

Differential expression of interleukin-32 in chronic rhinosinusitis with and without nasal polyps

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Abstract

Background: Chronic rhinosinusitis (CRS) is a heterogeneous disease characterized by local inflammation of the upper airways and sinuses and is frequently divided into polypoid CRS (CRSwNP) and nonpolypoid CRS (CRSsNP). However, the mechanism of inflammation in CRS has still not been fully elucidated. The aim of the study was to investigate the role of interleukin-32 (IL-32), a recently discovered proinflammatory cytokine, in CRS.

Methods: We collected nasal epithelial cells and nasal tissue from patients with CRS and control subjects. We assayed mRNA for IL-32 by real-time PCR and measured IL-32 protein using ELISA, Western blot, and immunohistochemistry.

Results: The expression of mRNA for IL-32 was elevated in epithelial cells from uncinat tissue from patients with CRSsNP compared with patients with CRSwNP ($P < 0.05$), control subjects ($P = 0.06$), and epithelial cells from nasal polyp (NP) tissue ($P < 0.05$). Production of IL-32 was induced by IFN- γ , TNF, and dsRNA in primary airway epithelial cells. In whole-tissue extracts, the expression of IL-32 protein was significantly elevated in patients with CRSwNP compared with patients with CRSsNP and control subjects. Immunohistochemistry data showed that IL-32 was detected in mucosal epithelial cells and inflammatory cells in the lamina propria. Levels of IL-32 were correlated with the levels of CD3 and macrophage mannose receptor in NP tissue. Immunofluorescence data showed IL-32 co-localization with CD3-positive T cells and CD68-positive macrophages in NPs.

Conclusion: Overproduction of IL-32 may be involved in the pathogenesis of CRS, although the role of IL-32 in the inflammation in CRSsNP and CRSwNP may be different.

Chronic rhinosinusitis (CRS) is a chronic inflammatory disease of the sinuses and upper airways that affects over 30 million Americans (1). It is a significant cause of morbidity in the United States. Clinically, CRS is a heterogenous symptom complex, often resistant to medical therapy, that is typically characterized by two or more of the following: mucopurulent drainage, nasal obstruction, facial pain/pres-

sure, and hyposmia/anosmia (1, 2). Current medications have variable efficacy, and over 250 000 surgical procedures are performed annually in the United States on patients who failed maximal medical therapy. CRS is clinically classified into CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). In general, CRSwNP is associated more closely with the clinical complaints of nasal obstruction and smell loss, and CRSsNP is associated with the symptoms of facial pain/pressure and headaches (3). Although CRS has been characterized by chronic inflammation, the mechanisms of inflammation in CRSsNP and CRSwNP are generally different. CRSwNP, characteristically, has more Th2 polarization and a greater degree of tissue eosinophilia (4, 5). In

Abbreviation

CRS, Chronic rhinosinusitis; CRSsNP, CRS without nasal polyps; CRSwNP, CRS with nasal polyps; GUSB, β -glucuronidase; IL-32, Interleukin-32; MMR, macrophage mannose receptor; NP, nasal polyp; UT, uncinat tissue.

contrast, CRSsNP demonstrates more Th1 polarization and collagen deposition in the nasal mucosa (4–6). However, the mechanism of inflammation in CRS has still not been fully elucidated.

Interleukin-32 (IL-32) is a recently described proinflammatory cytokine produced by T cells, NK cells, monocytes, endothelial cells, and epithelial cells (7, 8). It was cloned as a gene induced by IL-2 and initially named natural killer cell transcript 4, NK4, and its function remained unknown until 2005 (7, 9). There are six isoforms – α , β , γ , δ , ϵ , and ζ , – caused by mRNA splicing (8). There are potentially diverse roles for the different isoforms of IL-32; however, the functional differences are not well understood. Because the receptor for IL-32 and the ortholog of IL-32 in rodents have not been discovered, the function and regulation of IL-32 have been difficult to study. IL-32 is known to be induced by tumor necrosis factor (TNF), IL-1 β , interferon (IFN)- γ , dsRNA and LPS in monocytes, endothelial cells, epithelial cells, keratinocytes, and fibroblasts (7, 10–15). IL-32 activates the production of TNF and IL-8 through the activation of NF- κ B and p38 MAP kinase (7). IL-32 synergizes with nucleotide oligomerization domain ligands to stimulate IL-1 β and IL-6 release in a caspase-1-dependent manner (16). Recently, it has been reported that silencing endogenous IL-32 impairs IL-1 β - or LPS-dependent production of TNF, IL-6, and IL-8 in endothelial cells and the monocytic cell line THP-1 cells (12, 17). Other groups have shown that IL-32 is a key endogenous factor in TCR- and IFN- γ -dependent apoptosis in T cells and keratinocytes (13, 18). Therefore, IL-32 is now recognized as a dual-function protein that acts as a proinflammatory cytokine and an endogenous regulator of cytokine production and apoptosis.

IL-32 is known to play an important role in host defense against microorganisms including *Mycobacterium*, HIV, and influenza (10, 19–21). IL-32 has also been implicated in several inflammatory disorders, including rheumatoid arthritis, Crohn's disease, atopic dermatitis, and chronic obstructive pulmonary disease (8, 13, 15, 22, 23). IL-32 expression has

been found to correlate with disease severity in these conditions (13, 15, 24). Thus far, there have been no studies regarding IL-32 in sinus epithelial cells and in the disease states of CRS. In the present study, we investigated the expression of IL-32 and its potential role in the inflammatory response in CRSwNP and CRSsNP.

Methods

Patients and biopsies

Patients with CRS were recruited from the Allergy-Immunology Clinic and the Otolaryngology Clinic of the Northwestern Medical Faculty Foundation (NMFF) and the Northwestern Sinus Center at NMFF. Sinonasal and nasal polyp tissues were obtained from routine functional endoscopic sinus surgery in the patients with CRS. All subjects met the criteria for CRS as defined by the American Academy of Otolaryngology – Head and Neck Surgery Chronic Rhinosinusitis Task Force (1, 25). Patients with an established immunodeficiency, pregnancy, coagulation disorder, Churg–Strauss syndrome, diagnosis of classic allergic fungal sinusitis or cystic fibrosis were excluded from the study. Sinus tissues from inflammation-free control subjects undergoing endoscopic sinus surgery for surgical access to the anterior skull base were recruited from the otolaryngology practice at NMFF. All subjects signed informed consent forms, and the protocol governing procedures for this study was approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine. Details of subjects' characteristics are included in Table 1 and in Supporting Information.

Cell culture, real-time PCR, Western blot, immunohistochemistry, and ELISA

The general methods for culture of primary nasal epithelial cells (PNEC) (26), culture of normal human bronchial

Table 1 Subject characteristics

	Control			CRSsNP			CRSwNP		
Total no. of subjects	<i>n</i> = 24 (13M/11F)			<i>n</i> = 40 (15M/25F)			<i>n</i> = 54 (42M/12F)		
Age (years), median (range)	42 (16–63)			37 (23–64)			42 (26–74)		
	Y	N	U	Y	N	U	Y	N	U
Atopy	1	19	4	20	11	9	24	17	13
Asthma	0	23	1	5	30	5	23	29	2
Methodologies used	UT			UT			UT		
Nasal scraping cells	<i>n</i> = 15 (11M/4F)			<i>n</i> = 21 (8M/13F)			<i>n</i> = 14 (13M/1F)		
Age	44 (27–63)			36 (26–55)			42 (26–61)		
Tissue RNA	<i>n</i> = 9 (3M/6F)			<i>n</i> = 18 (8M/10F)			<i>n</i> = 19 (15M/4F)		
Age	39 (16–59)			40 (23–64)			41 (26–61)		
Tissue extract	<i>n</i> = 5 (1M/4F)			<i>n</i> = 10 (3M/7F)			<i>n</i> = 10 (9M/1F)		
Age	47 (25–63)			36 (24–51)			47 (30–74)		
							NP		
							<i>n</i> = 25 (23M/2F)		
							43 (26–68)		
							<i>n</i> = 26 (19M/7F)		
							44 (26–74)		
							<i>n</i> = 10 (9M/1F)		
							42 (26–63)		

Y, yes; N, no; U, unknown; CRSsNP, CRS without nasal polyps; CRSwNP, CRS with nasal polyps; NP, nasal polyp; UT, uncinat tissue.

epithelial cells (NHBE) (27), real-time PCR (28), Western blot (29), immunohistochemistry (30), and enzyme-linked immunosorbent assay (ELISA) (26) are described previously. The details of methods are described in Supporting Information.

Statistics

All data are reported as mean $\pm$ SEM unless otherwise noted. Differences between groups were analyzed using the Mann–Whitney *U*-test (in clinical samples) and the two-tailed paired Student's *t* test (in *in vitro* samples). Correlations were assessed using the Spearman's rank correlation. A *P* value of < 0.05 was considered significant.

Results

IL-32 expression in nasal epithelial scraping cells in CRS

To determine the relevance of IL-32 expression in CRS, sino-nasal and polyp tissues and nasal epithelial scraping cells were collected from 40 subjects with CRSsNP, 54 subjects with CRSwNP and 24 control subjects.

We assessed the expression of IL-32 in freshly isolated epithelial scraping cells from uncinat tissue (UT) from patients with CRSsNP, CRSwNP, and controls as well as epithelial scraping cells from nasal polyp (NP) tissue from patients with CRSwNP. IL-32 mRNA levels were increased in epithelial scraping cells from UT in patients with CRSsNP ($n = 21$) compared with UT from controls ($P = 0.063$, $n = 15$), CRSwNP ($P < 0.05$, $n = 14$), and epithelial cells from NP tissue ($P < 0.05$, $n = 25$) (Fig. 1).

Expression of IL-32 in primary human airway epithelial cells

Although we found an upregulation of IL-32 in epithelial cells from patients with CRSsNP, the regulation of IL-32 in primary cells has not been carefully studied. To our knowledge, no one has reported the regulation of IL-32 expression in primary airway epithelial cells. To study the regulation of IL-32 in primary airway epithelial cells, PNEC and primary NHBE were treated with known activating cytokines including TNF, IL-4, IL-17A, IFN- β and IFN- γ , and TLR ligands including peptidoglycan (PGN, TLR2 ligand), dsRNA (TLR3 ligand), and LPS (TLR4 ligand) for 24 h. Messenger RNA for IL-32 was significantly upregulated by stimulation with TNF (16-fold, 26-fold, $P < 0.05$), IFN- γ (79-fold, 188-fold, $P < 0.05$), and dsRNA (21-fold, 38-fold, $P < 0.05$) and not affected by IL-4, IL-17A, IFN- β , PGN, or LPS in both PNEC ($n = 7$) and NHBE ($n = 5$; Fig. 2A,B and data not shown). These suggest that the regulation of IL-32 in PNEC and NHBE is likely the same. We therefore focused on the regulation of IL-32 in NHBE for further studies.

It has been reported that stimulation of IL-32 mRNA is further enhanced by combining TNF and IFN- γ in intestinal epithelial cell lines (15). Therefore, we examined the effect of the combined stimulation of cytokines, TNF and IFN- γ , and the TLR3 ligand, dsRNA, on the expression of IL-32 in

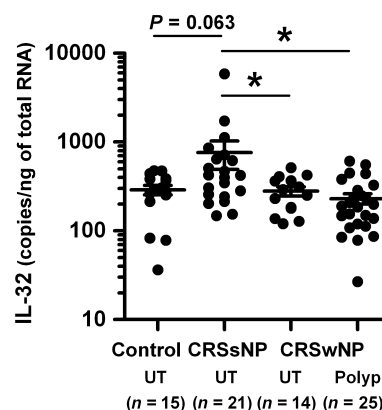


Figure 1 Expression of IL-32 in epithelial scraping cells. Total RNA was extracted from epithelial scraping cells from uncinat tissue (UT) and nasal polyps (Polyp), and the expressions of IL-32 were analyzed using real-time PCR. IL-32 mRNA levels were increased in epithelial scraping cells from UT in patients with CRS without nasal polyps compared with UT from controls ($P = 0.063$), CRS with nasal polyps ($P < 0.05$), and nasal polyps ($P < 0.05$, $n = 25$). * $P < 0.05$

NHBE. We found that TNF and dsRNA synergistically and significantly enhanced IFN- γ -dependent IL-32 mRNA expression in NHBE (4.3-fold and 4.9 fold, $n = 3-4$, $P < 0.05$) (Fig. 2C). We further confirmed this finding by Western blot. IL-32 protein was found in the cell lysate after stimulation with TNF, IFN- γ , and dsRNA (Fig. 2D). In addition, the expression of IL-32 in the cell lysates was further enhanced when NHBE were stimulated by the combination of IFN- γ and TNF or IFN- γ and dsRNA (Fig. 2D). We detected IL-32 protein in the supernatants only when NHBE were stimulated by the combinations (Fig. 2D). The IL-32 protein was detected as a 25-KDa band. This was larger than the recombinant IL-32 α protein, indicating that the major isoforms in airway epithelial cells are less likely to be the α isoform.

IL-32 expression in tissue samples in CRS

To further confirm at the protein level the elevation of IL-32 that we observed in nasal epithelial cells from UT from patients with CRSsNP, we used immunohistochemistry to detect IL-32 protein in UT and NP tissue. Immunohistochemistry demonstrated considerable IL-32 staining in the mucosal and the glandular epithelium as well as in submucosal cells (Fig. 3A and data not shown). However, we did not observe more intense staining of IL-32 in epithelial cells from CRSsNP compared with the levels in control subjects and patients with CRSwNP. In contrast, we found that IL-32 $^{+}$ inflammatory cells were elevated in NP tissue from patients with CRSwNP (Fig. 3A and data not shown). We therefore decided to determine the levels of IL-32 in sinus tissue. We assessed the expression of mRNA for IL-32 in UT from patients with CRSsNP, CRSwNP and controls, as well as NP tissue from CRSwNP. IL-32 mRNA was only significantly

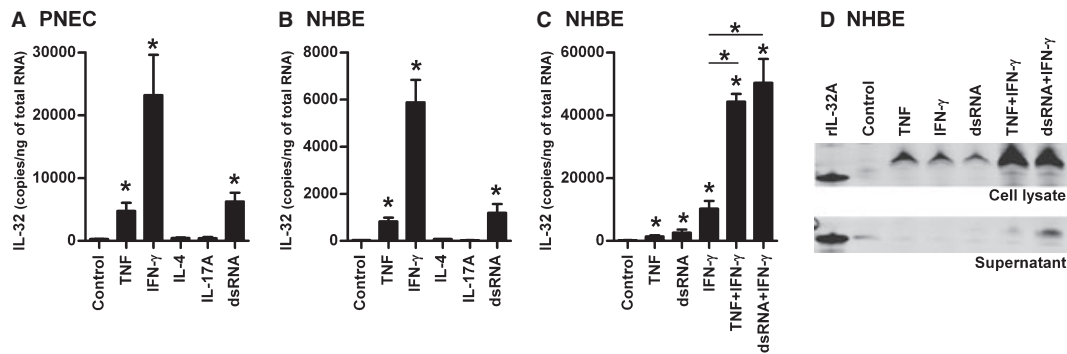


Figure 2 Induction of IL-32 in human primary epithelial cells. Primary nasal epithelial cells (PNEC, A) and primary normal human bronchial epithelial cells (NHBE, B and C) were incubated for 24 h (A–C) with 100 ng/ml TNF, 10 ng/ml interferon (IFN)- γ , 100 ng/ml IL-4, 100 ng/ml IL-17A, and 5 μ g/ml dsRNA. The expression of mRNAs for IL-32 was determined by real-time PCR (A–C). NHBE

were stimulated with 50 ng/ml TNF, 10 ng/ml IFN- γ , 5 μ g/ml dsRNA, and their combinations for 48 h, and then, protein expression in the cell lysates and supernatants was analyzed by Western blot (D). Results shown are mean $\pm$ SEM of seven (A) and four (B and C) independent experiments. The results are representative of four separate experiments (D). * P < 0.05.

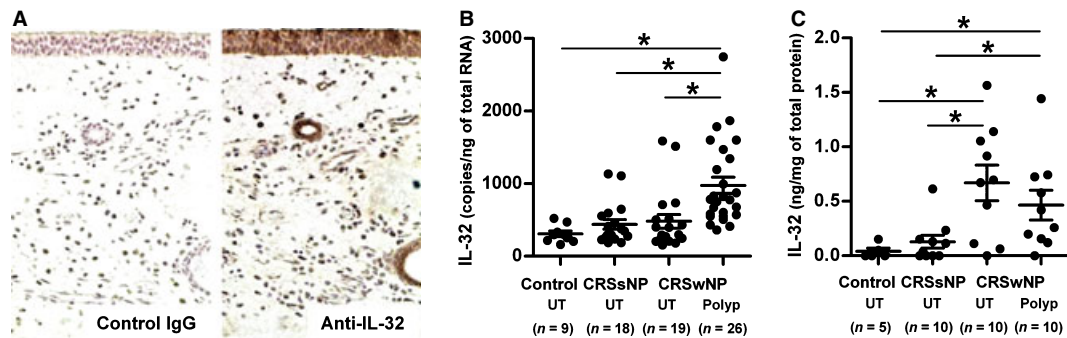


Figure 3 Expression of IL-32 in sinus tissue. Representative immunostaining for IL-32 and control IgG1 in nasal polyp (NP) tissue from a patient with CRS with NPs (A). IL-32 was detected in mucosal and glandular epithelium as well as in infiltrated inflammatory cells in NPs. Total RNA was extracted from uncinat tissue (UT) and NPs (Polyp), and the expression of IL-32 non-A (IL-32) was analyzed

by real-time PCR (B). IL-32 mRNA was significantly increased in NP tissue (P < 0.001). The concentration of IL-32 in tissue homogenates of UT and Polyp was measured using enzyme-linked immunosorbent assay (C). IL-32 protein was significantly increased in CRS with NPs. IL-32 concentration was normalized to the concentration of total protein. * P < 0.05.

increased in NP tissue from patients with CRSwNP (P < 0.001) in comparison with UT from patients with either form of CRS or control subjects (Fig. 3B). To confirm this observation at the protein level, we made detergent extracts from homogenates of UT and NP tissues and then measured the IL-32 concentration by ELISA. We initially assayed the concentration of IL-32 in tissue extracts by a commercial IL-32 α ELISA kit. However, the assay lacked adequate sensitivity to detect the IL-32 α protein in tissue homogenates (data not shown). In addition, we found that the levels of expression of IL-32 α were about 100-fold less than the levels of IL-32 non- α in epithelial cells and in whole-tissue extracts (Fig. S2). This suggests that IL-32 α is a minor isoform in nasal mucosa. We therefore decided to use an in-house ELISA system for pan-IL-32 isoforms. We found that the IL-32 protein was significantly increased in UT (n = 10, 0.67 ± 0.16 ng/mg) and NP tissue (n = 10, 0.46 ± 0.16 ng/mg) from patients with CRSwNP compared with UT from controls (n = 5, 0.04 ± 0.07 ng/mg) (Fig. 3C).

Detection of IL-32-producing cells in nasal polyps

We found that IL-32 $^{+}$ cells were significantly elevated in the submucosal region of NP tissue and that IL-32 mRNA and protein were significantly elevated in NP tissue (Fig. 3). We next proceeded to identify the IL-32-producing cells in the nasal mucosa of NP tissue. We first assessed whether the expression of IL-32 correlated with the levels of mRNA expression for markers of inflammatory cells in NP tissue by real-time PCR. We found that the levels of expression of IL-32 mRNA in NP tissue significantly and positively correlated with the expression of CD3 (r = 0.789, P < 0.001, n = 26), macrophage mannose receptor (MMR, r = 0.537, P = 0.005), CD1c (r = 0.481, P = 0.013), and tryptase (r = 0.489, P = 0.011) but not with CD20, CD56, CD138, or eosinophil cationic protein (ECP) (Fig. 4), indicating that IL-32 might be expressed in T cells, macrophages, dendritic cells, and mast cells. To identify the IL-32-producing cells in the submucosal tissue of NPs, we performed immunofluores-

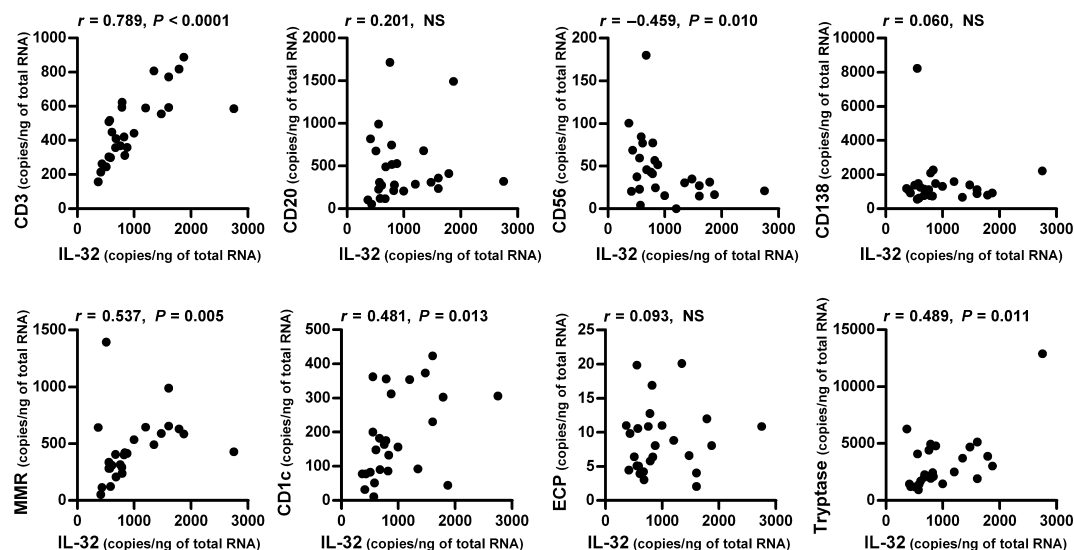


Figure 4 Correlation of IL-32 with cell-specific markers in nasal polyps (NP). Total RNA was extracted from NP tissue, and the expression of IL-32 and cell-specific markers was analyzed by real-time

PCR ($n = 26$). The correlations were assessed using the Spearman's rank correlation.

cence analysis using an anti-IL-32 antibody and antibodies against markers of these cells including CD3 (T cells), CD68 (macrophages), and tryptase (mast cells). We found IL-32 colocalization with CD3⁺ T cells and CD68⁺ macrophages but not with mast cells in NP tissue (Fig. 5).

Discussion

This study provides the first demonstration that IL-32 is increased in epithelial cells from patients with CRSsNP (Fig. 1) and that the levels of IL-32 in primary airway epithelial cells are regulated by the Th1 cytokine IFN- γ , the pro-inflammatory cytokine TNF, and the TLR3 ligand dsRNA (Fig. 2). Significant levels of IL-32 mRNA and protein were found in whole tissue from patients with CRSwNP (Fig. 3). The expression of IL-32 in NP tissue was positively correlated with the levels of CD3 and MMR (Fig. 4). IL-32-positive cells were more numerous in the submucosal tissue of NPs from patients with CRSwNP, and they were co-localized with CD3-positive and CD68-positive cells, indicating that T cells and macrophages are the major IL-32-producing cells in NPs (Figs 3 and 5).

CRS is characterized by chronic inflammation of the paranasal sinuses and is frequently divided into polypoid CRS (CRSwNP) and nonpolypoid CRS (CRSsNP). The lack of understanding of the mechanism for inflammation in CRS hinders the development of new treatments for this disease. IL-32 is a novel proinflammatory cytokine and also acts as an endogenous regulator of cytokine production and apoptosis. IL-32 is expressed in epithelial cells, and the overexpression of IL-32 has been shown to be involved in Th1-related inflammatory diseases, including rheumatoid arthritis, Crohn's disease, and chronic obstructive pulmonary disease (8, 15, 22, 23). In addition, there are very few studies of epithelial cells

taken from patients with CRS and controls, and most of those studies focus on epithelial cells from patients with CRSwNP. We therefore sought to examine the expression of IL-32 in epithelial cells from patients with CRSsNP, which is characterized by Th1 polarized inflammation. In this study, we report a very rare pattern of gene regulation in which expression is observed in epithelial cells only from patients with CRSsNP and not from those with CRSwNP. We demonstrated that mRNA encoding IL-32 was significantly increased in epithelial cells from patients with CRSsNP (Fig. 1). Although IL-32 was found to be regulated by the Th1 cytokine IFN- γ in epithelial cell lines, to our knowledge, no one has shown the regulation of IL-32 in primary airway epithelial cells. We demonstrated that primary nasal and bronchial epithelial cells produced IL-32 when stimulated with IFN- γ , TNF, and dsRNA with a synergistic enhancement by the combination of IFN- γ with either TNF or dsRNA (Fig. 2). Interestingly, there was no increased expression of IL-32 after treatment with the Th2 cytokine IL-4 or the Th17 cytokine IL-17A. Recently, Meyer et al. demonstrated that Th1 cells but not Th2, Treg, or Th17 cells induced the expression of IL-32 in epidermal keratinocytes in an IFN- γ -dependent manner (13). These results suggest that overexpression of IL-32 in epithelial cells from patients with CRSsNP may be induced by a Th1 environment.

We showed that IL-32 was upregulated in epithelial cells from patients with CRSsNP; however, it did not appear to be secreted, as demonstrated by low expression in nasal lavage fluids (data not shown). In *in vitro* studies, we did not see a substantial release of IL-32 protein in the culture supernatant of NHBE (Fig. 2). Several groups have reported that IL-32 is detected in the cytoplasm of keratinocytes, endothelial cells, T cells, and peripheral blood mononuclear cells but is not secreted from the cells (12, 13, 18, 19). Nold-

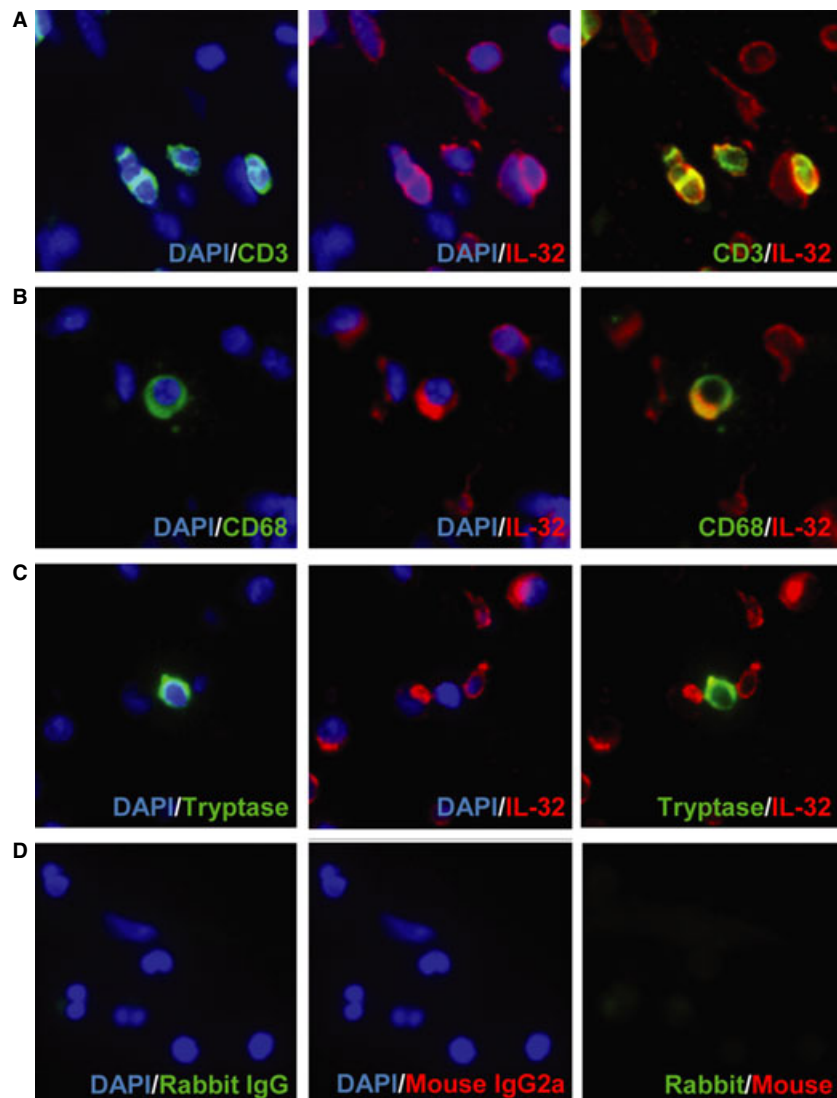


Figure 5 Identification of IL-32-producing cells by immunofluorescence assay. The immunofluorescence assay was performed using anti-human IL-32 mAb (red fluorescence), anti-CD3 mAb (green fluorescence) for T cells (A), anti-CD68 mAb (green fluorescence) for macrophages (B), anti-tryptase mAb (green fluorescence) for mast

cells (C) and control IgG (D). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue fluorescence). IL-32 was detected in CD3⁺ T cells and CD68⁺ macrophages in nasal polyps as demonstrated by co-localization (yellow fluorescence) of the antibody staining. The results are representative of three patients.

Petry et al. (12) showed that silencing of endogenous IL-32 results in the reduction in constitutive as well as IL-1 β -induced production of IL-1 α , IL-6, and IL-8 in endothelial cells. Other groups have shown that silencing of endogenous IL-32 prevents apoptosis in HeLa cells and keratinocytes (13, 18). These results suggest that IL-32 may act as an endogenous regulator of cytokine production and apoptosis in nasal epithelial cells. Future studies will be required to investigate whether IL-32 acts as a controller of proinflammatory cytokine production in epithelial cells or an inducer of epithelial damage in patients with CRSsNP.

Although we found clear evidence that IL-32 was elevated in epithelial cells from CRSsNP, we could not find more

intense staining of IL-32 in CRSsNP by immunohistochemistry. In addition, we could not obtain enough total protein from epithelial scraping cells from patients for our current ELISA (data not shown). It is recognized that immunohistochemistry is not a quantitative method. Future studies will be required to generate a high-sensitivity assay system for the detection of IL-32 protein and to confirm the elevation of IL-32 protein in epithelial cells from patients with CRSsNP.

In contrast to epithelial cells, we found increased expression of IL-32 in whole-tissue extracts from NPs but not in whole tissue from patients with CRSsNP (Fig. 3). We have recently reported a similar trend in the expression of S100A8/A9 in CRS. We showed that the expression of S100A8/A9 was

decreased in epithelial cells from CRSwNP; however, the expression of S100A8/A9 was increased in whole-tissue extracts from patients with CRSwNP (31). We also found that concentrations of S100A8/A9 were correlated with the concentration of neutrophil elastase, suggesting that the elevation of S100A8/A9 in NP tissue was reflected by the infiltration of S100A8/A9-producing neutrophils in the tissue (31). In our current study, immunohistochemistry data clearly showed that IL-32-positive inflammatory cells were more abundant in NP tissue (Fig. 3 and data not shown). This suggests that the infiltration of inflammatory cells may be responsible for the elevation of IL-32 in whole tissue from NPs.

Nasal polyps are generally characterized by eosinophilic inflammation. In addition, it has been reported that lymphocytes, macrophages, dendritic cells, and neutrophils also accumulate in the nasal mucosa of patients with CRSwNP (4, 5, 26, 30–33). Our immunofluorescence data clearly showed that IL-32 was detected in CD3⁺ T cells and CD68⁺ macrophages (Fig. 5) and that the expression of IL-32 was positively correlated with their markers, CD3 and MMR, in NPs (Fig. 4). These results suggest that T cells and macrophages are the major IL-32-producing cells in the nasal mucosa of NPs. Calabrese et al. (23) showed that IL-32 was elevated in the lungs of patients with chronic obstructive pulmonary disease and that IL-32 was detected in alveolar macrophages and CD8-positive T cells in addition to epithelial cells. These combined results suggest that macrophages and T cells are the major IL-32-producing inflammatory cells in airway inflammatory diseases.

Although there are six isoforms of IL-32, variations in the distribution and functional differences of those isoforms are not well described. IL-32 ϵ and IL-32 ζ were very minor isoforms and were found only in activated T cells (18). Alternative expression of the major isoform of IL-32 was reported in some cells; IL-32 α was the major isoform in myofibroblasts, IL-32 β in activated T cells, and IL-32 γ in epidermal keratinocytes (13, 14, 18). Collectively, these findings suggest that there are specific and subtle biological differences in the expression, and therefore likely the function, of the different isoforms. In the current study, we also detected IL-32 α and the other isoforms separately. We showed that the levels of expression of IL-32 α were 100-fold less than IL-32 non- α isoforms in PNEC, NHBE, nasal scraping cells, and nasal tissues, although the expression patterns were similar between the IL-32 α and the IL-32 non- α isoforms (Figs S1 and S2 and data not shown). Western blot data showed that the molecular weight of IL-32 was 25 kDa in NHBE (Fig. 2). This suggests that the major isoform was IL-32 β and/or IL-32 γ in NHBE. Choi et al. (11) demonstrated that IL-32 β and IL-32 γ have greater biological activity than IL-32 α and IL-32 δ in peripheral blood mononuclear cells. This suggests that the more active isoform of IL-32 is expressed in airway epithelial cells. Future studies will be required to determine the characterization of isoforms of IL-32 in CRS.

Although IL-32 is a proinflammatory cytokine that induces the production of IL-1 β , IL-6, TNF, and IL-8 in monocytes and peripheral blood mononuclear cells, the mechanism of secretion of IL-32 remains still unclear (7, 11). Goda et al. (18) showed that IL-32 was released from apoptotic T cells. In the present study, we showed that the release of IL-32 in primary epithelial cells occurred when cells were stimulated with the combination of IFN- γ and dsRNA, and the combination of IFN- γ and dsRNA induced the highest LDH release in NHBE (data not shown), indicating that epithelial cells might be able to release IL-32 only during cell death. Another source of IL-32 in NPs could be macrophages. Bai et al. (10) demonstrated that IL-32 controlled pathogen-dependent cytokine production endogenously in the differentiated THP-1 macrophage line. However, it is still unknown whether primary macrophages release IL-32 or IL-32 acts intracellularly in cells contained within NPs. Future studies will be required to determine whether IL-32 is released by cell activation or during cell death and whether IL-32 acts extracellularly in patients with CRS.

In summary, we report here that IL-32 is elevated in epithelial cells from patients with CRSsNP and that IL-32-producing T cells and macrophages are elevated in nasal tissue from patients with CRSwNP. These results support the conclusion that the role of IL-32 in inflammation associated with CRSsNP and CRSwNP may be different. Future studies will center on the function of IL-32 in CRS to determine its role in inflammation and the immune system.

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Conflict of interest

The authors have no conflict of interest to declare.

Supporting Information

Additional Supporting Information may be found in the online version of this article found at: <http://www.wileyonlinelibrary.com>

Data S1. Methods.

Figure S1. The structure of IL-32 isoforms.

Figure S2. Expression of IL-32 α in CRS.

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Supporting information

Differential expression of interleukin-32 in chronic rhinosinusitis with and without nasal polyps

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Methods

Patients and biopsies

CRS patients were recruited from the Allergy-Immunology clinic and the Otolaryngology clinic of the Northwestern Medical Faculty Foundation (NMFF) and the Northwestern Sinus Center at NMFF. Sinonasal and nasal polyp tissues were obtained from routine Functional Endoscopic Sinus Surgery (FESS) in the patients with CRS. All subjects met the criteria for CRS as defined by the American Academy of Otolaryngology-Head and Neck Surgery Chronic Rhinosinusitis Task Force. The presence of sinusitis or bilateral nasal polyps was confirmed by office endoscopy and computed tomography (CT) imaging. The presence of sinusitis or bilateral nasal polyps was confirmed by office endoscopy and computed tomography imaging. All patients scheduled for surgery had previously failed adequate trials of conservative medical therapy (prolonged antibiotic regimens, nasal steroid sprays, oral steroids, saline irrigations, and decongestants) for control of symptoms. Patients with an established immunodeficiency, pregnancy, coagulation disorder, Churg-Strauss syndrome, diagnosis of classic allergic fungal sinusitis or cystic fibrosis were excluded from the study. Details of subjects' characteristics are included in Table I. Sinus tissues from disease-free control subjects were obtained during endoscopic skull base tumor excisions as well as intranasal procedures for obstructive sleep apnea and facial fracture repairs on patients without a history of CRS or asthma, recruited from the Otolaryngology clinic at Northwestern University. Subjects were skin-tested to pollens,

dust mites, pets, molds, and cockroach using Hollister-Stier (Spokane, WA) extracts. A positive skin test response was defined as a wheal greater in size than that produced by the saline control by 3 mm or more. Histamine was used as a positive control. Atopic status was assessed in all subjects unless subjects declined or if the history did not suggest atopy. All subjects signed informed consent forms and the protocol governing procedures for this study has been approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine. A portion of each sample was transferred in RNAlater for isolation of RNA (Ambion, Austin, TX) and stored at -20°C.

Cell culture

Human primary nasal epithelial cells (PNEC) were collected from the uncinate tissue by curettage with a Rhinoprobe (Arlington Scientific, Inc., Springville, UT) under a Northwestern University Feinberg School of Medicine Institutional Review Board-approved human subject research protocol. Some of the cells obtained by nasal scraping were transferred in RNAlater (Qiagen, Valencia, CA) for isolation of RNA and stored at -20°C. In past studies, the purity of epithelial cells collected with this method has been determined to be generally more than 95% based on cytokeratin staining. PNEC for in vitro cell culture experiments were collected from patients with CRS and expanded in 6-well culture plates. The purity of PNEC for in vitro experiments has previously been found to exceed 99%. Primary normal human bronchial epithelial cells (NHBE) were obtained from Lonza (Walkersville, MD). PNEC and NHBE were maintained in serum-free bronchial epithelial cell growth medium (BEGM, Lonza).

PNEC and NHBE were plated in 24-well culture plates coated with collagen (Vitrogen; Collagen Biomaterials, Palo Alto, CA). Before stimulation, PNEC and NHBE were cultured in BEGM without hydrocortisone for at least 36 hours. PNEC and NHBE were stimulated with 10 μ g/ml PGN, 5 μ g/ml dsRNA, 1 μ g/ml LPS, 100 ng/ml TNF, 100 ng/ml IL-4, 100 ng/ml IL-17A, 1000 IU/ml IFN- β and 10 ng/ml IFN- γ for 24 hours.

Real-time PCR

Total RNA from nasal epithelial scraping cells was extracted using the RNeasy Micro Kit (Qiagen). Total RNA from sinus tissue was extracted using QIAzol (Qiagen) and was cleaned and treated with DNase I using RNeasy according to the manufacturer's instructions. The quality of total RNA from nasal scraping cells and sinus tissue was assessed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using a RNA 6000 Nano LabChip (Agilent Technologies). Single-stranded cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random primers. Semi-quantitative real-time RT-PCR was performed using the TaqMan method with an Applied Biosystems 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Primer and probe sets for IL-32 α isoform (IL-32A; sense, 5'- GCTTTTGTGACAAGTCCTACGGA-3'; antisense, 5'- AAGGTGGTGTGTCAGTATCTTCATTTTG -3'; FAM/MGB, 5'- CTCTGAACCCCAATCC -3'), IL-32 non α isoforms (IL-32nonA; sense, 5'- TCTTCATGTCCTCTTTCCAGTCCTA-3'; antisense, 5'- AAGGTGGTGTGTCAGTATCTTCATTTTG -3'; FAM/MGB, 5'- CTCTGAACCCCAATCC -3'), CD3E (Hs99999153_m1), CD20 (sense, 5'-

CACTCTTCAGGAGGATGTCTTCACT-3'; antisense, 5'- TCTGGACAGCCCCCAAAGT -
 3'; FAM/MGB, 5'- TCTTCATGAGGGAATCTA -3'), CD56 (Hs00941830_m1), CD138
 (sense, 5'- GCCTGGTGGGTTTCATGCT-3'; antisense, 5'-
 TCCTGTTTGGTGGGCTTCTG -3'; FAM/MGB, 5'- AGAAGGACGAAGGCAGC -3'),
 MMR (Hs00267207_m1), CD1c (Hs00233509_m1), ECP (Hs01923184_s1), TPSAB1
 (Hs02576518_gH), GUSB (Human β -glucuronidase endogenous control, PN;
 4326320E) and β -actin (ACTB; sense, 5'-CTGGCCGGGACCTGACT-3'; antisense, 5'-
 GCAGCCGTGGCCATCTC-3'; FAM/MGB, 5'- CACCACCACGGCCGA-3') were
 purchased from Applied Biosystems. To determine the exact copy number of the target
 genes, quantified aliquots of purified PCR fragments of the target genes were serially
 diluted and used as standards in each experiment. Aliquots of cDNA equivalent to 10 ng
 of total RNA were used for real-time PCR. The mRNA expression levels were
 normalized to the median expression of housekeeping genes, GUSB for clinical
 samples (nasal scraping cells and nasal tissue) and ACTB for in vitro samples (PNEC
 and NHBE).

Western blot analysis

NHBE were stimulated with 50 ng/ml TNF, 10 ng/ml IFN- γ , 5 μ g/ml dsRNA or their
 combinations for 48 hours. The supernatant was concentrated 10 times using Amicon
 Ultra-4 10,000 MWCO filters (Millipore). Cells were harvested in a cell lysis buffer (Cell
 Signaling Technology, Beverly, MA) supplemented with halt protease inhibitor cocktail
 (Thermo Fisher Scientific, Rockford, IL). Western blot analysis was performed as

described previously. Briefly, whole cell lysates and concentrated supernatants were dissolved in LDL sample buffer (Invitrogen). To detect IL-32, 30 µg of whole cell lysates or 20 µl of supernatants were resolved on a 4-12% NuPAGE Bis-Tris Gel (Invitrogen) in an MES buffer system and then proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). The membranes were blocked in blocking buffer for fluorescent western blotting (Rockland, Gilbertsville, PA) for 2 hours and subsequently incubated overnight at 4°C with 0.5 µg/ml mouse anti-IL-32 mAb (clone: KU32-52, BioLegend, San Diego, CA). Following primary antibody incubation, the membranes were washed repeatedly in PBS/0.1% Tween 20 and then labeled by a 45 minute incubation at room temperature with infrared secondary antibody Alexa Fluor 680 conjugated goat anti-mouse IgG (1:10,000, Invitrogen). Protein was detected on an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

Immunohistochemistry and immunofluorescence

Nasal tissue was dehydrated, infiltrated and embedded with paraffin, and tissue was sectioned at 3 µm using a Leica RM2240 Cryostat. Sections were rehydrated and blocked for endogenous peroxidase activity with 3% H₂O₂/methanol. After washing, tissue sections were blocked for non-specific binding with 5% horse serum/0.3% Tween-20/PBS. Tissue sections were then incubated with 0.2 µg/ml mouse anti-human IL-32 mAb (clone: KU32-09, IgG1, BioLegend) or 0.2 µg/ml mouse control IgG1 (clone: P3, eBiosciences) at 4°C overnight. After washing, sections were incubated with biotinylated secondary horse anti-mouse IgG antibody (Vector Laboratories, Burlingame,

CA) at a 1:500 dilution for 1 hour at room temperature. After another rinse, sections were incubated in ABC reagent (avidin-biotin-HRP complex; Vector Laboratories) for 1 hour at room temperature. Sections were rinsed again and incubated in DAB reagent (Invitrogen) for 5 minutes at room temperature. They were then rinsed in deionized H₂O, counterstained with hematoxylin, dehydrated, cleaned, mounted and coverslipped using Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI) in preparation for microscopic analysis. For the immunofluorescence assay, rehydrated sections were boiled in a citrate buffer (Dako, Carpinteria, CA) for 15 minutes to induce antigen retrieval and were then blocked with 5% goat serum/0.3% Tween-20/PBS and then were incubated with mouse anti-human IL-32 mAb (clone: A7-B1, IgG2a, YbdYbiotech, Korea) and rabbit anti-CD3 mAb (clone EP449E, IgG, Thermo scientific), mouse anti-human CD68 mAb (clone: PG-M1, IgG3, Thermo scientific) or mouse anti-human tryptase mAb (clone: AA1, IgG1, Thermo scientific) in blocking buffer at 4°C overnight. The same concentrations of isotype control IgG were used in control experiments. After washing, sections were incubated with Alexa Fluor 647 conjugated goat anti-mouse IgG2a (Invitrogen) and Alexa Fluor 488 conjugated goat anti-rabbit IgG, goat anti-mouse IgG3 (Invitrogen) or goat anti-mouse IgG1 (Invitrogen) for 1 hour at room temperature in the dark. After a final washing, coverslips were mounted onto slides using SlowFade Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Images from immunofluorescence slides were obtained with an Olympus IX71 inverted research microscope using 40x objective lens and images were collected by using SlideBook software (Olympus, Center Valley, PA).

Measurement of IL-32 in tissue homogenates

Freshly obtained tissue specimens were weighed, and 1 ml of PBS supplemented with 0.05 % Tween 20 (Sigma-Aldrich, St. Louis, MO) and 1% protease inhibitor cocktail (PN; P8340, Sigma-Aldrich) was added for every 100 mg of tissue. The tissue was then homogenized with a Bullet Blender Blue (Next Advance, Averill Park, NY) at setting 7 for 8 minutes at 4°C. After homogenization, the suspension was centrifuged at 4000 rpm for 20 minutes at 4°C and the supernatants were stored at -80°C until analyzed.

The concentrations of IL-32 protein in cell-free supernatants were measured using IL-32A ELISA kit (BioLegend, detection limits are 7.8 pg/ml) and in-house sandwich ELISA. Briefly, plates were coated with 1 µg/ml of mouse anti-human IL32 mAb (clone; A7, YbdYbiotech, Seoul Korea) and then 0.5 µg/ml of rabbit anti-human IL-32 (ligand affinity purified IgG) was used as a detection reagent. The minimal detection limits 82 pg/ml. The concentration of total protein was measured with a Bradford method-based Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Concentrations of IL-32 in the tissue homogenate were normalized to the concentration of total protein.

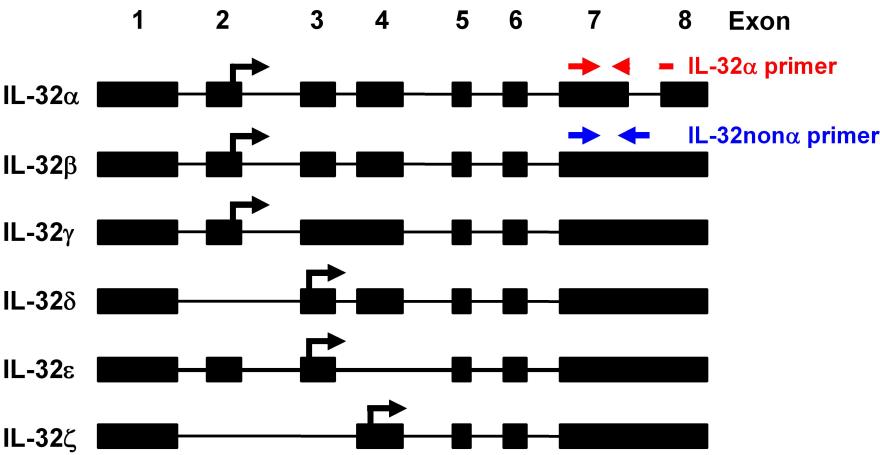


Figure S1 The structure of IL-32 isoforms.

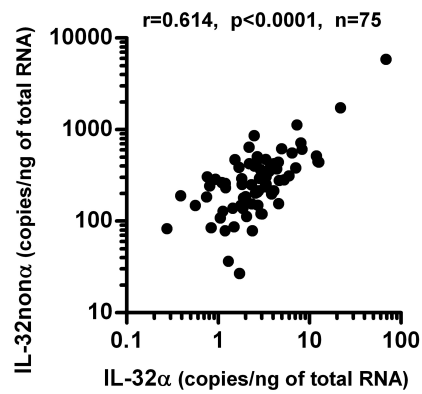
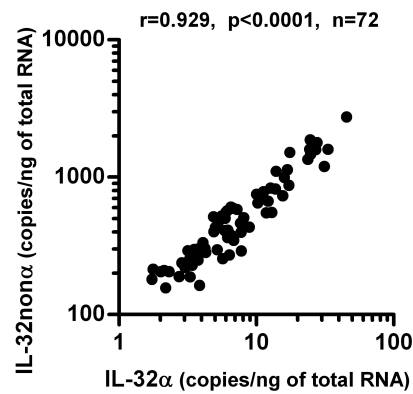
A. Nasal scraping cells**B. Sinus tissue**

Figure S2 Expression of IL-32 α in CRS.

Total RNA was extracted from epithelial scraping cells (A) and whole tissue (B) from uncinat
tissue (UT) and nasal polyps (Polyp) and expressions of IL-32 α and IL-32non α were analyzed
using real-time PCR. The correlations were assessed by using the Spearman rank correlation.