

Increased expression of the epithelial anion transporter pendrin/SLC26A4 in nasal polyps of patients with chronic rhinosinusitis

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Background: Chronic rhinosinusitis (CRS) is a multifactorial disease of unknown cause characterized by sinonasal inflammation, increased mucus production, and defective mucociliary clearance. Expression of Pendrin, an epithelial anion transporter, is increased in asthma and chronic obstructive pulmonary disease. Pendrin increases mucus production and regulates mucociliary clearance. **Objectives:** We sought to investigate the expression of pendrin and the mucus-related protein Muc5AC in sinonasal tissues of control subjects and patients with CRS and to evaluate the

regulation of pendrin expression in nasal epithelial cells (NECs) *in vitro*.

Methods: The expression and distribution of pendrin in sinonasal tissues was analyzed by using real-time PCR, immunoblot analysis, and immunohistochemistry. Differentiated NECs were used to study the regulation of pendrin expression.

Results: Increased pendrin expression was observed in nasal polyp (NP) tissue of patients with CRS. Immunohistochemistry analysis revealed that pendrin was largely restricted to the epithelial layer. Pendrin expression significantly correlated with inflammatory cell markers, suggesting that the factors made by these cells might induce pendrin expression. Furthermore, both pendrin and periostin levels (a biomarker in asthma) correlated with IL-13 levels, suggesting that pendrin can be induced by this cytokine in sinonasal tissues. Expression of the mucus component protein Muc5AC correlated weakly with pendrin expression, indicating that pendrin might modulate mucus production in NPs. In cultured NECs pendrin expression was induced by T_H2 cytokines and induced synergistically when T_H2 cytokines were combined with IL-17A. Interestingly, human rhinovirus had a potentiating effect on IL-13-induced pendrin expression. Dexamethasone suppressed pendrin expression, suggesting that the therapeutic benefit of dexamethasone in asthmatic patients and those with CRS might involve regulation of pendrin expression.

Conclusions: T_H2-mediated pendrin expression is increased in NPs of patients with CRS and might lead to increased inflammation, mucus production, and decreased mucociliary clearance. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Pendrin, SLC26A4, periostin, Muc5AC, chronic rhinosinusitis, nasal polyp, mucus, mucociliary clearance, nasal epithelial cells

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Chronic rhinosinusitis (CRS) is a common disease affecting 10% of the population in developed countries.¹ Patients with CRS have a poor quality of life comparable with that of patients with other chronic conditions, such as congestive heart failure, chronic obstructive pulmonary disease, angina, and back pain, yet have few therapeutic options.^{2,3} Most patients do not respond to medical interventions and often undergo surgery in an attempt to alleviate symptoms, which results in a significant cost burden.⁴

CRS is characterized by persistent inflammation of the nasal and paranasal sinus mucosa that lasts longer than 12 weeks. Diagnosis requires endoscopic confirmation of inflammation,

Abbreviations used

ALI:	Air-liquid interface
CRS:	Chronic rhinosinusitis
CRSsNP:	Chronic rhinosinusitis without nasal polyps
CRSwNP:	Chronic rhinosinusitis with nasal polyps
NEC:	Nasal epithelial cell
NP:	Nasal polyp
Poly(I:C):	Polyinosinic:polycytidylic acid
UT:	Uncinate tissue

purulent discharge, or edema in the middle meatus or ethmoid region; the presence of nasal polyposis; or radiographic evidence of paranasal sinus inflammation in the setting of rhinosinusitis symptoms (eg, purulent discharge, nasal obstruction, facial pain/pressure/fullness, and hyposmia).⁵

CRS can be further divided into 2 major subtypes: chronic rhinosinusitis without nasal polyps (CRSsNP) and chronic rhinosinusitis with nasal polyps (CRSwNP). Although the cytokines and cells driving CRSsNP are not clear, CRSwNP is often associated with an increased T_H2 cytokine profile and generally eosinophilic inflammation.⁶ Recent studies have proposed the existence of multiple endotypes of the disease, highlighting the complex nature of CRS.⁷ Although prior efforts have deepened our understanding of the inflammatory profiles and cells associated with CRS, the cause and pathogenesis of CRS remain largely unclear.^{8,9} One potential contributing factor highlighted in previous studies is an imbalance in mucociliary clearance and mucus production.¹⁰ Additional work has also demonstrated abnormalities in ion transport in the setting of CRS.^{11,12}

Pendrin/SLC26A4 is an apically expressed ion exchanger originally identified as the causative mutation in Pendred syndrome, a condition characterized by prelingual hearing loss and iodide organification.¹³ Pendrin is predominantly expressed in the inner ear, kidney, and thyroid gland but is also present in airways.¹³⁻¹⁶ A role for pendrin in regulating inflammation and mucus production in asthmatic patients, patients with CRS, and those with chronic obstructive pulmonary disease has been previously proposed.¹⁴⁻¹⁶ Pendrin has also been shown to regulate epithelial air-surface liquid levels and composition.^{15,17}

In this study we determined the expression and localization of pendrin in sinonasal tissues, including uncinate tissue (UT) and nasal polyp (NP) tissue, taken from patients with CRS and control subjects. We investigated the cellular and cytokine profiles associated with pendrin expression in sinonasal tissues. Finally, we assessed the regulation of pendrin expression in differentiated nasal epithelial cells (NECs) in response to inflammatory cytokines and glucocorticoid treatment *in vitro*.

METHODS**Patients and tissue samples**

Patients were recruited from the Sinus and Allergy Clinics at Northwestern Memorial Hospital by using protocols approved by the Institutional Review Board of Northwestern University. All patients with CRS (diagnosed by using task force guidelines^{18,19}) and control subjects signed informed consent forms. Tissue samples (both UTs and NPs) and nasal scraping cells were collected from the patients in whom conservative medical therapy (saline irrigations, decongestants, and prolonged treatment with steroids, antibiotics, or both) had failed at the time of sinus surgery. Control UTs were collected from

patients undergoing surgery for skull-based tumor excision. Control subjects did not have any history of inflammatory upper airway diseases. Patients with immunodeficiency, Churg-Strauss syndrome, or cystic fibrosis were excluded from this study. A detailed list of patients' characteristics is presented in Table I.

Cell culture

NECs were collected during surgery, expanded, and cultured under air-liquid interface (ALI) conditions.²⁰ A detailed protocol can be found in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Nasal protein extraction and immunoblot analysis

Proteins were isolated from tissues, as previously described.³ A detailed protocol can be found in the [Methods](#) section in this article's Online Repository.

Real-time PCR

Tissues were lysed in QIAzol (Qiagen, Valencia, Calif), and RNA extraction and real-time PCR were performed, as described previously.³ A detailed protocol can be found in the [Methods](#) section in this article's Online Repository.

Immunohistochemistry

The basic protocol for immunohistochemistry has been described previously.²¹ A detailed protocol for immunohistochemistry is submitted in the [Methods](#) section in this article's Online Repository.

Statistical analysis

Multiple comparisons were carried out with ANOVA. The Student *t* test was used to compare *in vitro* experimental data. All analyses were performed with GraphPad Prism software (GraphPad Software, La Jolla, Calif). A *P* value of less than .05 was considered statistically significant.

RESULTS**Increased expression of pendrin in NPs of patients with CRS**

T_H2 cytokine-mediated pendrin expression has been reported in asthmatic patients. Because nasal polyposis is often a T_H2 cytokine-dominated disease, we analyzed the expression of pendrin in 83 sinonasal tissues of control subjects (control UT, *n* = 18) and patients with CRS (CRSsNP UT, *n* = 23; CRSwNP UT, *n* = 22; and polyp, *n* = 20). Pendrin expression was profoundly increased in NPs of patients with CRSwNP (10-fold, 771.5 ± 254.5 copies/ng total RNA) and UTs of patients with CRSsNP (4-fold, 309.3 ± 74.62 copies/ng total RNA) compared with control UTs (71.68 ± 11.31 copies/ng total RNA). Although there was a trend for increased pendrin expression in UTs of patients with CRSwNP compared with control UTs, it was not statistically significant (Fig 1, A).

Next, we assessed whether mRNA data correlated to protein data by using immunoblot analysis in 48 sinonasal tissue samples (control UT, *n* = 9; CRSsNP UT, *n* = 12; CRSwNP UT, *n* = 11; and polyps, *n* = 16). Surprisingly, we did not detect pendrin protein at the expected molecular weight (80 kDa: native and 100-120 kDa: glycosylated pendrin). Our pendrin-specific antibody detected a cross-reacting band at a size of approximately 70 kDa. To confirm the specificity of the antibody, we performed peptide-blocking experiments. Incubation of pendrin antibody with pendrin peptide before incubating the blot completely

TABLE I. Subjects' characteristics

	Control subjects			Patients with CRSsNP			Patients with CRSwNP		
Total no. of subjects	37 (18 M)			47 (23 M)			84 (57 M)		
Age (y), median (range)	48.5 (16-78)			38 (20-71)			45 (23-74)		
	Yes	No	U	Yes	No	U	Yes	No	U
Atopy	4	31	2	24	15	8	42	27	15
Asthma	0	36	1	11	34	2	35	47	2
	UT			UT			UT		
							NP		
PCR									
No. of subjects	18 (8 M)			23 (8 M)			23 (17 M)		
Age (y), median (range)	46 (16-62)			36 (20-64)			43 (27-56)		
NEC PCR									
No. of subjects	13 (6 M)			15 (8 M)			15 (10 M)		
Age (y), median (range)	41 (29-75)			36 (21-64)			52 (35-67)		
Immunoblot									
No. of subjects	9 (5 M)			12 (7 M)			10 (6 M)		
Age (y), median (range)	61 (20-78)			46 (20-71)			49 (32-71)		
							45 (23-70)		

M, Male; U, unknown.

abrogated the 70-kDa band intensity, indicating that the 70-kDa band detected by the antibody is indeed pendrin (see Fig E1 in this article's Online Repository at www.jacionline.org).

Next, we investigated whether there was any difference in the 70-kDa form of pendrin in our tissue samples. We found that the 70-kDa band density was significantly increased (3-fold) in NPs of patients with CRS compared with that seen in control UTs (Fig 1, B and C). However, there was no significant difference between UTs of control subjects and those of patients with CRS. These data suggest that there is a specific increase in pendrin expression in NPs of patients with CRS compared with that in UTs from patients with CRS or control subjects.

Pendrin is expressed by the surface epithelial cells of the sinonasal mucosa

We used immunohistochemical analysis to determine the distribution of pendrin in sinonasal tissues. Pendrin expression was detected in surface epithelial cells, and in some cases, minor staining was also found in submucosal glands (Fig 2, A and B). Closer examination of surface epithelium revealed that pendrin was expressed mostly by ciliated epithelial cells (Fig 2, C). Because our immunohistochemical analysis indicated that pendrin was expressed by surface epithelial cells in sinonasal tissues, we performed real-time PCR on epithelial scrapings to specifically analyze whether epithelial cells contributed to the increased pendrin expression seen in NP tissue. Pendrin mRNA expression trended toward an increase (3-fold) in NP epithelial cells from patients with CRSwNP compared with that seen in epithelial cells from control UTs (see Fig E2 in this article's Online Repository at www.jacionline.org). Although not statistically significant, this trend suggests that the increased pendrin expression observed in NP tissue might be explained in part by an increase in epithelial pendrin expression.

Pendrin expression correlated with inflammatory cell marker expression in sinonasal tissues

In an ovalbumin-alum-induced allergic mouse model of inflammation, pendrin knockout animals had less inflammation

and inflammatory cell markers, suggesting that pendrin might regulate recruitment of inflammatory cells and therefore inflammation.¹⁵ Therefore we examined whether pendrin expression correlated with inflammatory cell markers in sinonasal tissues. Interestingly, we observed significant correlation between pendrin and CD3d (T cells), CD20 (B cells), CD1c (dendritic cells), tryptase (mast cells), CD68 (macrophages), CD163 (M2 macrophages) Charcot-Leyden crystal (eosinophils), and CXCR1 (neutrophils) (Fig 3). This suggests that pendrin might be involved in recruitment of these cells or that products of these cells might be inducing pendrin expression in sinonasal tissues.

Pendrin expression correlated with IL-13 levels in sinonasal tissues

Because previous studies have reported pendrin expression to be induced by T_H2 cytokines, we questioned whether the underlying factor or factors responsible for the increased pendrin expression in NPs might be the presence of increased levels of T_H2 cytokines, particularly IL-13. We confirmed an increased expression of IL-13 mRNA in NPs of patients with CRS compared with that seen in control UTs (Fig 4, B). mRNA levels of IFN- γ and IL-17A were slightly increased, although not significantly, in NPs of patients with CRS compared with control UTs (Fig 4, A and C). Pendrin mRNA correlated weakly but significantly with mRNA levels of IL-13 in sinonasal tissues (Fig 4, D). These observations suggest that pendrin expression in sinonasal tissues might be associated with the expression of IL-13.

Of note, we also determined the expression of periostin, a T_H2-induced protein used as a biomarker in asthmatic patients. As reported by others,^{22,23} periostin levels were significantly increased in NPs of patients with CRS (see Fig E3, A, in this article's Online Repository at www.jacionline.org). Interestingly, periostin mRNA also correlated strongly and significantly with IL-13 levels in sinonasal tissues (see Fig E3, B).

Taken together, our data suggest that IL-13 expression is associated with the expression of pendrin mRNA in sinonasal tissues.

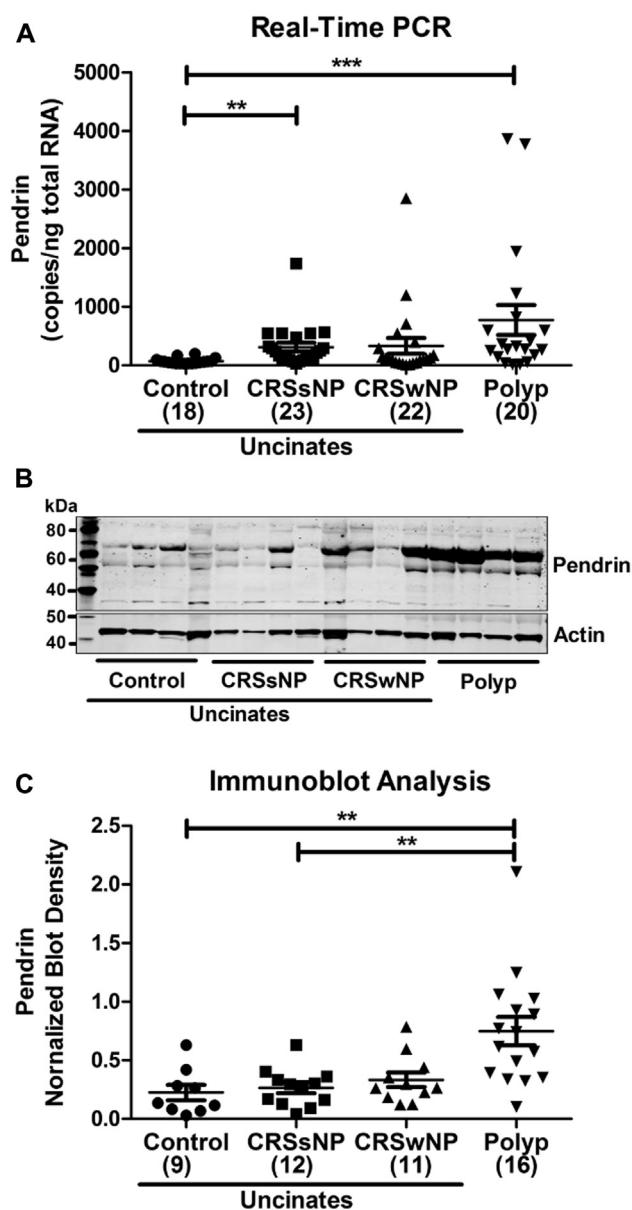


FIG 1. Increased pendrin expression in NPs of patients with CRS. **A**, Gene expression of pendrin determined by using real-time PCR in sinonasal tissues. Pendrin expression was normalized based on median expression of the housekeeping gene β -glucuronidase and expressed as copies per nanogram of total RNA. **B**, Representative immunoblot of pendrin and actin in sinonasal tissues. **C**, Immunoblot analysis of pendrin expression in sinonasal tissues. Pendrin band density was normalized to actin band density and represented as blot density. ** $P < .01$ and *** $P < .001$.

Pendrin is induced profoundly by T_H2 cytokines in differentiated epithelial cells *in vitro*

Next, we wanted to investigate whether there is a direct role for cytokines, especially T_H2 cytokines, in the induction of pendrin in NECs. To this end, differentiated NECs ($n = 5-9$) were stimulated with a Toll-like receptor 3 ligand (polyinosinic:polycytidylic acid [poly(I:C)], 10 μ g/mL) and T_H1 (IFN- γ , 10 ng/mL), T_H2 (IL-4 or IL-13, 10 ng/mL), T_H17 (IL-17A, 10 ng/mL), and T_H22 (IL-22, 10 ng/mL) cytokines, as well as the classic inflammatory cytokine TNF (10 ng/mL), for 7 days to mimic the chronic inflammation

found in sinonasal tissues of patients with CRS. Pendrin mRNA and protein expression were analyzed by using real-time PCR and immunoblotting, respectively. As expected, pendrin mRNA expression was increased dramatically by treatment with T_H2 cytokines (IL-4 and IL-13; Fig 5, A). We observed 169.2 ± 54.5 -fold and 183.4 ± 38 -fold induction of pendrin mRNA by treatment with IL-4 and IL-13, respectively. Interestingly, pendrin mRNA was also modestly induced by treatment with IL-17A (31.4 ± 13.8 -fold). Moreover, treatment of airway epithelial cells with poly(I:C), IFN- γ , and TNF induced pendrin mRNA, although to a lesser extent (10.9 ± 4.5 -, 18.2 ± 7.5 -, and 21.7 ± 9.1 -fold, respectively). IL-22, a cytokine involved in repair and epithelial proliferation, did not induce pendrin compared with control-treated cells. The induction of pendrin in NECs by T_H2 cytokines and IL-17A was dose dependent (see Fig E4 in this article's Online Repository at www.jacionline.org). Although induction of pendrin mRNA by T_H2 cytokines is well characterized, there are no data on the regulation of pendrin protein by these cytokines. Therefore we analyzed the expression of pendrin protein by means of immunoblot analysis in NECs from 6 donors. None of the cytokines or Toll-like receptor stimuli tested affected the expression of native pendrin (80 kDa). Interestingly, T_H2 cytokines (IL-4 and IL-13) were able to strongly induce an approximately 100-kDa glycosylated form of the pendrin protein (Fig 5, B). Although our mRNA data indicated that poly(I:C), IFN- γ , IL-17A, and TNF induced pendrin mRNA in airway epithelial cells, surprisingly, we did not observe appreciable band intensity corresponding to the size of glycosylated pendrin. This observation was also true for bronchial epithelial cells (data not shown), suggesting similar mechanisms of pendrin regulation between nasal and bronchial epithelial cells. Collectively, these results indicate that the glycosylated form but not native pendrin is induced profoundly by T_H2 cytokines in differentiated airway epithelial cells.

Synergistic induction of pendrin by T_H2 cytokines and IL-17A in differentiated airway epithelial cells

Because T_H2 and T_H17 cytokines are found to be expressed together and important in patients with some forms of asthma and CRS, we wanted to investigate whether these 2 cytokines can work in concert to increase pendrin expression in NECs. Differentiated NECs were stimulated with media alone or with IFN- γ , IL-4, IL-13, or IL-17A alone or in combination (IFN- γ /IL-4/IL-13 and IL-17A) for 24 hours, and mRNA expression was analyzed by using real-time PCR. Treatment with individual cytokines induced pendrin expression (IFN- γ , 1.9 ± 0.4 -fold; IL-4, 69.2 ± 15.4 -fold; IL-13, 78.5 ± 32.6 -fold; and IL-17A, 38.0 ± 17.3 -fold; $n = 11-15$). Surprisingly, combined treatment with either IL-4 or IL-13 and IL-17A profoundly induced pendrin mRNA expression (276.9 ± 70.3 - and 266.7 ± 93.6 -fold, respectively), indicating a synergistic effect of these cytokines in inducing pendrin (Fig 6, A). Moreover, even very low concentrations of IL-17A (1 ng/mL) along with IL-13 were able to induce synergy, suggesting an amplification effect by IL-17A on T_H2 -induced pendrin (data not shown). However, this synergy was observed to a lesser extent when IFN- γ and IL-17A were used in combination, suggesting some specificity of the underlying T_H2 and T_H17 cytokine signaling mechanisms in the induction of pendrin.

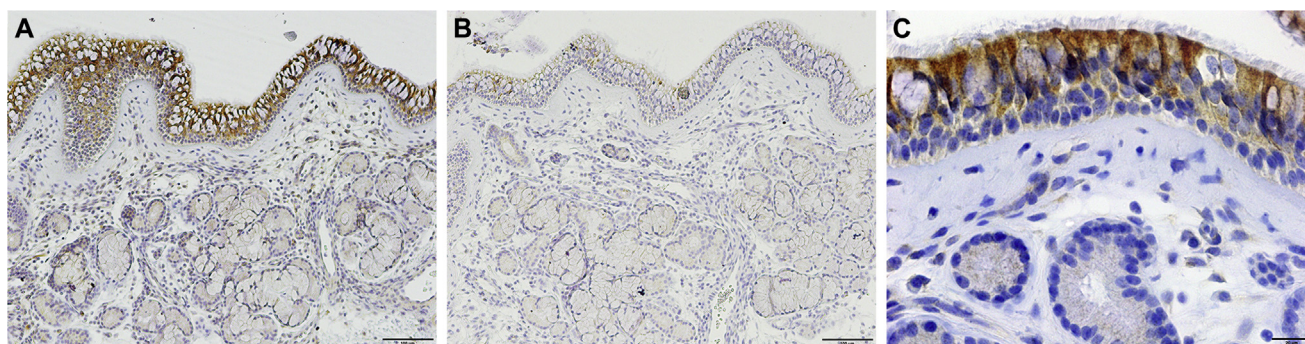


FIG 2. Pendrin is expressed by the surface epithelial cells of the sinonasal mucosa. **A** and **B**, Representative immunohistochemical analysis of pendrin (Fig 2, **A**) and control IgG (Fig 2, **B**). **C**, Magnified image of Fig 2, **A**. Shown are representative pictures of at least 4 different donors.

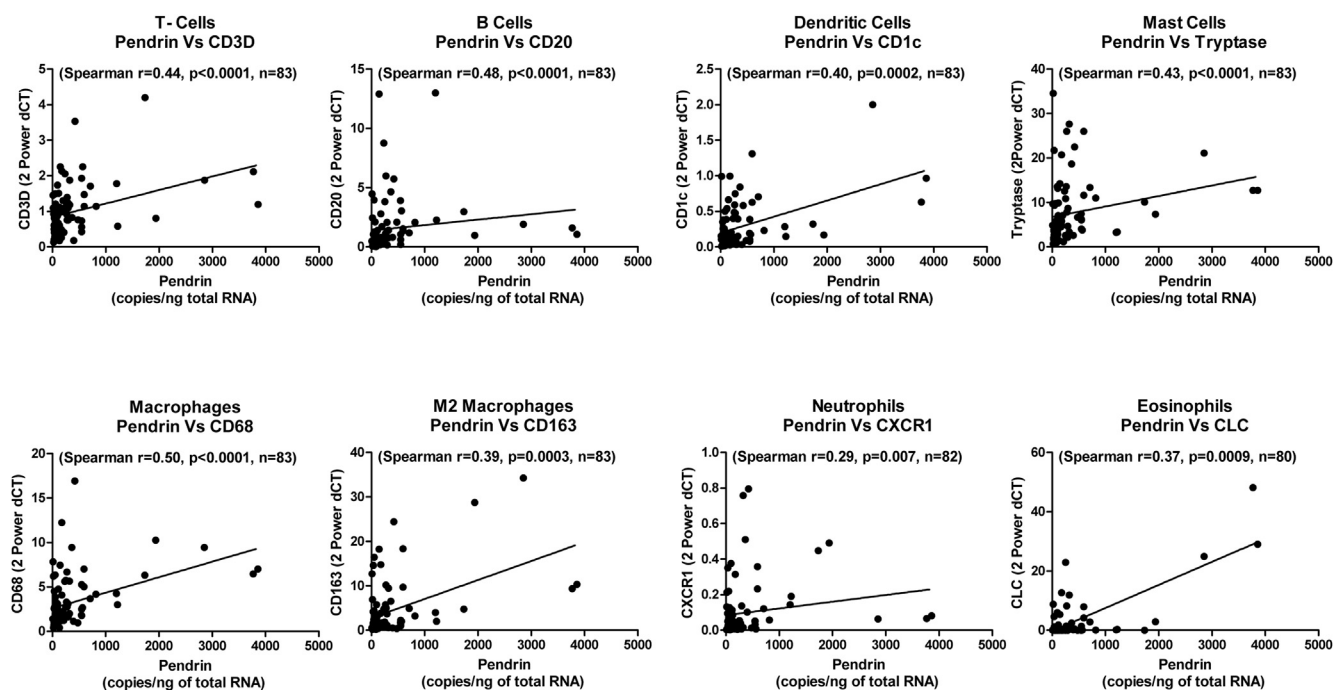


FIG 3. Pendrin expression correlated with inflammatory cell marker levels in sinonasal tissues. The relationship between pendrin expression and levels of various inflammatory cell markers (CD3d [T cells], CD20 [B cells], CD1c [dendritic cells], tryptase [mast cells], CD68 [macrophages], CD163 [M2 macrophages], Charcot-Leyden crystal [eosinophils], and CXCR1 [neutrophils]) was assessed by using Spearman Rank correlation. Data for pendrin was used from Fig 1, **A**.

Synergistic induction by combined treatment with T_H2 cytokines and IL-17A was not observed with expression of periostin. Surprisingly, periostin, a T_H2 -induced gene with expression that is increased in asthmatic patients, was suppressed by the combined treatment (see Fig E5 in this article's Online Repository at www.jacionline.org). Next, using immunoblot analysis, we confirmed the synergistic induction of pendrin at the protein level in NECs from 4 donors. In contrast with the extended treatment time we used earlier (Fig 5, 7 days), a shorter treatment of airway epithelial cells with IL-4, IL-13, and IL-17A alone (for 48 hours) induced very minimal levels of the glycosylated form of pendrin protein (Fig 6, **B**). However, glycosylated pendrin levels were increased dramatically by combined treatment of IL-17A with either IL-4 or IL-13, indicating that the synergistic induction of

mRNA was faithfully recapitulated at the protein level. Taken together, these data indicate that a combination of T_H2 cytokines and IL-17A induces pendrin in a synergistic manner in NECs.

Muc5AC expression is increased in NPs of patients with CRS, and pendrin expression correlated with Muc5AC expression in sinonasal tissues

Because pendrin expression has been linked to increased mucus production, we wanted to test whether the expression of the major mucus protein component Muc5AC was increased in NPs of patients with CRSwNP and whether expression of pendrin and Muc5AC was associated in sinonasal tissues. As reported by others,^{24,25} we confirmed the increased expression of Muc5AC in

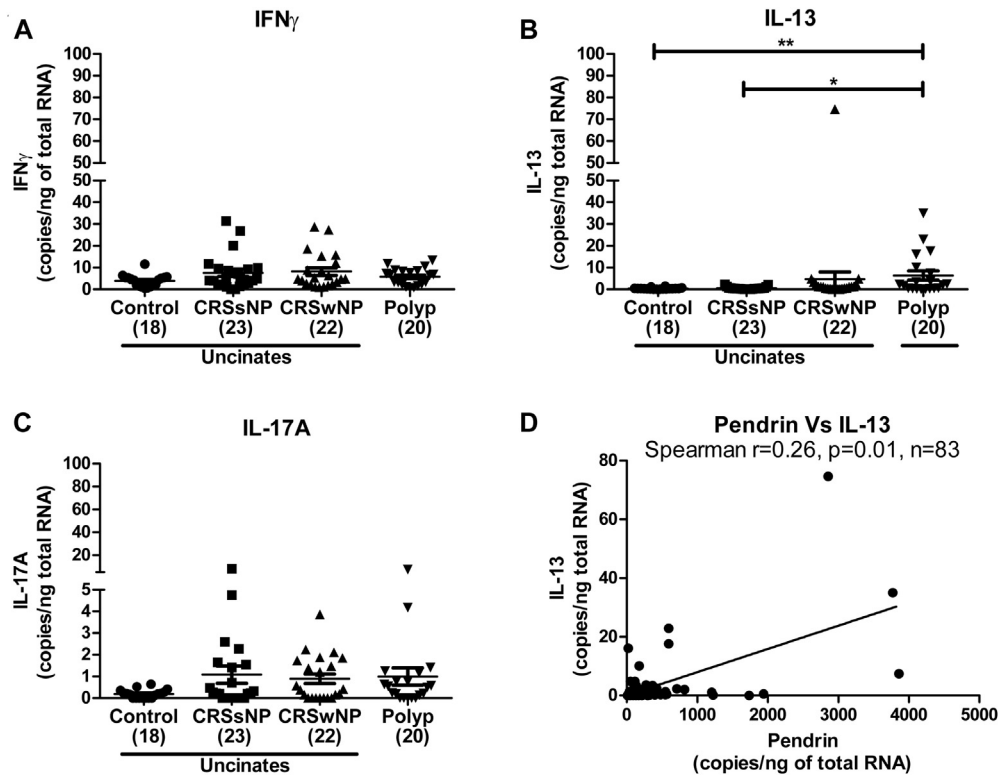


FIG 4. Pendrin expression correlated with IL-13 mRNA expression in sinonasal tissues. **A-C**, Gene expression of IFN- γ (Fig 4, A), IL-13 (Fig 4, B), and IL-17A (Fig 4, C) in sinonasal tissues was determined by using real-time PCR. **D**, The relationship between pendrin expression and IL-13 expression was analyzed in mRNA samples by using Spearman rank correlation. Data for pendrin was used from Fig 1, A. * $P < .05$ and ** $P < .01$.

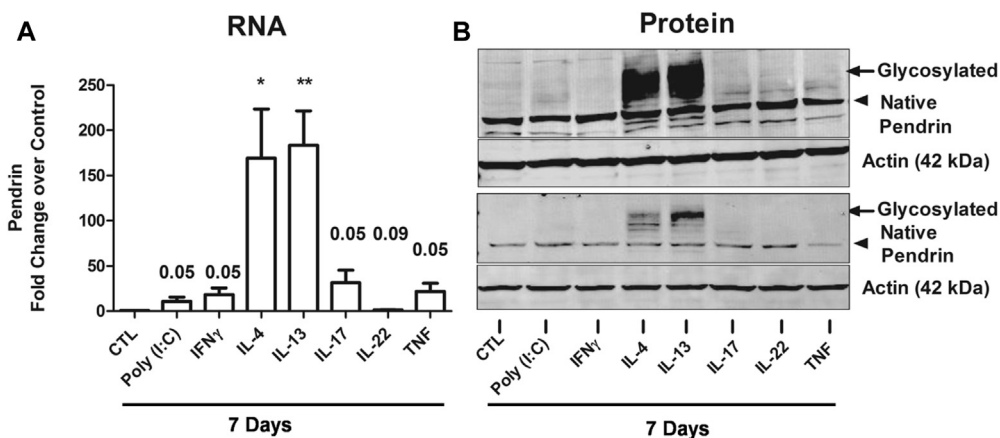


FIG 5. Pendrin is induced by TH2 cytokines and IL-17A in airway epithelial cells *in vitro*. **A**, Differentiated NECs were untreated (CTL) or stimulated with Toll-like receptor 3 ligand (Poly [I:C]) or cytokines as indicated for 7 days. Pendrin gene expression was quantified by using real-time PCR ($n = 5-9$). Gene expression was normalized to actin and expressed as fold change over untreated control samples. **B**, Pendrin protein was verified by using immunoblot analysis in stimulated ALI-cultured NECs. Arrowheads and arrows indicate native (80 kDa) and glycosylated (100 kDa) pendrin, respectively. Shown are representative blots for pendrin and actin from 2 of 6 donors.

NPs of patients with CRSwNP compared with that seen in UTs from control subjects or patients with CRS (Fig 7, A). Expression of Muc5AC mRNA was increased by 4-fold in NPs compared with that seen in control UTs ($36,654 \pm 10,790$ vs $8,553 \pm 2,238$, $P < .05$, $n = 83$). This indicates that NPs have increased expression of Muc5AC that might contribute to the

increased mucus production in polyps of patients with CRSwNP. We correlated the levels of gene expression of pendrin with Muc5AC mRNA expression in sinonasal tissues using a Spearman rank correlation. There was a weak but statistically significant correlation between pendrin and Muc5AC expression in sinonasal tissues ($r = 0.24$, $P = .02$, $n = 83$; Fig 7, B). One

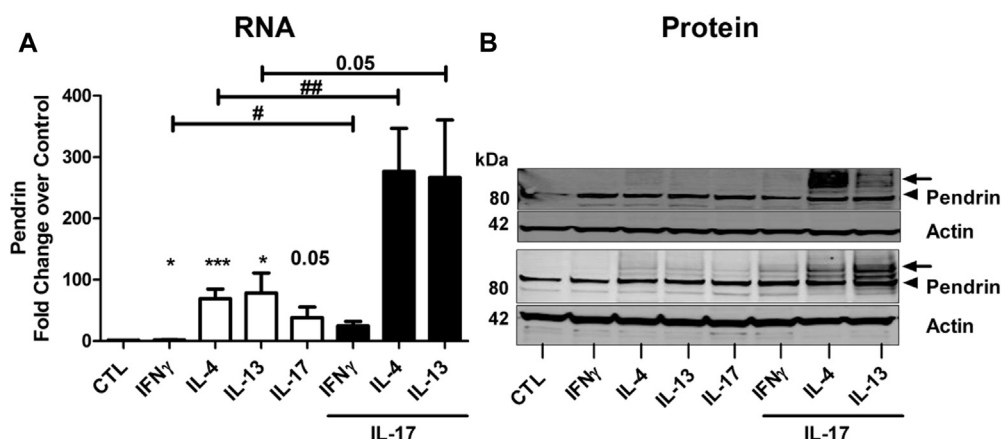


FIG 6. Synergistic induction of pendrin by T_H2 cytokines and IL-17A in airway epithelial cells. Real-time PCR analysis (**A**) and immunoblot analysis (**B**) of pendrin expression in differentiated NECs stimulated with cytokines alone (10 ng/mL) or in combination (10 ng each) as indicated are shown. Fig 6, A, shows data from 11 to 15 experiments. Arrowheads and arrows indicate native (80 kDa) and glycosylated (100 kDa) pendrin, respectively. Representative blots for pendrin and actin from 2 experiments of 4 experiments are shown in Fig 6, B. # and * $P < .05$, ## and ** $P < .01$, and *** $P < .001$, paired t test. *Comparing control with treated.

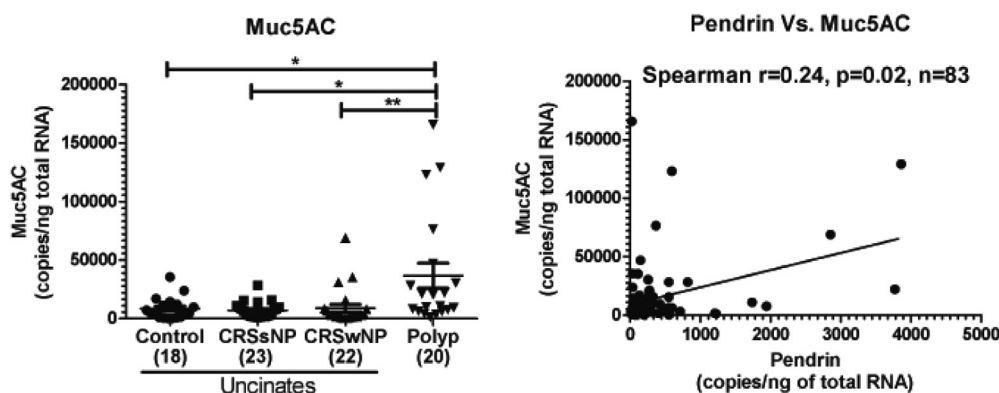


FIG 7. Muc5AC expression is increased in NPs of patients with CRS, and pendrin expression correlated with Muc5AC mRNA in sinonasal tissues. Muc5AC mRNA expression was measured in sinonasal tissues by using real-time PCR. The relationship between pendrin and Muc5AC expression was analyzed in mRNA samples by using real-time PCR. Correlations were assessed by using Spearman rank correlation. Data for pendrin was used from Fig 1, A. * $P < .05$ and ** $P < .01$.

interpretation of these data is that Muc5AC expression and pendrin expression in sinonasal tissues are associated and that an increase in pendrin expression might promote increased mucus production in patients with CRS.

Glucocorticoids suppressed pendrin expression in airway epithelial cells

Glucocorticoids are leading drugs for the treatment of diseases of airway inflammation in which mucus expression is prominent. Therefore we investigated whether dexamethasone treatment can suppress pendrin expression in NECs. Differentiated NECs ($n = 5$) were pretreated with dimethyl sulfoxide or dexamethasone for 1 hour before treatment with cytokines. Cells were then stimulated with medium, IL-4, IL-13, or IL-17A alone or in combination for 24 hours. RNA was extracted and subjected to real-time PCR. As expected, stimulation of NECs with IL-4, IL-13, and IL-17A induced pendrin expression. Interestingly,

pretreatment with dexamethasone suppressed basal and cytokine-induced pendrin expression by 50% in NECs (Fig 8, A). As a positive control for steroid treatment, we analyzed the expression of glucocorticoid-induced leucine zipper (GILZ), a gene that is induced by dexamethasone. As expected, dexamethasone induced significant quantities of GILZ in airway epithelial cells (Fig 8, B).

Potential effect of IL-13/IL-17A and poly(I:C)/human rhinovirus infection on pendrin expression in differentiated airway epithelial cells

Because our experiments suggest that pendrin is induced by poly(I:C) in NECs, we next investigated whether poly(I:C) or rhinovirus infection has a potentiating effect on cytokine-induced pendrin expression. NECs were either untreated or treated with poly(I:C) or cytokines alone or in combination for 24 hours (RNA, $n = 8-12$) and 48 hours (protein [$n = 2$] and rhinovirus infection [$n = 4$]). RNA was analyzed for pendrin by using

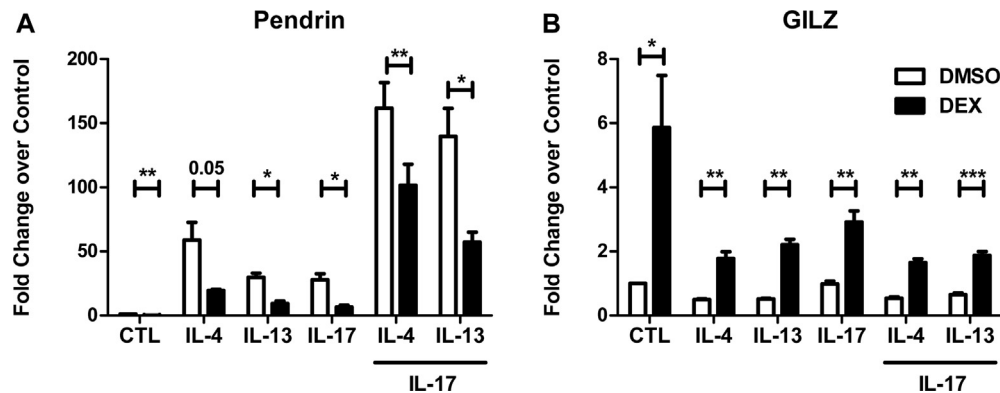


FIG 8. Glucocorticoids suppressed pendrin expression in airway epithelial cells. Differentiated NECs were pretreated with 100 nmol/L dexamethasone (DEX) or dimethyl sulfoxide (DMSO) for 1 hour before cytokine treatment for 24 hours. RNA was analyzed by using real-time PCR for pendrin (A) and glucocorticoid-induced leucine zipper (GILZ), a positive control, induced by glucocorticoid treatment (B). Shown are data from 5 donors. * $P < .05$, ** $P < .01$, and *** $P < .001$, paired t test.

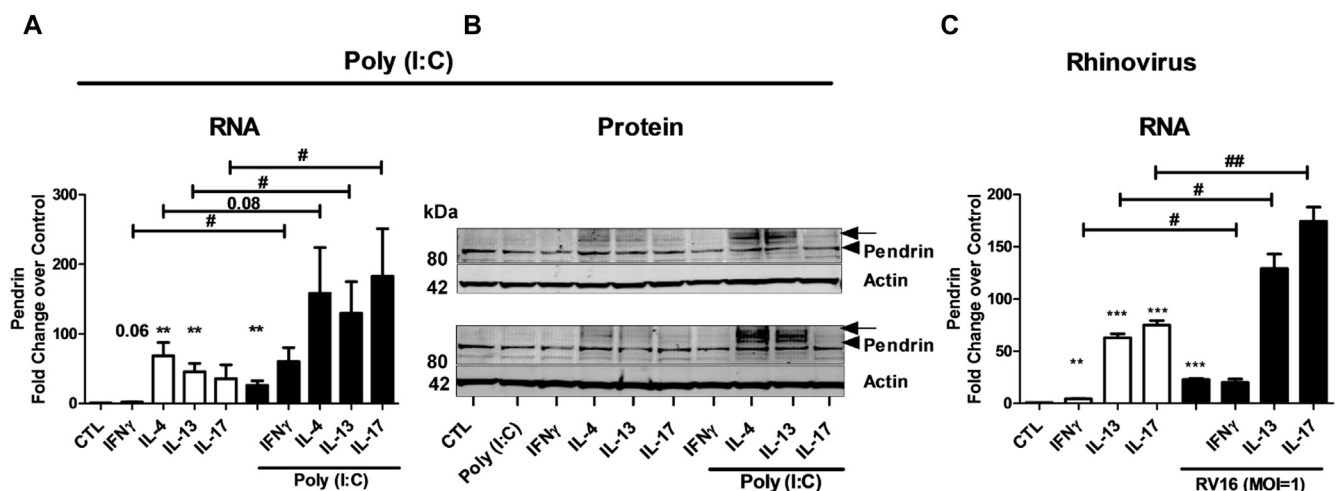


FIG 9. Potentiation effect of IL-13/IL-17A and Poly(I:C)/human rhinovirus infection on pendrin expression in airway epithelial cells. Differentiated NECs were stimulated with cytokines and/or Poly(I:C; A and B) or cytokines and/or human rhinovirus infection (C). Cells were harvested at 24 (Fig 9, A) or 48 (Fig 9, B and C) hours after stimulation. RNA (Fig 9, A and C) was analyzed by using real-time PCR. Pendrin mRNA expression was normalized to actin and expressed as fold change over control. Data from 8 to 12 experiments (Fig 9, A), 2 experiments (Fig 9, B), and 4 experiments (Fig 9, C) are shown. In Fig 9, B, arrowheads and arrows indicate native (80 kDa) and glycosylated (100 kDa) pendrin, respectively. # and * $P < .05$, ## and ** $P < .01$, and *** $P < .001$, paired t test. *Comparing control with treated.

real-time PCR. As observed previously, treatment with poly(I:C) and other cytokines alone minimally induced pendrin mRNA. This induction was further enhanced by the combined treatment of poly(I:C) and T_H1/T_H2/T_H17 cytokines (Fig 9, A). Moreover, treatment with IL-4, IL-13, or IL-17A along with poly(I:C) induced high levels of pendrin mRNA. We confirmed pendrin protein expression using immunoblot analysis. As observed earlier, treatment with poly(I:C) or T_H1/T_H2/T_H17 cytokines alone did not induce the glycosylated form of pendrin. Glycosylated pendrin was only induced by the combined treatment with poly(I:C) and T_H2 cytokines. Although combined treatment with IL-17A and poly(I:C) induced pendrin mRNA, we did not observe induction of the glycosylated form of pendrin (Fig 9, B). This suggests that among T_H cell cytokines, T_H2 cytokines are the only ones that strongly induce glycosylated pendrin.

Next, we used rhinovirus (RV16) infection to test the relevance of the poly(I:C) data to infection. RV16 infection and treatment of NECs with individual cytokines (IL-13 and IL-17A) induced pendrin mRNA, which was further enhanced by the combined influence of IL-13/IL-17A with rhinovirus infection (Fig 9, C). This suggests that pendrin is induced by T_H2/T_H17 cytokines and the induction is further enhanced by rhinovirus infection, implying a role for pendrin either during cold virus infections, asthma exacerbations, or both.

DISCUSSION

CRS is a complex multifactorial disease of the upper airways, and very little is known about its cause and pathogenesis. Several theories have been proposed to explain the pathogenesis of CRS,

but there is no direct evidence linking a single phenomenon/process to the cause of this disease.^{26,27} Among several possible factors contributing to the pathogenesis of CRS, dysregulated mucociliary clearance^{28,29} and increased mucus production¹⁰ are important events that might increase susceptibility to recurrent sinus infections. In this regard we became interested in pendrin, a T_H2 cytokine-induced gene that has been shown to be involved in these processes.^{14,15} Studies by Nakao et al¹⁴ showed that enforced expression of pendrin in airway epithelial cells causes increased mucus production. Moreover, pendrin knockout animals have decreased inflammatory cell recruitment and decreased inflammation in an ovalbumin-alum-induced asthma model.¹⁵ Pendrin also functions as an anion transporter, exchanging bicarbonate, thiocyanate, and iodide for chloride in different tissues. Pendrin, cystic fibrosis transmembrane conductance regulator, and sodium channels regulate chloride secretion in airway epithelial cells, thereby regulating air-surface liquid levels. In this regard epithelial cells from pendrin knockout animals have increased air-surface liquid, possibly because of dysregulation of ion transport activity in absence of pendrin. Because CRSwNP is associated with heavy secretions of mucus and T_H2 inflammation,⁶ we hypothesized that T_H2 cytokines would increase pendrin expression and function, thereby causing a defect in mucociliary clearance through the previously described functions of pendrin.

We found that pendrin expression was dramatically increased in NP tissues of patients with CRSwNP. It was notable that the size of the immunoreactive band was smaller than would be expected from the native protein. This finding could be due to several factors, including the presence of alternative splice variants in sinonasal tissues, abnormal migration of pendrin during electrophoresis, or cleavage of pendrin either *in vivo* or during the protein extraction procedure. More investigation into the specific cause of the size variance we observed is warranted.

We and others have found that pendrin is localized mainly at the sinonasal epithelial cells by using immunohistochemical analysis.^{14,16} Consistently, pendrin mRNA expression in NP scraping cells was increased but not significant compared with that seen in control UT scraping cells, indicating that the increase in pendrin expression seen in NP tissue might not be solely contributed by surface epithelial cells. An important goal of future work is identification of the pendrin-expressing cells in NPs of patients with CRSwNP.

To study the factors responsible for increased pendrin expression in NP tissue, we evaluated various T_H cell cytokines, such as IFN- γ , IL-13, and IL-17A. Levels of IL-17A mRNA were not significantly increased in patients with CRS, as we have shown previously.²¹ We have also not found increased levels of IFN- γ or other interferons in patients with CRS (W. Stevens et al, in preparation). Pendrin did not correlate with IFN- γ expression and showed a weak correlation with IL-17A expression (data not shown). Pendrin mRNA expression also correlated weakly with IL-13 levels, as would be expected by the ability of IL-13 to induce its expression *in vitro* (Fig 4). In cultured NECs both IL-13 and IL-17A induced pendrin expression, although only T_H2 cytokines induced the glycosylated form. We speculate that glycosylation might lead to increased membrane targeting, a necessary step in pendrin's function; if this is the case, then IL-13 might be the main cytokine among those tested that induces functionally active pendrin. Although it is well established that T_H2 cytokines induce pendrin through a signal transducer and

activator of transcription 6-dependent mechanism,³⁰ the induction of pendrin by IL-17A demonstrated in our study is a novel observation. Because IL-17A plays a critical role in lung innate host defense, further studies are needed to elucidate the mechanism by which IL-17A induces pendrin and the role of IL-17A-induced pendrin in epithelial innate host defense.

Furthermore, combined treatment of T_H2 cytokines and IL-17A synergistically induced pendrin, but not periostin, in NECs. Because IL-13, IL-17A, or both contribute to the pathogenesis of some forms of asthma^{31,32} and some groups implicate both in patients with CRS,³³⁻³⁶ we speculate a role of pendrin in these diseases. Although other studies have shown the presence of IL-17A in NPs of patients with CRS correlating positively with symptom severity and radiologic score,^{33,35} our previous study did not detect the presence of IL-17A in sinonasal tissues²¹; therefore we reanalyzed the presence of IL-17A protein using a more sensitive Luminex assay (EMD, Millipore, Mass). We detected measurable IL-17A in only 25% of the samples, suggesting that the synergy between T_H2 cytokines and IL-17A is not likely to explain the increased pendrin expression in all NPs. However, we cannot rule out the role of IL-17 in synergistic expression of pendrin in NPs because very minute quantities (less than the detection limit of ELISA) might be sufficient to induce synergistic expression *in vivo*. Additionally, the timing of when the polyps were obtained can also play an important role in the detection of IL-17A in sinonasal tissues. Interestingly, supporting a role for IL-17A, recent studies have shown that IL-17A is also induced by diesel exhaust particles, which might be potentially important in patients with T_H2 diseases, such as asthma and CRS.³² Of note, we found that IL-1 β can also cooperate additively with IL-13 to induce pendrin in epithelial cells (see Fig E6 in this article's Online Repository at www.jacionline.org). Interestingly, IL-1 β mRNA expression correlates moderately but significantly with pendrin mRNA (data not shown). Because IL-1 β is a pleiotropic cytokine involved in infection and inflammation, we speculate that IL-1 β -induced pendrin might play a role in inflammatory and infectious diseases of the upper and lower airways. We have some evidence that inflammasome activation is occurring in NPs (S. Seshadri, unpublished observations), and therefore it is possible that IL-1 β is contributing to the CRS phenotype.

Because pendrin expression has been shown to be increased in asthmatic patients and because rhinovirus infections are the leading cause of asthma exacerbations leading to emergency department visits, we analyzed pendrin expression on treatment of NECs with human rhinovirus infection in the presence of IL-13 or IL-17A. Interestingly, both IL-13 and IL-17A, along with rhinovirus infection, led to the induction of pendrin in an additive manner, suggesting that pendrin expression might be increased during rhinovirus infection and contribute to the profound mucus secretion observed with the common cold. Thus in asthmatic patients pendrin might play a role in rhinovirus-induced asthma exacerbations.

Lastly, we show that the glucocorticoid significantly suppressed pendrin mRNA at rest and after stimulation with IL-4, IL-13, or IL-17A alone; dexamethasone also suppressed the synergistic induction by these cytokines. This suggests that the therapeutic efficacy of glucocorticoids in asthmatic patients and those with CRS might be due in part by suppressing pendrin expression. Although we did not test dexamethasone against the combination of rhinovirus and cytokines, if it suppresses synergistic activation of pendrin in that situation, it might be

relevant to the well-known ability of glucocorticoids to suppress asthma exacerbations.

When our study was in progress, Ishida et al¹⁶ reported increased pendrin expression in NPs of patients with CRS and those with aspirin-exacerbated respiratory disease. We confirmed and extended their findings to show the inflammatory cells and cytokines associated with pendrin expression in sinonasal tissues. Interestingly, the increase in pendrin expression in NPs seems to be a common phenomenon observed in polyps from both Japanese and American subjects, although the pathology of NPs might differ.^{18,37} We speculate that the increased pendrin expression found in polyps from both Japanese and American subjects might be an independent phenomenon depending on the cytokine milieu present in these tissues. Also, our data identify previously unknown regulators of pendrin expression in epithelial cells, such as induction of pendrin by IL-17A, synergistic induction by the combination of T_H2 and T_H17 cytokines, and the potentiating effect of human rhinovirus infection in T_H2 induced pendrin.

There are few studies that have attempted to elucidate the function of pendrin in airway epithelial cells. On the basis of these, we can presume that pendrin plays a role in innate immunity, inflammation, mucus production, and ion transport. Pendrin expression correlated with inflammatory cell marker levels, suggesting either that pendrin can be induced by products secreted from these cells or can regulate recruitment of these cells and therefore inflammation through other mechanisms. Because overexpression of pendrin in epithelial cells increases mucus production and mucus production is a hallmark feature of patients with CRS, we examined the association of pendrin with Muc5AC. Our data suggest that pendrin expression and Muc5AC expression are weakly associated in sinonasal tissues and that increased pendrin expression might contribute to increased mucus production. This weak association might be due to small sample size, variability of human samples, and variability introduced because of sample isolation and processing and, importantly, suggests alternate modes of regulation and function. Because pendrin is also involved in regulating ion transport in airway epithelial cells, we speculate that increased pendrin expression in NPs might lead to dysregulated ion transport activity (increased Cl⁻ absorption), as proposed by others.^{11,12}

In conclusion, this study demonstrates increased pendrin expression in NPs of patients with CRS that is associated with inflammatory cytokine levels. Increased pendrin expression might contribute to the chronic inflammatory response by increasing mucus production and decreasing mucociliary clearance, leading to frequent bacterial infection and colonization. Further understanding of the molecular mechanisms involved in the regulation of pendrin expression and function will help in the design of novel therapeutics for treating patients with CRS and/or asthma.

Clinical implications: Increased pendrin expression can lead to increased mucus production and defective mucociliary clearance in patients with T_H2-mediated diseases, such as asthma and nasal polyposis.

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METHODS

Cell culture

NECs were collected during surgery and cultured under ALI conditions.^{E1} NECs were first plated in a single well of a 6-well culture plate coated with collagen (Vitrogen; Collagen Biomaterials, Palo Alto, Calif) for expansion. After reaching confluence, cells were trypsinized and plated in 0.4- μ m pore membrane inserts of a 12-well transwell plate (Costar, Corning, NY). Cells were grown in Bronchial Epithelial Growth Medium (Lonza, Basel, Switzerland) with medium on the apical and basal side until they reached confluence. Confluent cells were then cultured under ALI conditions. Apical medium was removed, and the basal medium was replaced with ALI medium containing Bronchial Epithelial Cell Growth Medium/DMEM supplemented with Bronchial Epithelial Growth Medium SingleQuot kit (Lonza) and additional BSA (1.5 μ g/mL) and retinoic acid (5×10^{-8} mol/L; Sigma-Aldrich, St Louis, Mo) to induce differentiation. The ALI medium was changed 3 times a week for 3 weeks. After 21 days, cells were changed to ALI medium without hydrocortisone for 48 hours and then used for experiments. For stimulation with cytokines, ALI medium with cytokines was added apically (50 μ L) and basally (950 μ L), and cells were stimulated for 24 hours for experiments evaluating RNA and 48 hours for experiments evaluating protein. In some experiments, NECs were stimulated with cytokines for 1 week to mimic chronic cytokine exposure. Medium containing cytokines was changed on days 2 and 4, and the cells were harvested on day 7 for assessment of RNA and protein. For dexamethasone experiments, cells were first pretreated with dexamethasone or dimethyl sulfoxide for 1 hour and then stimulated with cytokines as indicated for a further 24 hours. Cells were then harvested in RNA lysis buffer containing β -mercaptoethanol.

Real-time PCR

Total RNA from sinonasal tissue was extracted with QIAzol (Qiagen) by using the RNeasy purification system, according to the manufacturer's instructions. NECs (scrapings and ALI cultured) were lysed in RNA lysis buffer supplemented with β -mercaptoethanol, and RNA was extracted according to the manufacturer's protocol (Clontech, Mountain View, Calif). DNA contamination was eliminated by treatment of RNA with DNase I during purification. Good quality RNA from scraping cells, as determined by using an Agilent bioanalyzer (Agilent Technologies, Santa Clara, Calif), was used for real-time PCR. One microgram of RNA was converted to single-strand cDNA by using the SuperScript II reverse transcriptase system (Invitrogen, Carlsbad, Calif) with random primers. Semiquantitative real-time RT-PCR was performed with a TaqMan method by using an Applied Biosystems 7500 Sequence Detection System (Applied Biosystems, Foster City, Calif) in 15- μ L reactions (7.5 μ L of $2\times$ TaqMan Gene Expression Master mix [Applied Biosystems], 0.75 μ L of $20\times$ primer/probe mix, and 5 μ L of 2 ng/ μ L cDNA). All primers/probes were purchased from Applied Biosystems.

Median expression of GUSB (human β -glucuronidase endogenous control, PN; 4326320E) was used for normalizing gene expression. The exact copy number of the target genes was determined by running serial dilutions of quantified PCR product of the target gene as standards in the PCR reaction. Target gene expression was expressed as copies per nanogram ng of total RNA. In some experiments the Δ cycle threshold method was used to assess gene expression.

Extraction of proteins from sinus tissue and cultured epithelial cells

Tissue proteins were extracted, as described by Peters et al.^{E2} Briefly, tissues were cut into small pieces and resuspended in PBS containing 0.05% Tween-20 supplemented with a protease inhibitor cocktail (Sigma). Tissues were lysed in a Bullet Blender (Next Advance, Averill Park, NY)

with 1.6-mm stainless steel beads for 4 minutes twice at setting 7. Tissue lysates were centrifuged at 1968g to clear the debris. Tissue lysates were stored at -20°C until further use. Proteins from cultured cells were extracted with RIPA buffer (50 mmol/L Tris [pH 7.4], 1% SDS, 1 mmol/L EDTA, 150 mmol/L NaCl, and 0.25% sodium deoxycholate with protease and phosphatase inhibitors). Protein concentrations in all samples were determined by using a BCA assay (Thermo Scientific, Waltham, Mass), according to the manufacturer's protocol. Proteins in the preparation were resolved on 4-12% Bis-Tris gel (Life Technologies, Carlsbad, Calif) and transferred to a polyvinylidene difluoride membrane for immunoblotting.

Membranes were incubated with affinity-purified pendrin PP1 (1.0 μ g/mL) and actin antibody (1:10,000; clone C4; MP Biomedicals, Santa Ana, Calif). The PP1 antibody is directed against the first 15 amino acids at the N-terminal of the protein. This antibody has been previously described in detail and has been used by other groups to characterize pendrin.^{E3,E4}

After washing, blots were incubated with appropriate secondary antibody (Li-Cor Biosciences, Lincoln, Neb). Immunoblots were digitalized and quantified with Odyssey imaging software (Li-Cor Biosciences) to quantify pendrin protein in tissue extracts. Densitometric values for pendrin were normalized to those of the housekeeping gene β -actin.

Immunohistochemistry

Immunohistochemistry was performed, as described by Peters et al.^{E2} Briefly, nasal tissue was dehydrated, infiltrated, and embedded in paraffin, and tissue was sectioned at 3 μ m by using a Leica RM2245 Cryostat (Leica Microsystems, Bannockburn, Ill). All sections were deparaffinized and rehydrated; tissue sections were boiled for 20 minutes in citrate-based buffer for antigen unmasking (pH 6.0; Vector Laboratories, Burlingame, Calif). All sections were then blocked for endogenous peroxidase activity with 3% H_2O_2 /methanol. After rinsing, sections were treated to block nonspecific binding with 5% goat serum/0.3% Tween-20/PBS. Tissue sections were then incubated overnight at 4°C with 0.03 μ g/mL rabbit anti-human pendrin antibody or control rabbit IgG. Sections were rinsed and then incubated with biotinylated secondary goat anti-rabbit antibody (Vector Laboratories) at a 1:500 dilution for 1 hour at room temperature. After another rinse, sections were incubated in ABC reagent (avidin-biotin-horseradish peroxidase complex, Vector Laboratories) for 1 hour at room temperature. Sections were rinsed again and incubated in diaminobenzidine reagent (Invitrogen) for 10 minutes at room temperature. They were then rinsed in deionized H_2O , counterstained with hematoxylin, dehydrated, cleared, mounted, and placed in coverslips by using Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, Mich) in preparation for microscopic analysis.

Sections were observed with an Olympus IX71 Inverted Microscope at a $\times 200$ magnification and a MicroFire AR digital microscope camera (Optronics, Goleta, Calif).

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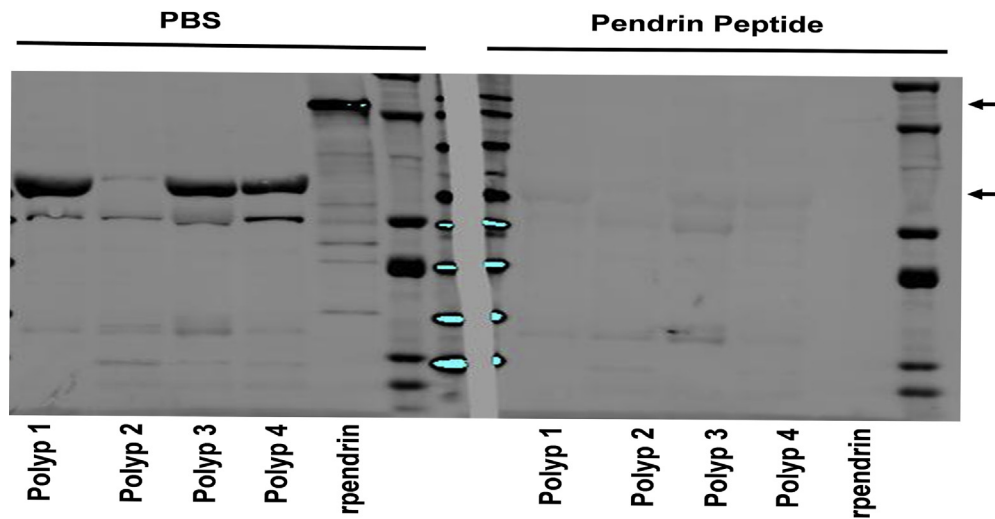


FIG E1. Pendrin peptide blocked detection of pendrin by pendrin-specific antibody in sinonasal tissues. Total protein (30 μ g) from sinonasal tissues and recombinant pendrin were separated by means of gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membrane. Membranes were then incubated with pendrin antibody premixed with PBS or pendrin peptide overnight. Blots were developed according to the normal immunoblot protocol. The blot shown is representative of at least 2 experiments using different sinonasal tissues from multiple donors.

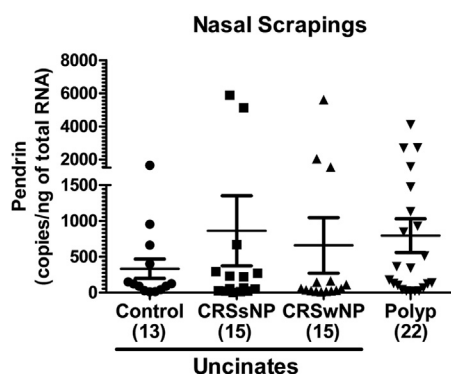


FIG E2. A trend toward increased pendrin expression in polyp epithelial cells compared with UT control epithelial cells. NECs from control subjects, patients with CRSsNP, and patients with CRSwNP (UTs) and polyps were obtained during surgery. RNA was isolated, and pendrin expression in NECs was analyzed by using real-time PCR.

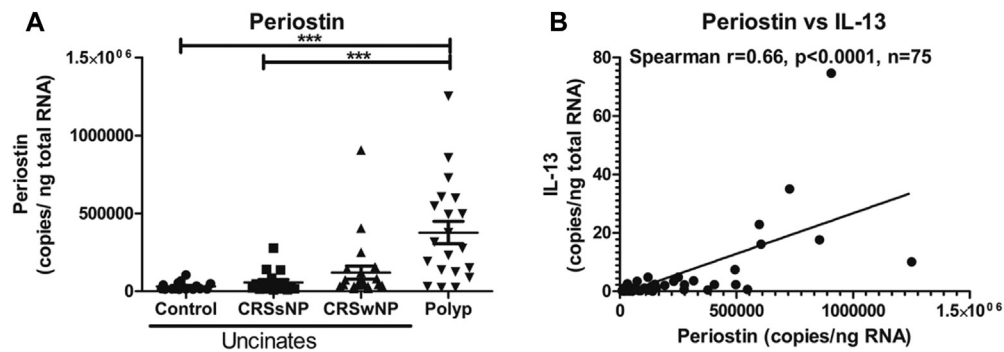


FIG E3. Periostin expression was increased in NPs of patients with CRS and correlated with IL-13 expression in sinonasal tissues. **A**, Periostin expression in sinonasal tissues was analyzed by using real-time PCR. **B**, The relationship between periostin expression and IL-13 expression in sinonasal tissues was analyzed by using Spearman rank correlation. Data for IL-13 were used from Fig 4. *** $P < .001$.

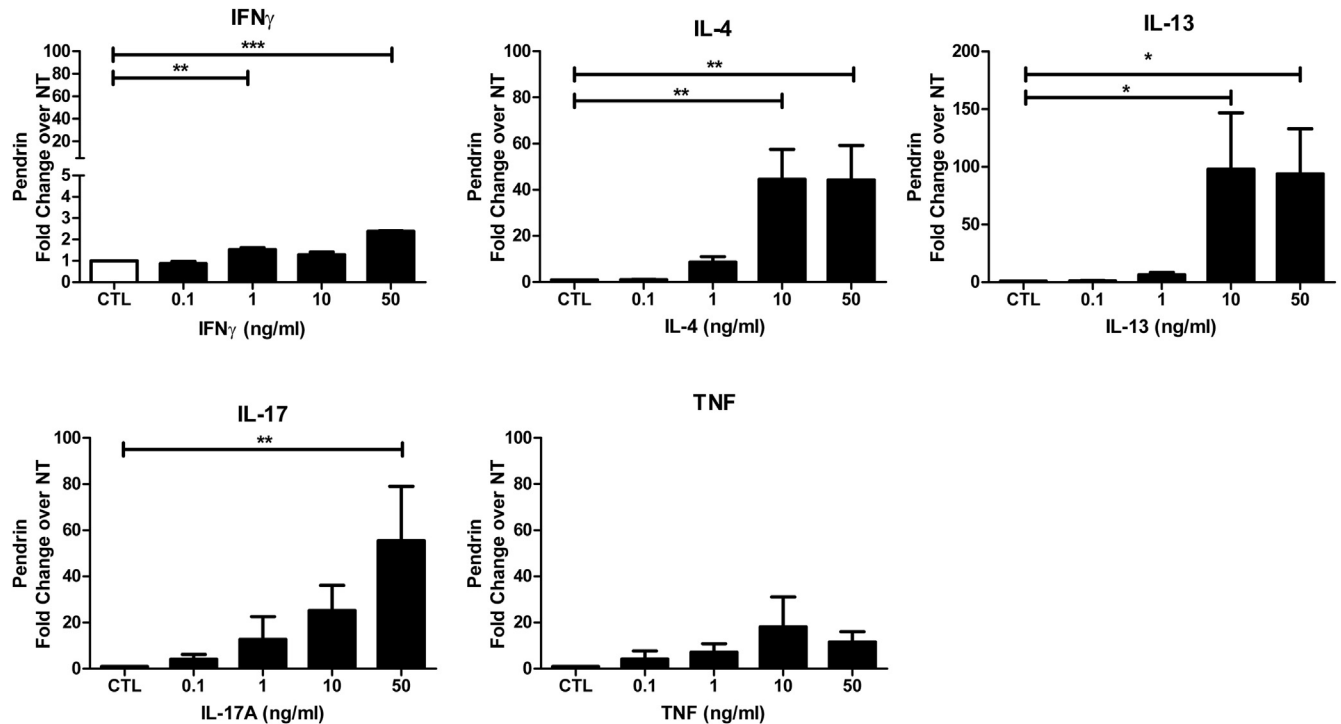


FIG E4. Expression of pendrin mRNA was increased by T_H2 cytokines and IL-17 stimulation in a dose-dependent manner in differentiated airway epithelial cells. Differentiated NECs were untreated (CTL) or stimulated with increasing concentrations (0.1-50 ng/mL) of cytokines as indicated for 24 hours. Pendrin gene expression was quantified by using real-time PCR ($n = 3-12$). Gene expression was normalized to actin and expressed as fold change over untreated control samples. * $P < .05$, ** $P < .01$, and *** $P < .001$.

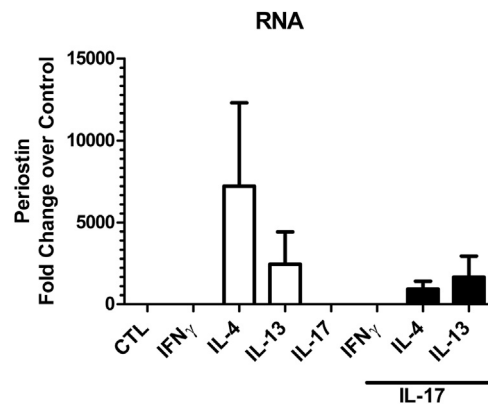


FIG E5. Periostin expression was not synergistically induced by combined treatment with T_H2 and IL-17A in differentiated airway epithelial cells. Real-time PCR analysis of periostin expression in differentiated NECs stimulated with cytokines alone (10 ng/mL) or in combination (10 ng each) as indicated for 24 hours is shown. Shown are data from 6 experiments. *CTL*, Control.

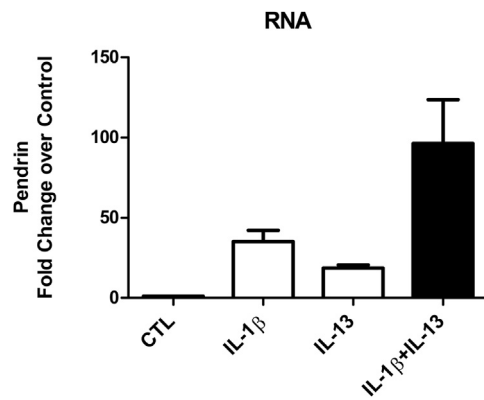


FIG E6. Pendrin expression was additively induced by combined treatment with IL-13 and IL-1 β in differentiated airway epithelial cells. Real-time PCR analysis of pendrin expression in differentiated NECs stimulated with cytokines alone (10 ng/mL) or in combination (10 ng each) as indicated for 24 hours is shown. Shown are data from 2 experiments. *CTL*, Control.