, showed dose-dependent inhibitory effects on IL-13-induced eotaxin-3 protein secretion, indicating a class effect of PPIs (Fig 4, A).⁴² Moreover, when the extrapolated relative potencies of PPIs for inhibiting IL-13-induced eotaxin-3 were compared with their published potencies as inhibitors of gastric acid secretion,⁴² there was a strong positive correlation between these 2 different effects ($r = 0.91$, $P = .03$;

Fig 4, B). We further found that SCH-28080 also significantly inhibited IL-13-induced eotaxin-3 levels (Fig 4, C). SCH-28080 is mechanistically unrelated to PPIs in that it inhibits H,K-ATPases through competitive interactions with K^+ ,^{43,44} whereas PPIs function through binding to sulfhydryl groups of the H,K-ATPase.⁴³ Given these findings, we postulated that H,K-ATPase activity might regulate IL-13-induced eotaxin-3 expression.

Nongastric H,K-ATPase: Implication for IL-13-induced responses and effect of PPIs

In human subjects, P-type ATPases comprise numerous ion pumps, but only 2 H,K-ATPases have been described. The gastric H,K-ATPase (gH,K-ATPase, encoded by the *ATP4A* gene) is the

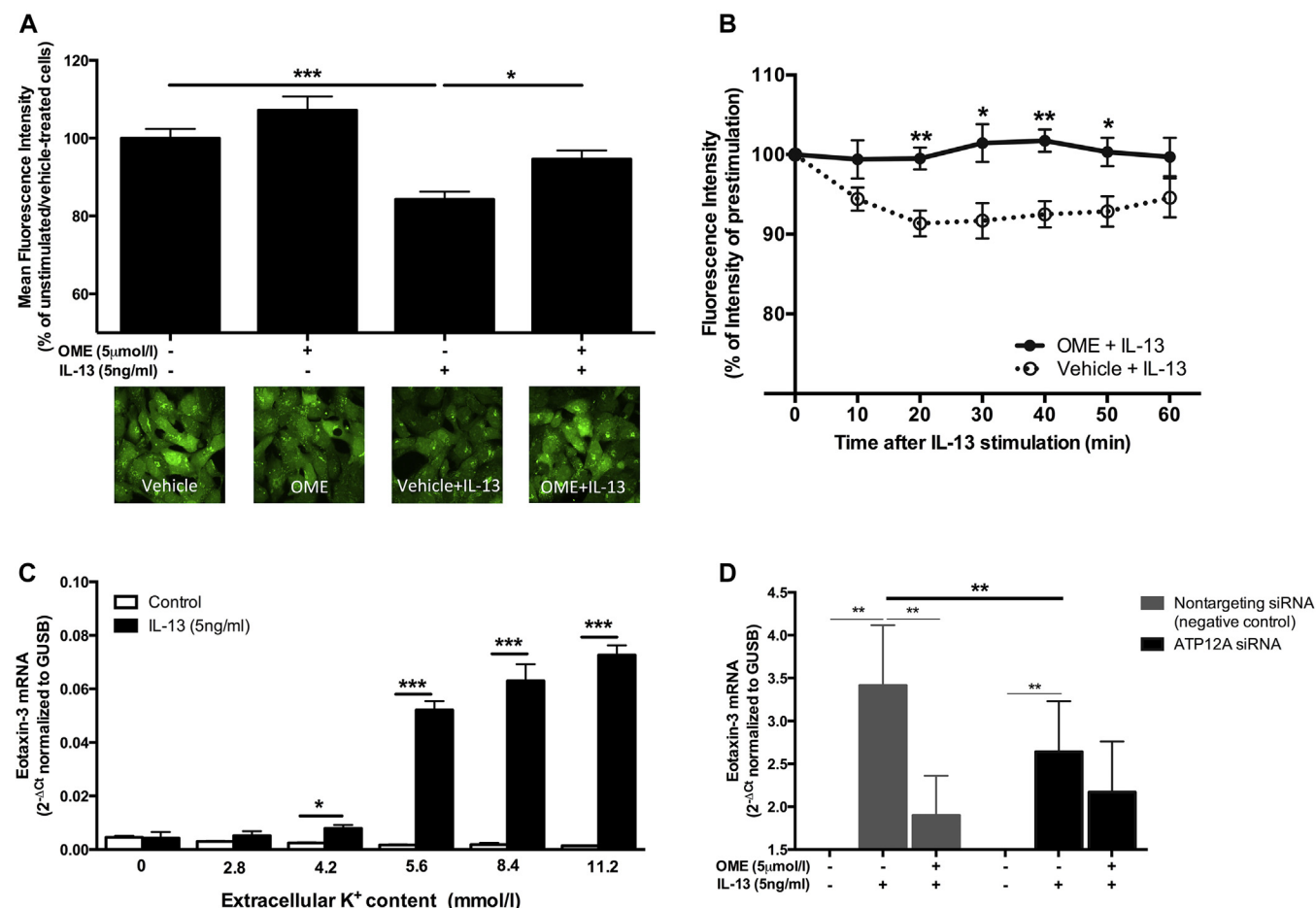


FIG 5. IL-13-induced responses are mediated by the ngH,K-ATPase. **A**, After 6 hours of IL-13 stimulation with omeprazole or vehicle in BEAS-2B cells, fluorescence intensity was measured in confocal microscopic images ($\times 60$ objective). **B**, Time course changes in fluorescence intensity in omeprazole (OME)- or vehicle-pretreated BEAS-2B cells after IL-13 stimulation. **C** and **D**, IL-13-induced eotaxin-3 mRNA expression was measured in BEAS-2B cells cultured in various $[K^+]_e$ -containing solution (Fig 5, C) and ATP12A or nontargeting siRNA-transfected HNECs (Fig 5, D). Data represent means $\pm$ SEMs of 3 (Fig 5, C) or 8 (Fig 5, D) independent experiments. * $P < .05$, ** $P < .01$, and *** $P < .001$.

classic target of PPIs in the stomach but was not expressed by airway epithelial cells (data not shown). In contrast, the nongastric H,K-ATPase (ngH,K-ATPase, encoded by the *ATP12A* gene) has been found in the kidney, prostate, lung, and nasal epithelium and represented a possible candidate.⁴⁵⁻⁴⁷ We confirmed the presence of the catalytic α -subunit of ngH,K-ATPase in BEAS-2B cells and HNECs (see Fig E3 in this article's Online Repository at www.jacionline.org). Given that the ngH,K-ATPase exchanges extracellular K^+ for intracellular H^+ ,⁴⁴ activated ngH,K-ATPase might induce intracellular alkalization. To test this, we measured pH_i and found that IL-13-stimulated cells showed significantly decreased fluorescence compared with unstimulated cells, indicating IL-13-induced increased pH_i (Fig 5, A). Moreover, omeprazole significantly attenuated this effect compared with vehicle (Fig 5, A). In kinetic studies, intracellular alkalization became apparent as early as 20 minutes after IL-13 stimulation and was blunted in omeprazole- or SCH-28080-treated cells (Fig 5, B, and see Fig E4 in this article's Online Repository at www.jacionline.org, respectively).

Additionally, we hypothesized that IL-13-mediated responses would depend on $[K^+]_e$ to facilitate ngH,K-ATPase activity. As

demonstrated in Fig 5, C, IL-13-mediated eotaxin-3 mRNA induction was influenced by $[K^+]_e$ and was completely eliminated in $[K^+]_e$ -free conditions, further supporting the role of ngH,K-ATPase in mediating IL-13-induced gene expression.

Knockdown of ATP12A

To reinforce the observed findings, we directly disrupted the expression of *ATP12A* using an siRNA knockdown approach. Overall, knockdown efficiency for *ATP12A* mRNA was 71% in HNECs (see Fig E5 in this article's Online Repository at www.jacionline.org). As hypothesized, induction of eotaxin-3 by IL-13 was significantly reduced in *ATP12A* siRNA-transfected cells compared with that seen in nontargeting siRNA-transfected cells ($P < .01$), but no additive effect of omeprazole were observed in *ATP12A* siRNA-transfected cells (Fig 5, D).

Effect of omeprazole on STAT6 phosphorylation and eotaxin-3 mRNA stability

Because transcriptional regulation of IL-13-induced eotaxin-3 mRNA is known to be mediated through STAT6 signaling,^{17,48} we

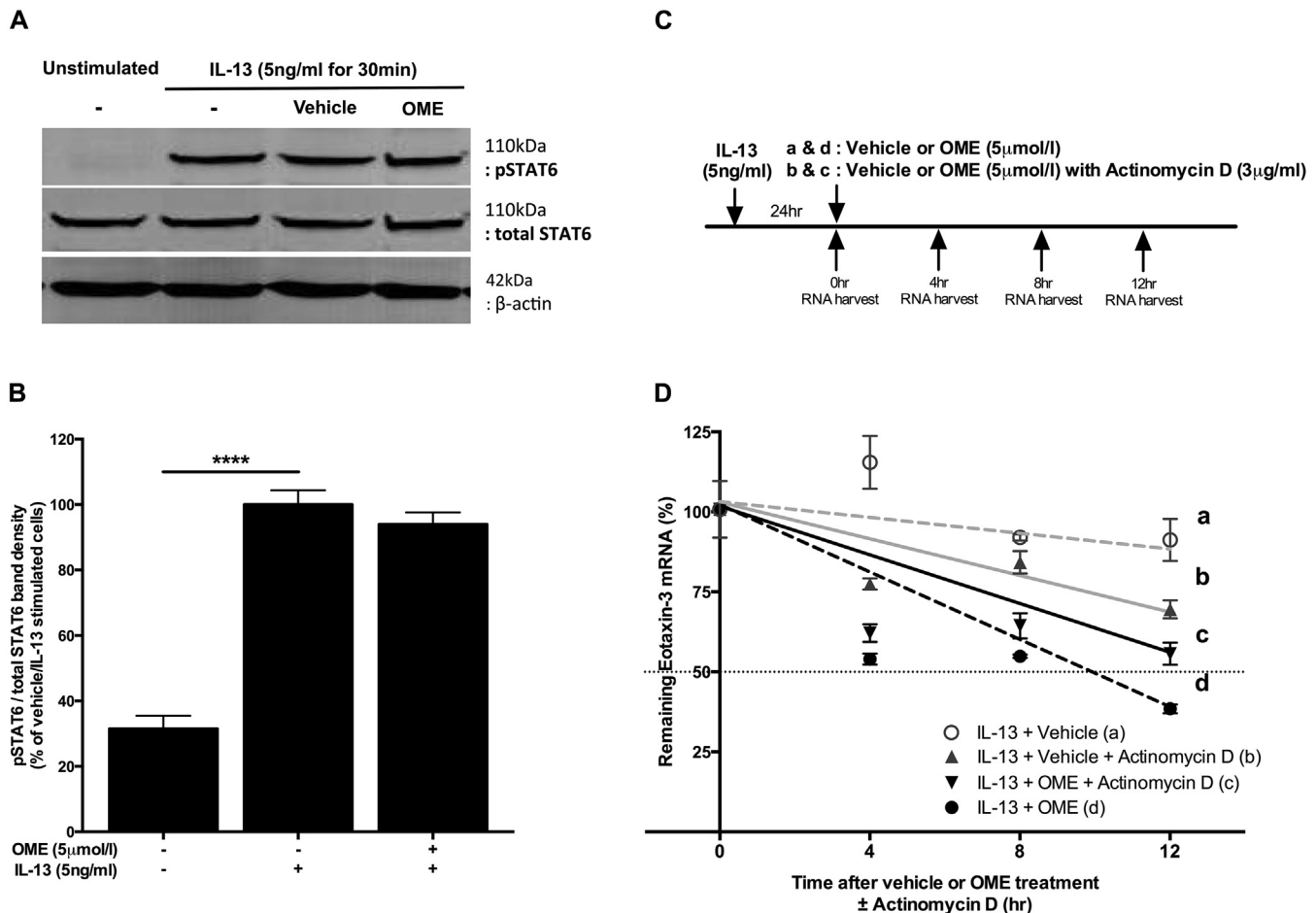


FIG 6. Effects of omeprazole (OME) on IL-13-induced STAT6 phosphorylation and eotaxin-3 mRNA stability. **A**, In IL-13-stimulated BEAS-2B cells with omeprazole or vehicle, phosphorylated STAT6 (pSTAT6) and total STAT6 protein expression were measured by using Western blotting. **B**, Semiquantitative densitometry data for Fig 6, A (mean $\pm$ SEM, $n = 3-6$). **C**, Experimental protocol for eotaxin-3 mRNA stability assessment by using real-time PCR. **D**, Relative eotaxin-3 mRNA expression levels after treatment with actinomycin D, omeprazole, or both. Data represent means $\pm$ SEMs ($n = 3$ each). **** $P < .0001$.

evaluated the effect of omeprazole on STAT6 phosphorylation. IL-13-induced phosphorylated STAT6 was not significantly inhibited by omeprazole (Fig 6, A and B).

We next assessed whether omeprazole influenced IL-13-induced eotaxin-3 mRNA stability by using actinomycin D, which inhibits *de novo* transcription (Fig 6, C).⁴⁸ IL-13-induced eotaxin-3 mRNA expression was relatively stable without omeprazole or actinomycin D (Fig 6, D, line a). Omeprazole significantly accelerated the decrease of eotaxin-3 mRNA levels over the following 12 hours (Fig 6, D, line a vs line d; $P < .001$ at 12 hours). In the presence of actinomycin D, omeprazole had a lesser effect but still enhanced eotaxin-3 mRNA decay compared with vehicle (Fig 6, D, line c vs line b; $P < .05$, all at each time point), suggesting posttranscriptional regulation by omeprazole. However, when comparing the effect of omeprazole with or without actinomycin D, a lesser magnitude of eotaxin-3 mRNA decay was observed in the presence of actinomycin D (Fig 6, D, line c) compared with that of omeprazole alone (Fig 6, D, line d; $P < .05$ after 8 hours), indicating that inhibition of eotaxin-3 mRNA by omeprazole might in part be related to decreased *de novo* transcription, as well as increased posttranscriptional degradation.

DISCUSSION

It is well established that enhanced tissue eosinophilia plays a role in both the pathogenesis and prognosis of CRSwNP.^{5,6} Thus recent pharmacotherapeutic approaches are focused on controlling type 2 inflammatory mediators.^{26,28,49} In this study, we showed that eotaxin-3 is a potential biomarker for tissue IL-13 levels, eosinophilia, and radiographic severity in patients with CRS (Table 1 and see Table E3). We then comprehensively evaluated *in vitro* profiles of the eotaxins by IL-13-stimulated HNECs and BEAS-2B cells and found that both cell types, but particularly HNECs, predominantly expressed eotaxin-3 (Fig 2). Given recent findings that PPIs had direct antieosinophilic effects in patients with esophageal conditions, we hypothesized and confirmed that PPIs had similar inhibitory effects on IL-13-induced eotaxin-3 expression by HNECs *in vitro* (Fig 2) and that PPIs can have similar effects on patients taking these medications (Fig 3). Furthermore, we provide the first demonstration that potential mechanisms underlying the observed effect of PPIs can occur through inhibition of ngH,K-ATPase activity, which is activated by IL-13 (Fig 5).

To date, there are only a few recent reports evaluating the eotaxins in patients with CRS.⁵⁰⁻⁵² These studies reported that

tissue eotaxin-3 mRNA expression was correlated with clinical symptoms and eosinophilia⁵⁰ and that eotaxin-2 levels in nasal secretions correlated with radiographic and endoscopic scores.⁵¹ Our *in vivo* analysis supports these studies but also demonstrates that eotaxin-2, eotaxin-3, and IL-13 levels were intercorrelated in tissues and secretions and further positively correlated with tissue eosinophilia and radiographic severity in patients with CRS (Table I). Additionally, we found that the eotaxins could be measured in nasal secretions and significantly reflected tissue eosinophilia (Table I) and IL-13 levels (see Table E3), suggesting their potential value as noninvasive biomarkers. Although these measures were increased in both UT and NPs in patients with CRSwNP and were actually higher in NPs, the significant correlations between mediators and radiographic and eosinophilic severity were only found within UT (Table I and see Table E4). This suggests that the extent of type 2 inflammation in UT might be more reliably representative of disease burden of CRS. The reasons for the discrepancies in NPs are unclear, but one possible hypothesis is that the dense fibrin deposition in the stroma of NPs can alter chemotaxis, resulting in discordance between measures derived from different cellular sources (eg, group 2 innate lymphoid cell-derived IL-13 and eosinophil-produced ECP).⁵³

Using *in vitro* experiments, we found that eotaxin-3 was the predominant eotaxin produced by HNECs (Fig 2, A and B). Although eotaxin-2 *in vivo* levels were highly increased in tissue extracts from patients with CRSwNP, it was only modestly induced in IL-13-stimulated HNECs. This suggests that the majority of eotaxin-2 can be attributable to nonepithelial inflammatory cells, which has been previously reported in patients with other diseases.^{16,54} Among the eotaxins, recent studies converge on a critical role for eotaxin-3 in human eosinophilic diseases. Provost et al²⁰ found that effects of eotaxin-3 on eosinophil migration were greater than those of the other eotaxins in asthmatic patients. Another study reported that eotaxin-3 was the only CC chemokine to be highly induced by IL-13-treated HBECs and that correlations between eotaxin-3 levels and eosinophil counts within the sputum were significant, supporting our observations.⁸ Although this study also showed that IL-13-stimulated eotaxin-3 release by HBECs from patients with severe asthma was increased compared with that in control HBECs, we do not find similar differences comparing HNECs from control subjects versus patients with CRSwNP.⁸ In patients with EoE, eotaxin-3 was shown to be the most highly upregulated gene (53-fold) compared with control values, whereas eotaxin-2 and eotaxin-1 were only modestly induced (<2-fold) in a genome-wide microarray analysis,⁵⁵ and its protein levels strongly correlated with eosinophilia.²¹ Altogether, given that eotaxin-3 was most highly induced *in vitro*, was highly expressed *in vivo*, and positively correlated with surrogate markers of disease severity, we postulated that therapeutic approaches modulating HNEC-produced eotaxin-3 might improve CRSwNP management.

Safe systemic options for long-term medical management of CRSwNP are currently lacking. Although corticosteroids are the mainstay of medical management in patients with CRSwNP, their effects are short lived, and long-term treatment is limited by systemic side effects.^{28,56,57} Recent innovative biologics targeting type 2 mediators have demonstrated promising therapeutic benefits, but access still limits their availability as options for treating CRSwNP.²⁶⁻²⁸ In eosinophilic esophageal conditions, PPIs are

increasingly recognized to have antieosinophil properties. They currently serve as first-line therapy in patients with symptomatic esophageal eosinophilia, leading to histologic remission with greater than 50% efficacy.^{55,58} PPIs are thought to block the gH,K-ATPase in parietal cells and have a well-established record as orally available medications for GERD.^{42,59} Their antieosinophil effects in the esophagus were previously assumed to result from PPIs suppression in gastric acid and GERD resolution. However, the greatest resolution of eosinophilia was observed in the proximal esophagus, where gastroesophageal reflux is less likely to reach, and patients who respond to PPIs frequently did not show abnormal esophageal pH.³¹ Furthermore, PPIs blocked IL-4/IL-13-induced eotaxin-3 expression in esophageal epithelial cells.^{29,30} Together, these observations have raised the possibility that the antieosinophil effects of PPIs might occur through mechanisms that are more direct and unrelated to GERD resolution.

We showed here that IL-13-induced eotaxin-3 protein secretion was reduced by 57.9% in BEAS-2B cells and 37.1% in HNECs by 5 μ mol/L omeprazole (Fig 2, C and D) *in vitro*. Notably, these *in vitro* anti-inflammatory effects were specific to type 2 cytokine-mediated responses (see Fig E2). Furthermore, we made striking observations that patients with CRS who were taking PPIs at the time of surgery had significantly lower eotaxin-3 and eotaxin-2 levels in nasal tissue compared with patients not receiving PPIs (Fig 3). These results show promise that our *in vitro* results might be replicated *in vivo*, but further studies, including clinical trials, are needed to prospectively evaluate their efficacy in patients with CRSwNP. Prior studies have shown mixed efficacy of PPIs for treating asthma, but analysis was targeted at comorbid GERD resolution but not for type 2 asthma.⁶⁰⁻⁶²

We also present novel evidence indicating that the mechanism by which PPIs inhibit IL-13-induced eotaxin-3 involves inhibition of ngH,K-ATPase activity. Specifically, PPIs inhibited IL-13-induced eotaxin-3 expression with the same rank order as inhibition of gastric acid secretion,⁴² suggesting a near-perfect structure-activity relationship of PPIs for these 2 effects ($r = 0.91$; Fig 4, B), and furthermore, IL-13-induced eotaxin-3 expression was suppressed by SCH-28080, a mechanistically distinct H,K-ATPase inhibitor (Fig 4, C). Because gH,K-ATPase, the known target of PPIs, is not expressed in airway epithelium, our data led us to consider the ngH,K-ATPase, the only other P-type ATPase with H^+ , K^+ -antiporting activity. It should be noted that the ngH,K-ATPase shares approximately 65% sequence homology with the gH,K-ATPase and Na,K-ATPase and is moderately sensitive to their inhibitors.^{44,63-65} Although the inhibitory effects of PPIs on P-type ATPases other than gH,K-ATPase are largely unknown, a recent study demonstrates that omeprazole blocked another P-type ATPase, ATP7A (Menkes protein), in human epidermal melanocytes, supporting our hypothesis.⁶⁶ Additionally, our results might explain recent findings that PPI responsiveness in esophageal biopsy specimens of patients with EoE was strongly associated with expression of *KCNJ2* (gene encoding the K^+ channel, Kir2.1), which colocalizes with and counteracts H,K-ATPase activity.⁵⁵

Another major finding is that expression of IL-13-responsive genes, such as eotaxin-3, might require ngH,K-ATPase activity for optimal expression (summarized in Fig 7). This hypothesis is supported by findings that IL-13 stimulation induced rapid

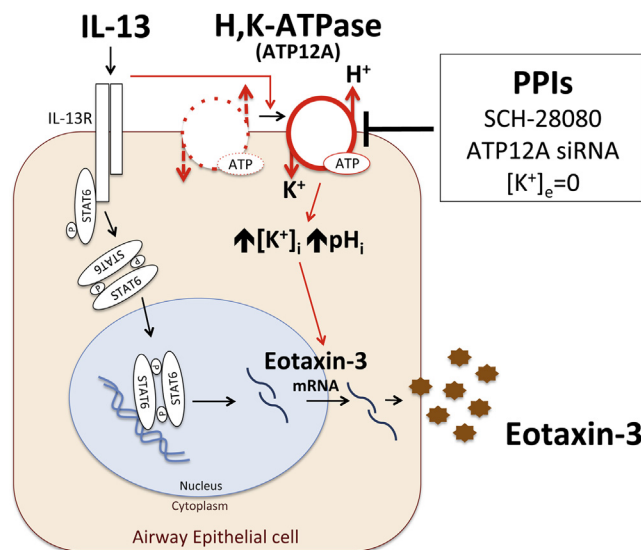


FIG 7. Hypothetical model showing the potential role of ngH,K-ATPase in facilitating inhibitory effects of PPIs on IL-13-mediated eotaxin-3 expression. In addition to the canonical IL-13/STAT6 pathway, IL-13-mediated eotaxin-3 expression might be affected by the ngH,K-ATPase activity. The ngH,K-ATPase can be blocked by PPIs and other inhibitors, including SCH-28080, *ATP12A* siRNA, and $[K^+]_e$ -free solution, resulting in H^+ , K^+ -flux and pH_i changes, which might affect expression of IL-13-mediated eotaxin-3.

intracellular alkalization, which was blocked by omeprazole (Fig 5, A and B) and SCH-28080 (see Fig E4); eotaxin-3 mRNA induction by IL-13 was highly sensitive to $[K^+]_e$ and was completely eliminated in $[K^+]_e$ -free solution (Fig 5, C); and knockdown of *ATP12A* significantly blunted IL-13 induction of eotaxin-3 mRNA (Fig 5, D).

Although the ngH,K-ATPase exists in airway epithelium and plays a role in airway surface liquid acidification,⁶⁷ its role in IL-13 signaling is unknown. A fascinating recent study demonstrates that human subjects normally express 10- to 100-fold higher baseline levels of airway ngH,K-ATPase than mice, and the pH gradient generated by this ion pump is counteracted by CFTR secreting bicarbonate. Overexpression of the ngH,K ATPase in CFTR^{-/-} mice, led to uncompensated airway acidification that increased bacteria at the airway surface, giving these mice a phenotype closer to the human disease.⁶⁸ Further investigation is needed to evaluate whether IL-4 and IL-13 similarly acidifies the airway surface, but prior reports respectively demonstrate that these cytokines induce basolateral secretion of H^+ by glomerular epithelial cells⁶⁹ and reduced K^+ secretion by HBECS.⁷⁰

Limitations of our data are that we have not yet established direct mechanisms through which the IL-13 signaling pathway, intracellular alkalization, eotaxin-3 expression, and ngH,K-ATPase interact. It should be noted that pH_i or $[K^+]_e$ can affect cytokine-induced gene expression, transcription factor DNA-binding activity, or cellular enzyme activity.^{71,72} These ionic effects in airway epithelial cells might explain the previously reported decrease in STAT6 binding to the eotaxin-3 promoter,³⁰ although detailed biochemical studies of the effects of pH_i and intracellular K^+ concentration on promoter binding will be required to directly implicate this mechanism. Other limitations include noteworthy findings that eotaxin-3 protein adheres to cell surfaces and can only be fully released by different cell extraction protocols⁷³ from those used in the numerous previous

studies,^{8,18,29,30,48} including our own. These studies might underestimate the total amount of eotaxin-3 released by cells.

Other potential limitations to the value of PPIs for treating CRSwNP include their decreased bioavailability and reduced potency outside acidic spaces, such as the stomach.⁵⁹ However, given the availability of extended-release PPI formulations and the evidence that airway inflammatory conditions, including CRS, lead to airway acidification⁷⁴⁻⁷⁶ might make these surmountable concerns. We also note that the peak concentrations of omeprazole used for our *in vitro* studies are achievable *in vivo* by using conventional oral dosing of omeprazole, with published peak mean plasma concentrations ranging from 3.2 $\mu\text{mol/L}$ (20 mg/d for 8 days) to 6.0 $\mu\text{mol/L}$ (60 mg/d for 7 days).^{77,78} Additionally, other previous studies have shown significant improvement in postnasal drip, a component symptom of CRS by lansoprazole⁷⁹ or rabeprazole⁸⁰ compared with placebo, thus reinforcing the potential therapeutic benefit for PPIs outside the stomach.

Taken together, we suggest here that inhibitors of the ngH,K-ATPase might be of significant therapeutic value in the IL-13-mediated responses found in patients with CRSwNP, and further studies are needed to elucidate their potential.

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Key messages

- Tissue levels of type 2 inflammatory mediators, including IL-13, eotaxin-2, and eotaxin-3, were correlated with tissue eosinophilia and radiographic severity in patients with CRS.
- Eotaxin-3, the most highly induced eotaxin after IL-13 stimulation in human airway epithelial cells, was inhibited by PPIs *in vitro*. Lower *in vivo* levels of eotaxin-3 were observed in patients with CRS taking PPIs compared with those without PPIs.
- The inhibitory effect of PPIs *in vitro* occurred through multiple mechanisms, including inhibition of ngH,K-ATPase activity.

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METHODS

Subjects

All patients with CRS met the clinical criteria for CRS, as defined by the American Academy of Otolaryngology–Head and Neck Surgery Chronic Rhinosinusitis Task Force.^{E1,E2} Patients with established immunodeficiency, pregnancy, coagulation disorder, classic allergic fungal sinusitis, isolated antrochoanal polyps, or cystic fibrosis were excluded from the study. The presence of sinusitis or NPs was confirmed by means of office endoscopy and CT imaging. Control subjects without a history of CRS undergoing nasal surgery were recruited from the otolaryngology clinic at Northwestern University.

Sample collection and preparation

Standardized nasal tissue samples consisting of UT and NPs were obtained during routine functional endoscopic sinus surgery. Specimens from control subjects were obtained during endoscopic skull-base tumor excisions, intranasal procedures for deviated septum or obstructive sleep apnea, and facial fracture repairs for patients without a history of sinonasal inflammation. After removal, weighed tissue samples were placed in PBS-Tween supplemented with a cocktail of protease inhibitors (Sigma-Aldrich) added at a 1:100 dilution. For total protein extraction, samples were homogenized with a Bullet Blender Blue (Next Advance, Averill Park, NY), according to the manufacturer's instructions. The samples were then centrifuged at 4000 rpm for 20 minutes at 4°C, and the supernatants were frozen at –20°C until analysis. Nasal lavage fluid was also obtained before initiating surgery from control subjects and patients with CRS. After suctioning the nasopharynx, 8 mL of PBS was sprayed through a syringe toward the middle meatus, and resultant fluid was collected with a Lukens trap (Covidien, Mansfield, Mass). Collected nasal lavage fluid was spun down for 10 minutes at 3000 rpm, with the resulting supernatant concentrated by means of centrifugation in an Amicon Ultra-4 10K Centrifugal Filter Unit (EMD Millipore) at 3000 rpm for 10 minutes at 4°C. The supernatant was stored at –20°C until use.

Cell culture

HNECs collected by means of epithelial scrapings of IT and NPs with the Rhinoprobe (Arlington Scientific, Springville, Utah) were seeded in collagen (Vitrogen; Collagen Biomaterials, Palo Alto, Calif)–coated 6-well plates and maintained in serum-free bronchial epithelial cell growth medium (BEGM; Lonza, Walkersville, Md) at 37°C with 5% CO₂ in humidified air. Before cytokine stimulation or treatment with reagents, including PPIs and SCH-28080, HNECs were cultured in BEGM without hydrocortisone for at least 24 hours. Similarly, BEAS-2B cells were seeded (1×10^6 cells/plate) in 12-well plates and cultured in Dulbecco modified Eagle medium (Biofluids, Rockville, Md) with 5% FBS, 50 U/mL penicillin, and 50 ng/mL streptomycin (GIBCO BRL, Gaithersburg, Md) at 37°C in a 5% CO₂ atmosphere in humidified air. For all cell-culture experiments, cells were stimulated and/or treated according to experimental protocols when they reached 80% confluence. For experiments with PPIs, PPIs were reconstituted in culture medium at pH 5.5 for 30 minutes to be converted into the active sulfonamide form and then mixed with an equal volume of culture media at neutral pH, as described previously.^{E3-E6} SCH-28080 was reconstituted in dimethyl sulfoxide (0.1%). In each experiment, matched vehicle controls were used for comparison. Cells treated with equal volume of acidic culture media at pH 5.5 without drug mixed with culture media at neutral pH was used as matched vehicle control for PPI-treated cells and with dimethyl sulfoxide alone was used as matched vehicle control for SCH-28080–treated cells. PPIs, SCH-28080, or matched vehicle remained in medium throughout the period of cytokine stimulation. For mRNA stability assessment, BEAS-2B cells were stimulated with IL-13 (5 ng/mL) for 24 hours to generate significant amounts of eotaxin-3 mRNA, and the cells were then treated with vehicle or omeprazole (5 μ M) with or without adding actinomycin D (3 μ g/mL) for 4, 8, and 12 hours. Total RNAs were harvested at each time point (0, 4, 8, and 12 hours) after treatment, as described previously.^{E7} The cytotoxicity of each condition was monitored with the Vybrant MTT Cell Proliferation Assay Kit (V-13154, Life Technologies), showing that no significant toxicity was observed in any of the experiments presented.

ELISA

The concentration of each protein was assayed by using commercially available human CCL11/eotaxin (DY320), CCL24/eotaxin-2 (DY343), and CCL26/eotaxin-3 (DY346) DuoSet (R&D Systems, Minneapolis, Minn), according to the manufacturer's instructions. The standard used in the kit is recombinant human eotaxin, eotaxin-2, or eotaxin-3. The minimal detection limit is 15.6 pg/mL for CCL11/eotaxin kit, 31.2 pg/mL for CCL24/eotaxin-2 kit, and 62.5 pg/mL for CCL26/eotaxin-3 kit. The absorbance of each well was read at 450 nm by using the Bio-Rad Spectrophotometer Model 680 Microplate Reader (Bio-Rad Laboratories, Hercules, Calif) with associated software applied to the sandwich enzyme immunoassay technique.

Real-time PCR

Total RNAs were isolated from whole-cell extracts, and cDNA was prepared, as previously described.^{E8} Briefly, total RNAs were isolated from whole-cell extracts and treated with DNase I by using NucleoSpin RNA II (MACHEREY-NAGEL, Bethlehem, Pa), according to the manufacturer's instructions. Single-strand cDNA was synthesized from 0.5 μ g of total RNAs with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, Calif) and random primers. Semiquantitative real-time PCR was performed with the TaqMan method by using an Applied Biosystems 7500 Sequence Detection System (Applied Biosystems, Foster City, Calif) in 20 μ L reactions (10 μ L of 2 $\times$ TaqMan Master mix [Applied Biosystems], 400 nmol/L of each primer, and 200 nmol/L of TaqMan probe plus 10 ng of cDNA). Primer and probe sets for β -glucuronidase (human β -glucuronidase endogenous control, Hs00939627_m1), CCL11 (Hs00237013_m1), CCL24 (Hs00171082_m1), CCL26 (Hs00171146_m1), CXCL1 (Hs00236937_m1), CXCL10 (Hs01124252_g1), ATP12A (Hs01060284_m1), and ATP4A (Hs00167575_m1) were purchased from Applied Biosystems. All expression values were normalized to the housekeeping gene β -glucuronidase (GUSB), and expressed as $2^{-\Delta C_t}$, except for the mRNA stability assessment. For mRNA stability assessment, the amount of eotaxin-3 mRNA remaining at each time point was quantitated by using TaqMan real-time PCR and expressed as the mean percentage of the initial amount of eotaxin-3 mRNA present at time zero.

Western blotting

Cells were harvested at the indicated time points and then dissolved in M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) supplemented with Halt Protease Inhibitor Cocktail and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Ten micrograms of total protein from whole-cell lysates was dissolved in NuPAGE LDS sample buffer (Invitrogen) with NuPAGE sample reducing reagent (Invitrogen) and resolved in a 4–12% NuPAGE Bis-Tris Gel (Life Technologies) to detect total STAT6, phosphorylated STAT6, and ATP12A.^{E9} Proteins were transferred onto a polyvinylidene difluoride membrane (EMD Millipore) by using a semidry transfer apparatus (Bio-Rad Laboratories). The membranes were blocked in blocking buffer (LI-COR Biosciences, Lincoln, Neb) for at least 1 hour at room temperature and subsequently incubated overnight at 4°C on a shaker with primary rabbit anti-human STAT6 antibodies (1:1000; Cell Signaling, Beverly, Mass), phosphorylated STAT6 antibodies (1:1000, Cell Signaling), or ATP12A antibodies (1:1000; Abcam, Cambridge, Mass) with mouse anti-human β -actin (1:10,000, Sigma-Aldrich). After incubation with primary antibodies, the membranes were labeled with fluorescent secondary anti-rabbit antibodies (1:10,000, LI-COR Biosciences) or anti-mouse antibodies (1:10,000, LI-COR Biosciences) for 1 hour at room temperature. Images were obtained on an Odyssey infrared scanner (LI-COR Biosciences), and band density was quantitated with Image J software. K-RasVal12–transformed normal rat kidney cells were used as a positive control for ATP12A.

Data analyses for comparison of the relative potency of PPIs

For comparison of the relative potency of PPIs on inhibiting IL-13–induced eotaxin-3 expression, the concentration at which the respective PPI inhibited 50% of IL-13–induced eotaxin-3 was extrapolated by using curves generated

from experimentally measured values. Each curve was generated with data from 9 different concentrations, including those demonstrated in Fig 4, A (0, 0.1, 1, 5, 50, and 100 $\mu\text{mol/L}$), and an additional 3 different concentrations (0.3, 3, and 30 $\mu\text{mol/L}$) by using nonlinear regression on GraphPad Prism 6 software (GraphPad Software, La Jolla, Calif). We correlated inhibitory concentration of 50% levels with corresponding median effective dose data for suppression of intragastric acid secretion, as published by Kirchheiner et al.^{E10}

Statistical analyses

Differences between groups were analyzed by using the Mann-Whitney *U* test and a paired (for HNECs) or unpaired Student *t* test (for BEAS-2B cells). For multiple comparisons, the Kruskal-Wallis test with the Dunn test or 1-way ANOVA with the *post hoc* Holm-Sidak test was performed. Correlations were assessed by using Spearman rank correlation. All analyses were performed with GraphPad Prism 6 software.

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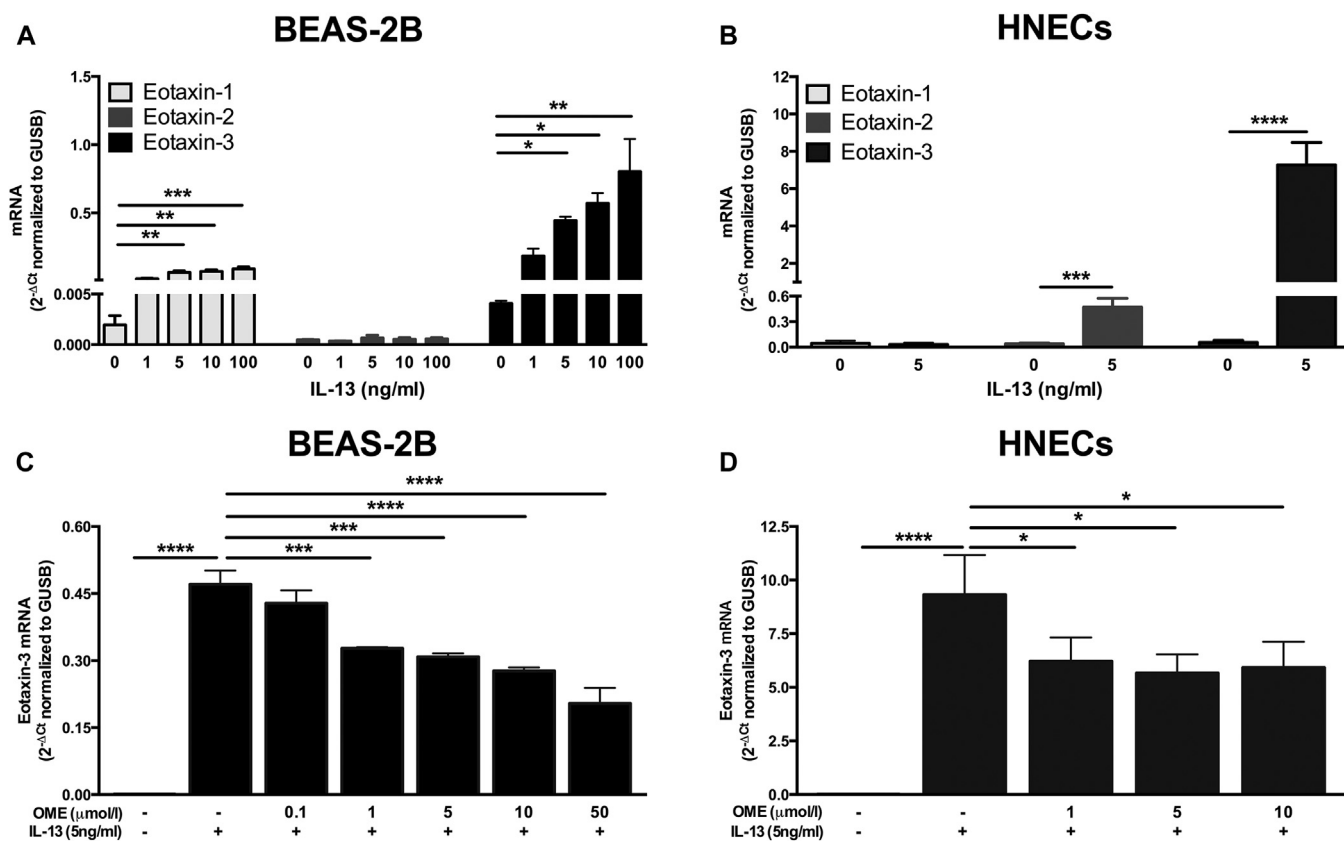


FIG E1. IL-13-induced eotaxin-1, eotaxin-2, and eotaxin-3 gene expression in cultured airway epithelial cells and dose-dependent inhibition of IL-13-induced eotaxin-3 expression by omeprazole (OME). **A** and **B**, BEAS-2B cells (Fig E1, A) and HNECs (Fig E1, B; $n = 15$) were stimulated for 48 hours with IL-13 at escalating doses. **C** and **D**, BEAS-2B cells (Fig E1, C) and HNECs (Fig E1, D) were pretreated with omeprazole for 2 hours and stimulated for 48 hours with IL-13 (5 ng/mL). Eotaxins (Fig E1, A and B) or eotaxin-3 (Fig E1, C and D) mRNA expression levels in total RNA from whole-cell extracts were measured by using real-time PCR. Data represent means $\pm$ SEMs of 3 (Fig E1, A and C), 15 (Fig E1, B), or 9 (Fig E1, D) independent experiments. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$ compared with unstimulated cells (Fig E1, A and B) or vehicle-treated/IL-13-stimulated cells (Fig E1, C and D).

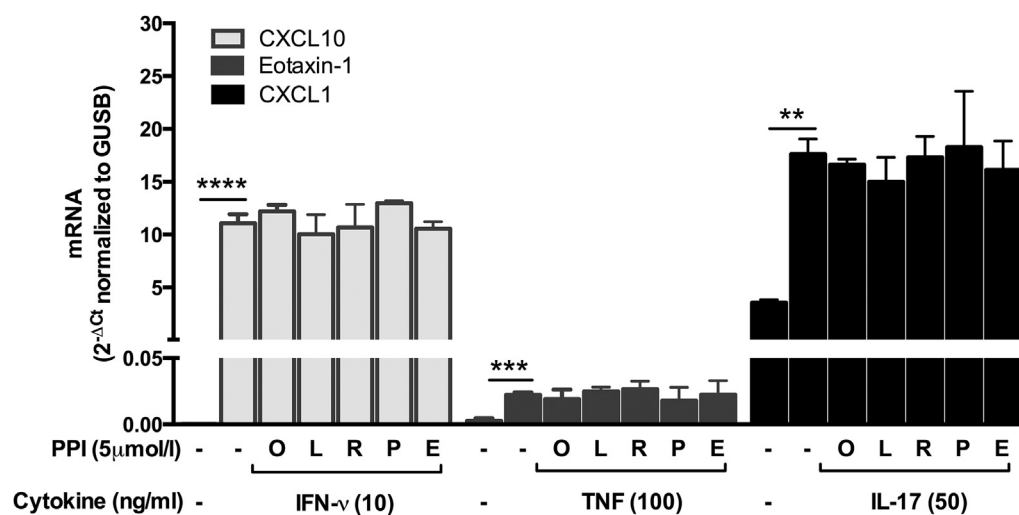


FIG E2. PPIs did not inhibit mRNA expression of IFN- γ -induced CXCL10, TNF-induced eotaxin-1, and IL-17-induced CXCL1 in BEAS-2B cells. Cells were pretreated with various PPIs for 2 hours and stimulated for 6 hours with IFN- γ (10 ng/mL), TNF (100 ng/mL), or IL-17 (50 ng/mL). IFN- γ -induced CXCL10, TNF-induced eotaxin-1, and IL-17-induced CXCL1 mRNA expression levels in total RNA from cells were measured by using real-time PCR. Data represent means $\pm$ SEMs of 3 independent experiments. ** P < .01, *** P < .001, and **** P < .0001 compared with vehicle-treated/cytokine-stimulated cells. E, Esomeprazole; L, lansoprazole; O, omeprazole; P, pantoprazole; R, rabeprazole (all at 5 μ mol/L).

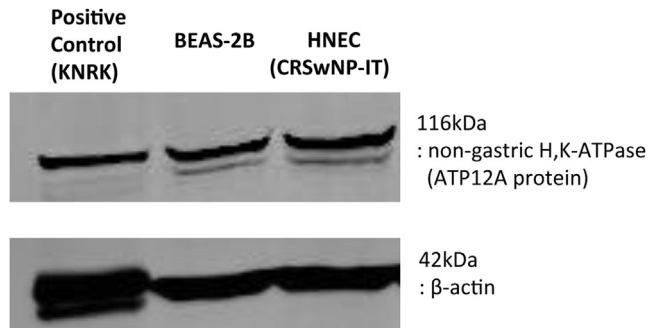


FIG E3. Representative Western blot films of ATP12A protein (ngH,K-ATPase) expression in BEAS-2B cells, HNECs, and K-RasVal12-transformed normal rat kidney (*KNRK*) cells (positive control).

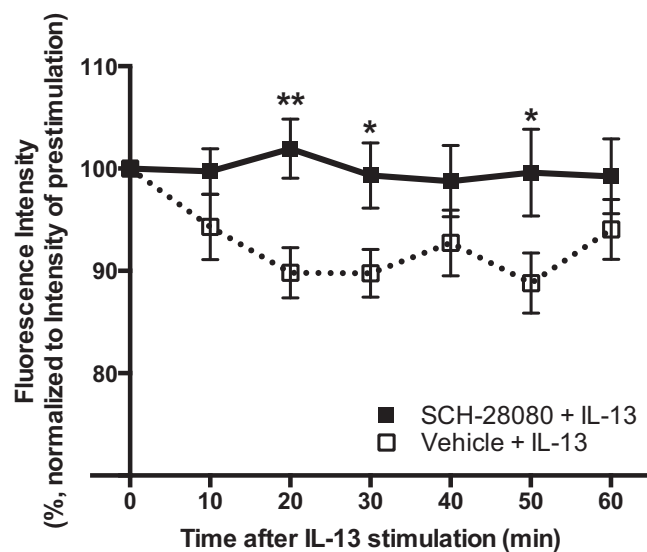


FIG E4. SCH-28080 blocked IL-13-induced intracellular alkalization in BEAS-2B cells. Cells were pretreated with SCH-28080 or vehicle for 2 hours before pHrodo Green dye staining. After staining, cells were stimulated with IL-13 at 5 ng/mL. Fluorescence intensities were measured at various times before and after IL-13 stimulation up to 1 hour by using spectrofluorometry. Data represent mean $\pm$ SEM percentages of initial values at time zero from 3 independent experiments. * $P < .05$ and ** $P < .01$.

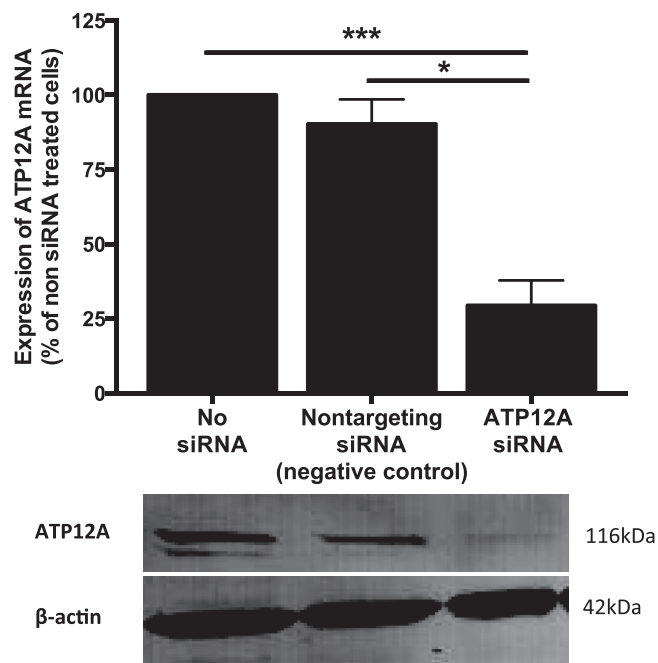


FIG E5. Overall knockdown efficiencies of *ATP12A* mRNA and protein expression in HNECs. Cells were transfected with 25 pmol of ON-TARGETplus *ATP12A* siRNA or nontargeting siRNA for 96 hours. *ATP12A* mRNA expression levels in total RNA from cells were measured by using real-time PCR. Representative Western blot films of *ATP12A* protein expression in HNECs is shown. The level of *ATP12A* mRNA was expressed as a percentage of non siRNA transfected value. Data represent means $\pm$ SEMs of 7 independent experiments. * $P < .05$ and *** $P < .001$.

TABLE E1. Clinical characteristics of subjects

	Control subjects	Patients with CRSsNP	Patients with CRSwNP	
Total no. of subjects (M/F)	13 (7/6)	18 (9/9)	72 (47/25)	
Age (y), median (range)	41 (19-78)	40 (20-69)	43 (19-72)	
Atopy (Y/N/U)	0/12/1	10/4/4	46/16/10	
Asthma (Y/N/U)	1/12/0	8/10/0	37/34/1	
Prior nasal surgery (Y/N)	0/13	1/17	25/47	
Methodology used				
Tissue extract				
Tissue type, no. (M/F)	UT, 7 (3/4)	UT, 18 (9/9)	UT, 39 (26/13)	NP, 47 (34/13)
Age (y), median (range)	44 (27-78)	42 (20-69)	45 (22-71)	43 (19-72)
Nasal lavage fluid				
No. (M/F)	7 (3/4)	18 (9/9)	56 (38/18)	
Age (y), median (range)	44 (27-78)	42 (20-69)	45 (19-72)	
Cultured HNECs				
Origin, no. (M/F)	IT, 6 (4/2)	—	IT, 10 (5/5)	Polyp, 6 (4/2)
Age (y), median (range)	34 (19-59)	—	46 (32-63)	38 (35-40)

CRSsNP, Chronic rhinosinusitis without nasal polyps; HNECs, human sinonasal epithelial cells; F, female; M, male; N, no; U, unknown; Y, yes.

TABLE E2. Composition of solutions

Component	Solution 1 (K ⁺ -free RS)	Solution 2	Solution 3	Solution 4 (RS)	Solution 5	Solution 6
KCl (mmol/L)	0	2.82	4.23	5.63	8.45	11.26
NaCl (mmol/L)	116.86	114.04	112.63	111.23	108.41	105.6
CaCl ₂ (mmol/L)	2.25	2.25	2.25	2.25	2.25	2.25
NaHCO ₃ (mmol/L)	2.38	2.38	2.38	2.38	2.38	2.38
pH	7.4	7.4	7.4	7.4	7.4	7.4

RS, Ringer solution.

TABLE E3. Correlation between type 2 inflammatory mediators and IL-13 in nasal tissues

Type 2 inflammatory mediators	IL-13 in UT (total subjects, n = 32)	
	<i>r</i>	<i>P</i> value
In UT		
Eotaxin-1	0.39	.03
Eotaxin-2	0.79	<.0001
Eotaxin-3	0.61	<.001
ECP	0.84	<.0001
In nasal lavage fluid		
Eotaxin-1	0.24	.19
Eotaxin-2	0.57	<.001
Eotaxin-3	0.68	<.0001
ECP	0.25	.32

Text in boldface indicates statistically significant correlations for that mediator and IL-13 levels in UT ($P < .05$).

TABLE E4. Correlations between type 2 inflammatory mediators in NPs and tissue eosinophilia or radiographic severity

Type 2 inflammatory mediators	ECP in NPs (patients with CRSwNP*)		CT scores (patients with CRSwNP†)	
	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
In NPs				
IL-13	0.30	.22	0.09	.56
Eotaxin-1	0.08	.75	0.12	.43
Eotaxin-2	0.13	.62	0.08	.61
Eotaxin-3	−0.35	.16	−0.06	.69
ECP	—	—	0.34	.03

Text in boldface indicates statistically significant correlations for that mediator in NPs and tissue eosinophilia or CT scores ($P < .05$).

CT, Computed tomography.

*N = 18 for correlations between ECP with measures in NPs.

†N = 42 to 47 for correlations between CT scores with measures in NPs.