

Increased expression of CC chemokine ligand 18 in patients with chronic rhinosinusitis with nasal polyps

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Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is associated with T_H2-dominant inflammation, including eosinophilia, which is in contrast to chronic rhinosinusitis (CRS) without nasal polyps (NPs). CC chemokine ligand 18 (CCL18)/pulmonary and activation-regulated chemokine is known to recruit naive T cells, B cells, and immature dendritic cells, as well as to activate fibroblasts. CCL18 is thought to be involved in T_H2-related inflammatory diseases, including asthma and atopic dermatitis.

Objective: The objective of this study was to investigate the expression of CCL18 in patients with CRS.

Methods: Using NP tissue and uncinata tissue (UT) from control subjects and patients with CRS, we examined the expression of CCL18 mRNA using real-time PCR and measured CCL18 protein using ELISA, Western blotting, and immunofluorescence.

Results: Compared with UT tissue from control subjects, CCL18 mRNA levels were significantly increased in NPs ($P < .001$) and UT ($P < .05$) from patients with CRSwNP but not in UT from patients with CRS without NPs. Similarly, CCL18 protein levels were increased in NPs and UT from patients with CRSwNP, and levels were even higher in patients with Samter's triad. Immunohistochemical analysis revealed CCL18 expression in inflammatory cells, and CCL18⁺ cell numbers were significantly increased in NPs. Immunofluorescence data showed colocalization of CCL18 in CD68⁺/CD163⁺/macrophage mannose receptor–positive M2 macrophages and tryptase-positive mast cells in NPs. Levels of CCL18 correlated with markers of M2 macrophages but not with tryptase levels, suggesting that M2 macrophages are major CCL18-producing cells in NPs.

Conclusion: Overproduction of CCL18 might contribute to the pathogenesis of CRSwNP through its known activities, which include recruitment of lymphocytes and dendritic cells, activation of fibroblasts, and initiation of local inflammation. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Chronic rhinosinusitis, nasal polyps, Samter's triad, CCL18, pulmonary and activation-regulated chemokine, M2 macrophages, mast cells

Chronic rhinosinusitis (CRS) is a chronic disease that afflicts roughly 10% of the US population.¹⁻³ CRS is characterized by inflammation of the sinonasal mucosa. Symptoms of CRS include anterior and/or posterior rhinorrhea, nasal obstruction, decreased sense of smell, and nasal pressure, at least 2 of which persist for 12 weeks despite medical management.¹⁻³ The pathogenesis of CRS is not fully understood at this time; however, allergy, bacterial and fungal infections, and structural anomalies have all been theorized to play a role.²⁻⁵ CRS is often divided into 2 groups based on histology and physical examination: chronic rhinosinusitis with nasal polyps (CRSwNP) and chronic rhinosinusitis without nasal polyps (CRSsNP). In general, CRSwNP is associated more closely with clinical complaints of nasal obstruction and olfactory loss and is characterized by eosinophilia and T_H2-related inflammation, especially in Western countries.^{5,6} It is also known that B cells, plasma cells, macrophages, neutrophils, and dendritic cells (DCs) are present in high concentrations in patients with CRSwNP, making inflammatory cell recruitment a contributor to the pathogenesis of this disease.⁵⁻¹³

Chemokines are a large group of proteins that participate in recruitment of inflammatory cells into tissue sites by binding G protein-coupled receptors on their target cells.^{14,15} They can be classified into several groups based on their molecular structure. Although some chemokines are involved in housekeeping functions of the adaptive immune system, others are produced under pathological conditions to mount immune and inflammatory responses and initiate wound healing by selectively recruiting immune cells to the inflamed site.¹⁴⁻¹⁶ Exaggerated expression of chemokines can result in tissue damage and inflammatory diseases through excessive recruitment of leukocytes. Therefore chemokine receptors serve as potential targets for the treatment of many inflammatory diseases.¹⁵⁻¹⁸

CC chemokine ligand 18 (CCL18), which is also known as pulmonary and activation-regulated chemokine (PARC), MIP-4, AMAC-1, and DC-CK1, is a 7.8-kDa protein composed of 69

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Abbreviations used

CCL18:	CC chemokine ligand 18
CLC:	Charcot-Leyden crystal protein
CRS:	Chronic rhinosinusitis
CRSsNP:	Chronic rhinosinusitis without nasal polyps
CRSwNP:	Chronic rhinosinusitis with nasal polyps
DC:	Dendritic cell
ECP:	Eosinophil cationic protein
HPF:	High-powered field
IMDM:	Iscove modified Dulbecco medium
MMR:	Macrophage mannose receptor
NMFF:	Northwestern Medical Faculty Foundation
NP:	Nasal polyp
PIC:	Protease inhibitor cocktail
STAB1:	Stabilin 1
UT:	Uncinate tissue

amino acids.¹⁹⁻²² CCL18 is thought to have been generated by the fusion of 2 CCL3-like genes, likely occurring after the diversification of rodents and primates.²²⁻²⁴ Therefore the ortholog of CCL18 in rodents has not been discovered. Although its receptor is also unknown at this time, CCL18 is known to be chemotactic for many cell types, including naive T cells, skin-homing memory T cells, T_H2 cells, and immature DCs.^{19,23,25} CCL18 is known to be expressed in the lung, thymus, and lymph nodes and is secreted by monocytes, macrophages, DCs, and mast cells.^{22,26} Its expression is regulated by the T_H2 cytokines IL-4 and IL-13, as well as by IL-10.^{19,21,22} CCL18 is highly expressed in patients with a number of inflammatory diseases, including atopic dermatitis, asthma, rheumatoid arthritis, and pulmonary fibrosis.^{22,25,27-29} Although levels of several chemokines are known to be increased in patients with CRS, particularly in patients with CRSwNP, expression of CCL18 has been minimally studied in this disease process. In this study we found that CCL18 levels were increased in patients with CRSwNP and examined the cell types that produce it.

METHODS**Patients and biopsy specimens**

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 (NP) tissues were obtained during routine functional endoscopic sinus surgery in 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 Patients with an established immunodeficiency, pregnancy, coagulation disorder, diagnosis of Churg-Strauss syndrome, classic allergic fungal sinusitis, or cystic fibrosis were excluded from the study. Details of subjects' characteristics are included in Table 1 and in Tables E1 to E3 and the Methods section in this article's Online Repository at www.jacionline.org. 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.

Real-time PCR

Real-time RT-PCR was performed by using the TaqMan method, as described previously.³⁰ Primer and probe sets for CCL18 (sense, 5'-CCAGCTCACTC TGACCACTTCTC-3'; antisense, 5'-GGTGCAGACGAGGACAAGGA-3';

probe, 5'-CTGCCAGCATCATGAAGGGCCTT-3') were purchased from Integrated DNA Technologies (Coralville, Iowa). The mRNA expression levels were normalized to the median expression of the housekeeping gene β -glucuronidase. Details can be found in the Methods section in this article's Online Repository.

ELISA, Western blotting, and cell culture

The concentration of CCL18 in cell-free supernatants was determined by using specific ELISA kits. The minimal detection limit for these kits is 3.9 pg/mL (R&D Systems, Minneapolis, Minn). The concentration of CCL18 in tissue homogenate was normalized to the concentration of total protein. Western blot analysis was performed as described previously.³¹ Human mast cells were obtained as described previously.²⁶ Further details can be found in the Methods section in this article's Online Repository.

Immunofluorescence assay

Immunofluorescent localization of CCL18 was performed as described previously.¹³ Briefly, blocked sections were incubated with anti-CCL18 antibody (LifeSpan Biosciences, Seattle, Wash) and either anti-CD68 mAb (Thermo Fisher Scientific, Waltham, Mass), anti-eosinophil cationic protein (ECP) mAb (Diagnostics Development, Uppsala, Sweden), or anti-tryptase mAb (Thermo Fisher Scientific) at 4°C overnight. After washing, sections were incubated with Alexa Fluor-conjugated secondary antibodies for 1 hour in the dark. After a final washing, cover slips were mounted onto slides by using SlowFade Gold antifade reagent with 4',6-diamidino-2-phenylindole (Invitrogen, Carlsbad, Calif). Details can be found in the Methods section in this article's Online Repository.

Statistics

All data are reported as means $\pm$ SEMs unless otherwise noted. Differences between groups were analyzed with the Mann-Whitney *U* test and the 1-way ANOVA Kruskal-Wallis test. Correlations were assessed by using Spearman rank correlation. A *P* value of less than .05 was considered significant.

RESULTS**CCL18 expression in patients with CRS**

We assessed the expression of CCL18 in uncinate tissue (UT) from patients with CRSsNP, patients with CRSwNP, and control subjects, as well as in NPs from patients with CRSwNP. CCL18 mRNA levels were significantly increased in NPs from patients with CRSwNP ($P < .001$) in comparison with those seen in UT from patients with either form of CRS or control subjects ($P < .001$, Kruskal-Wallis test; Fig 1, A). CCL18 mRNA levels were also significantly increased in UT from patients with CRSwNP ($P < .001$) in comparison with those seen in UT from control subjects (Fig 1, A). To confirm this observation at the protein level, we made detergent extracts from homogenates of UT and NPs and then measured the concentration of CCL18 using ELISA. CCL18 protein levels were significantly increased in NPs from patients with CRSwNP (4.75 ± 1.13 ng/mg; median, 1.58 ng/mg; $n = 39$) compared with those seen in UT from patients with CRSsNP (0.52 ± 0.06 ng/mg; median, 0.46 ng/mg; $n = 32$), patients with CRSwNP (1.52 ± 0.40 ng/mg; median, 0.59 ng/mg; $n = 29$), and control subjects (0.52 ± 0.12 ng/mg; median, 0.42 ng/mg; $n = 15$; $P < .001$, Kruskal-Wallis test; Fig 1, B). Although not statistically significant, CCL18 protein levels were also increased in UT from patients with CRSwNP ($P = .057$) compared with that seen in UT from control subjects (Fig 1, B). We further confirmed this finding by means of Western blotting. The CCL18 protein was detected as a 7.8-kDa protein and found

TABLE I. Subjects' characteristics

	Control subjects			Patients with CRSsNP			Patients with CRSwNP			Patients with Samter's triad		
Total no. of subjects (male subjects)	28 (14)			55 (26)			92 (65)			12 (6)		
Age (y), median (range)	48 (16-63)			38 (18-64)			43 (22-74)			47 (26-61)		
	Y	N	U	Y	N	U	Y	N	U	Y	N	U
Atopy	0	24	4	24	25	6	42	40	10	7	2	3
Asthma	0	27	2	12	33	10	40	51	1	12	0	0
Methodologies used	UT			UT			UT			NP		
Tissue RNA, total no. of subjects (male subjects)	9 (3)			18 (8)			17 (14)			24 (18)		
Age (y)	41 (16-59)			36 (23-64)			43 (26-61)			44 (26-74)		
Tissue extract, total no. of subjects (male subjects)	15 (9)			32 (15)			29 (20)			39 (28)		
Age (y)	49 (19-63)			37 (18-64)			41 (18-67)			41 (22-72)		
Immunofluorescence, total no. of subjects (male subjects)	11 (8)			11 (6)			11 (9)			17 (11)		
Age (y)	53 (19-62)			38 (21-61)			48 (34-72)			52 (22-70)		

Values are presented as medians (ranges), where shown.

N, No; U, unknown; Y, yes.

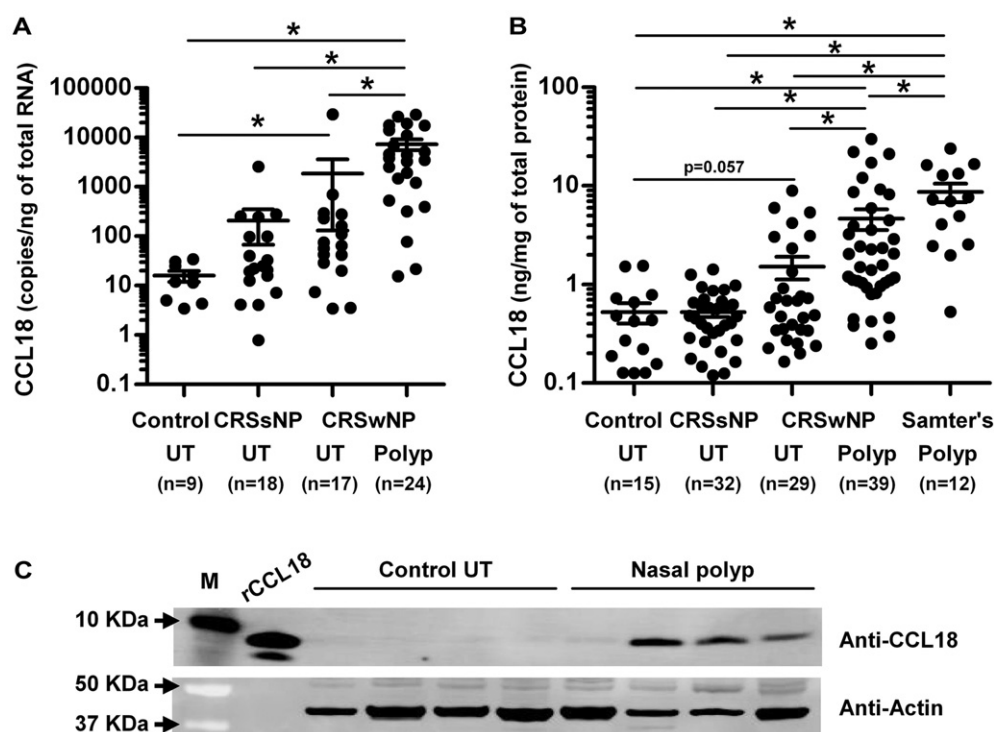


FIG 1. Increased expression of CCL18 in NPs. **A**, Total RNA was extracted from UT and NPs (*Polyp*), and expression of CCL18 RNA was analyzed by using real-time PCR. **B** and **C**, Expression of CCL18 protein in tissue homogenates of UT and NPs from patients with aspirin-tolerant CRSwNP and patients with Samter's triad was measured by using ELISA (**B**) and Western blotting (**C**). CCL18 concentration was normalized to the concentration of total protein (**B**). The results are representative of 2 separate experiments (**B** and **C**). * $P < .05$.

only in NPs but not in UT from patients with CRSsNP or control subjects (**Fig 1, C**, and data not shown).

NPs in aspirin-sensitive patients generally have more inflammation than those in aspirin-tolerant patients.³² Patients with clinical features including bronchial asthma, aspirin sensitivity, and NPs are referred to as having Samter's triad.³²⁻³⁴ Interestingly, we found that the concentration of CCL18 in NPs was significantly higher in patients with Samter's triad ($9.92 \pm$

1.93 ng/mg; median, 7.13 ng/mg; $n = 12$; $P = .0015$) compared with that seen in NPs from patients with aspirin-tolerant CRSwNP (**Fig 1, B**).

Identification of CCL18-producing cells in NPs

To examine the distribution of CCL18 in nasal mucosa, we used immunohistochemistry to detect CCL18⁺ cells in UT and

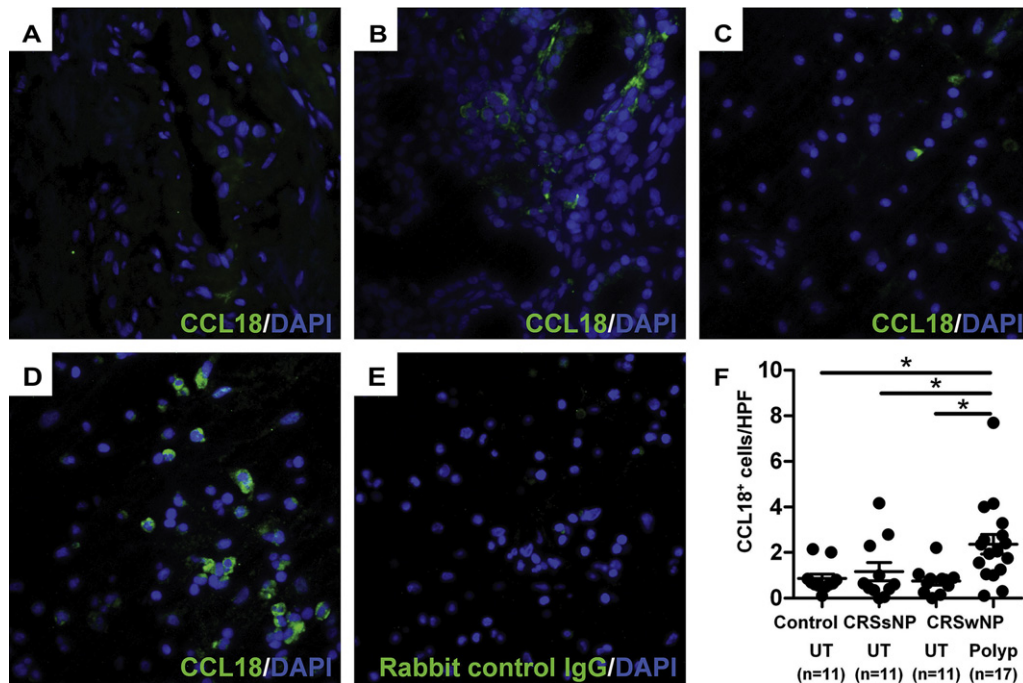


FIG 2. Immunofluorescence of CCL18 was performed with an anti-human CCL18 antibody. **A-D**, Representative immunostaining (green) for CCL18 in UT from a control subject (Fig 2, A), a patient with CRSsNP (Fig 2, B), a patient with CRSwNP (Fig 2, C), and NP tissue (Fig 2, D). **E**, Negative control antibody staining in NP tissue from a patient with CRSwNP. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). **F**, The number of CCL18⁺ cells in UT from control subjects (n = 11), patients with CRSsNP (n = 11), and patients with CRSwNP (n = 11) and in NPs (n = 17) was counted by using the National Institutes of Health-issued Image J software. **P* < .05.

NPs. As shown in Fig 2, CCL18 staining was observed mainly in submucosal inflammatory cells. Although CCL18 staining was also observed in some mucosal and glandular epithelium, levels of mRNA for CCL18 in epithelial cell scrapings from NPs were more than 500-fold lower than levels in whole NP tissue, and we could not find an increase CCL18 protein levels in nasal lavage fluid from patients with CRSwNP (Figs 1 and 2, see Fig E1, and data not shown). We therefore excluded epithelial cells as major CCL18-producing cells and focused on inflammatory cells in this study. We found that CCL18⁺ inflammatory cell numbers were highly increased in NPs (Fig 2, D). We counted the number of CCL18⁺ inflammatory cells using a semiquantitative method and found that CCL18⁺ inflammatory cell numbers were significantly increased in NPs from patients with CRSwNP (2.37 ± 0.43 CCL18⁺ cells/high-powered field [HPF], n = 17) compared with those seen in UT from patients with CRSsNP (1.16 ± 0.40 CCL18⁺ cells/HPF, *P* < .05, n = 11), patients with CRSwNP (0.75 ± 0.18 CCL18⁺ cells/HPF, *P* < .05, n = 11), and control subjects (0.86 ± 0.19 CCL18⁺ cells/HPF, *P* < .05, n = 11; *P* < .01, Kruskal-Wallis test; Fig 2, F).

We next attempted to identify the CCL18-producing cells in the nasal mucosa of NPs. We first assessed whether the expression of CCL18 correlated with the levels of mRNA expression for markers of inflammatory cells in NPs using real-time PCR. We found that the levels of expression of CCL18 mRNA in NPs were significantly and positively correlated with the expression of macrophage mannose receptor (MMR; *r* = 0.796, *P* < .0001) and Charcot-Leyden crystal protein (CLC; *r* = 0.695, *P* = .0002) but not with levels of CD3, CD20, CD56, CD138, CD1c, tryptase, or

CXCR1 (Fig 3, A, and data not shown), indicating that CCL18 might be expressed in macrophages and eosinophils.

We next performed dual-immunofluorescence analysis using anti-CCL18 and antibodies against markers of macrophages (CD68) and eosinophils (ECP). We found CCL18 colocalization with CD68⁺ macrophages (Fig 3, B) but not with ECP⁺ eosinophils (Fig 3, C) in NPs. In addition to macrophages, we also found colocalization of CCL18 with tryptase-positive mast cells (Fig 3, D). Although there was variability from patient to patient, 20% to 50% of CCL18⁺ cells were macrophages, 10% to 60% of CCL18⁺ cells were mast cells, and another 10% to 50% of CCL18⁺ cells were unidentified inflammatory cells in NPs (n = 8, data not shown).

We have previously shown that mast cells are able to produce CCL18 during IgE-mediated reactions.²⁶ However, the effect of cytokines on the production of CCL18 is still unknown. Because we observed CCL18⁺ mast cells in NPs (see above), we stimulated IgE-sensitized human peripheral blood-derived mast cells with the cytokines TNF, IL-1β, IL-4, IL-13, IFN-γ, and IL-17A alone or in combination with anti-IgE antibody in the presence of protease inhibitor cocktail (PIC) or dimethyl sulfoxide (vehicle control) for 48 hours to assess production of CCL18. We found that the cytokines alone did not induce CCL18 production, but IL-4, IL-13, and IFN-γ significantly enhanced IgE-mediated CCL18 production only in the presence of PIC (see Fig E2 in this article's Online Repository at www.jacionline.org and data not shown). However, levels of CCL18 in mast cell culture supernatants were very low. In addition, levels of CCL18 mRNA did not correlate with tryptase levels in NPs

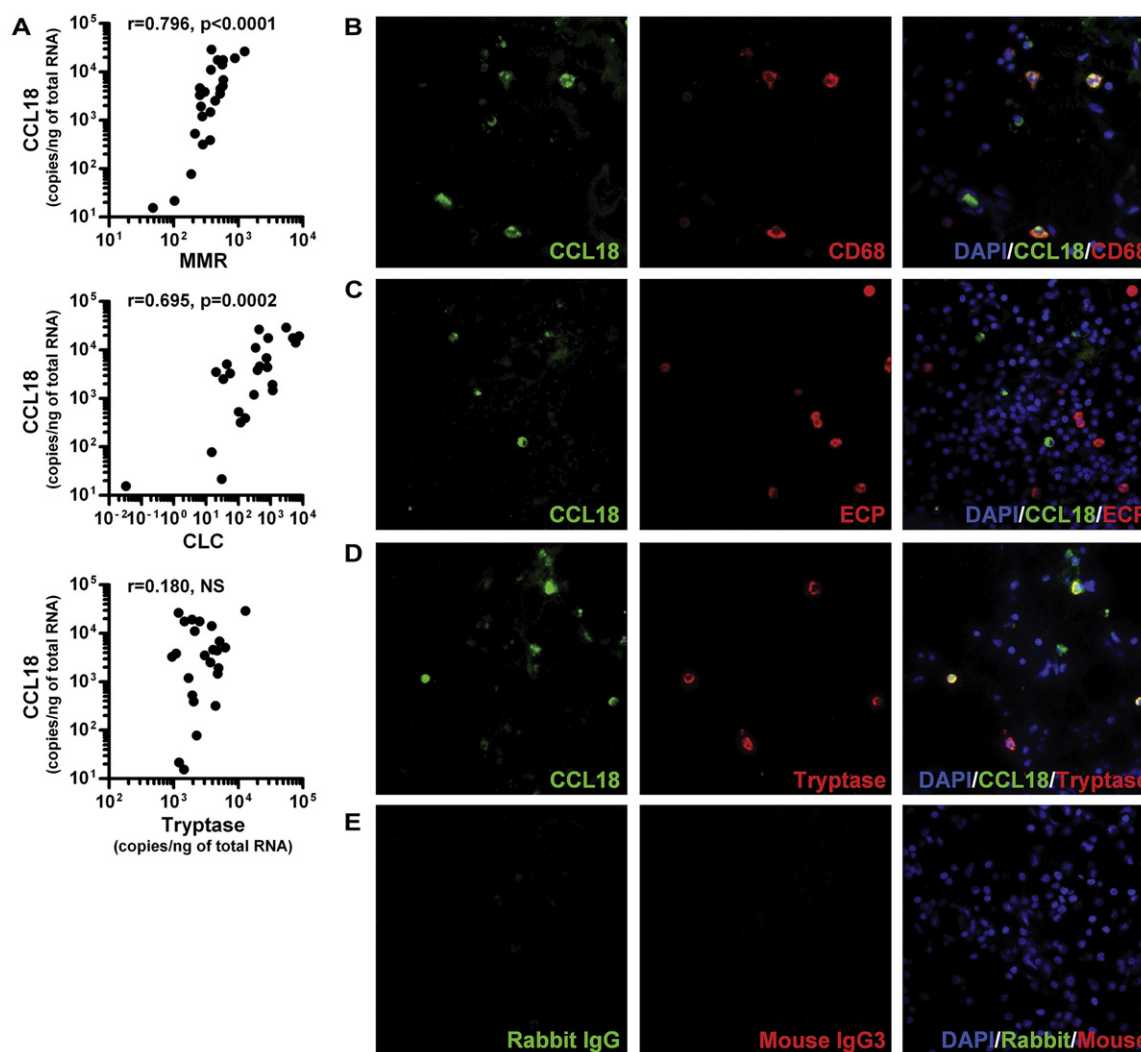


FIG 3. Detection of CCL18-producing cells in NPs. **A**, Total RNA was extracted from NP tissue, and the expression of CCL18 and cell-specific markers was analyzed by using real-time PCR ($n = 24$). The correlations were assessed by using Spearman rank correlation. **B-E**, An immunofluorescence assay was performed with anti-CCL18 (green), anti-CD68 mAb (red) for macrophages (Fig 3, **B**), anti-ECP mAb (red) for eosinophils (Fig 3, **C**), anti-tryptase mAb (red) for mast cells (Fig 3, **D**), and control IgG (Fig 3, **E**). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). The results are representative of 5 to 8 separate patients.

(Fig 3). This suggested to us that mast cells might not be a major CCL18-producing cell type in NPs, and we therefore focused on macrophages.

Detection of CCL18 in M2 macrophages

Macrophages are now widely recognized to be polarized by their microenvironment, especially by T_H cytokines and pathogens.³⁵⁻³⁹ Classically activated macrophages (also known as M1 macrophages) are developed by stimulation with the T_H1 cytokine IFN- γ and microbial products, such as LPS. In contrast, alternatively activated macrophages are primed by the T_H2 cytokines IL-4 and IL-13 and therefore are called M2 macrophages. Importantly, CCL18 is known as a useful marker of M2 macrophages.³⁶⁻³⁹ *In vitro*-generated M2 macrophages or IL-4-stimulated monocytes/macrophages are known to release large

amounts of CCL18. In addition, we found that expression of CCL18 was positively correlated in NPs with MMR, which is another well-known marker of M2 macrophages (Fig 3, **A**). Therefore we next examined whether M2 macrophages were major CCL18-producing cells in NPs. We first determined levels of MMR and the other M2 macrophages markers, CD163 and stabilin 1 (STAB1), in NPs using real-time PCR. Levels of mRNA for MMR, CD163, and STAB1 were significantly upregulated in NPs ($P < .05$) in comparison with those seen in UT from either patients with CRS or control subjects (Fig 4, **A**). We also found that expression of CCL18 significantly and positively correlated with expression of CD163 ($r = 0.630$, $P = .001$) and STAB1 ($r = 0.481$, $P = .017$; Fig 4, **B**). Importantly, levels of MMR also significantly correlated with levels of CD163 ($r = 0.704$, $P < .001$) and STAB1 ($r = 0.593$, $P = .002$; $n = 24$; Fig 4, **B**, and data not shown).

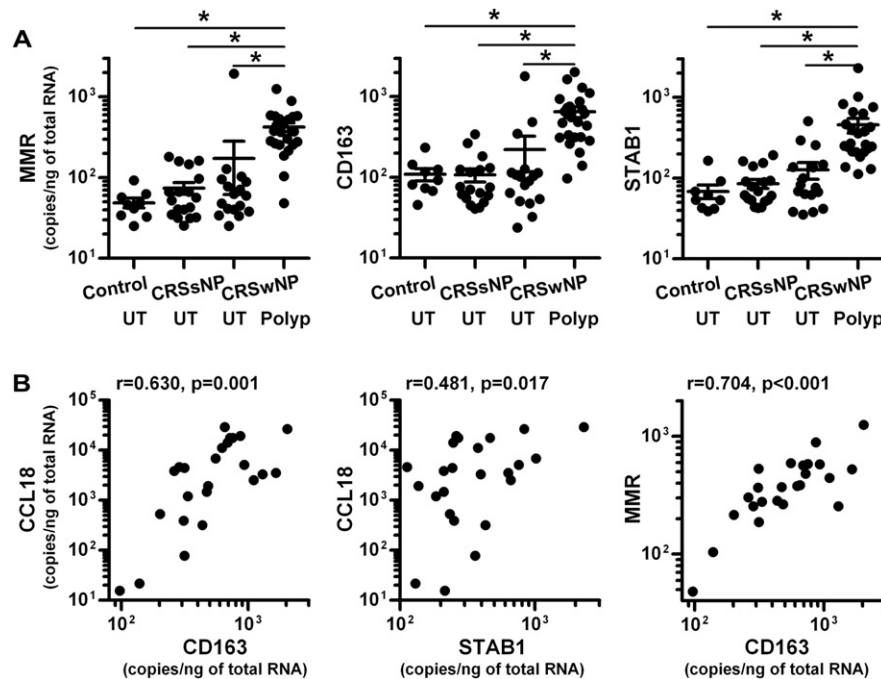


FIG 4. Correlation of CCL18 with markers of M2 macrophages in NPs. **A**, Total RNA was extracted from UT from control subjects ($n = 9$), patients with CRSsNP ($n = 18$), and patients with CRSwNP ($n = 17$) and NP tissue ($n = 24$). The expression of CCL18 and the M2 macrophage markers MMR, CD163, and STAB1 was analyzed by using real-time PCR. **B**, The correlations in NPs were assessed by using Spearman rank correlation. * $P < .05$.

To further investigate whether M2 macrophages were CCL18-producing cells in NPs, we performed triple-immunofluorescence analysis using anti-CCL18 and antibodies against markers of M2 macrophages, including MMR and CD163. We detected CCL18 in CD68⁺, MMR⁺, and CD163⁺MMR⁺ cells in NPs (Fig 5). These data suggest that M2 macrophages are major CCL18-producing cells in NPs.

DISCUSSION

It has been reported that CCL18 levels are increased in affected tissues in patients with T_H2-related inflammatory diseases and that expression of CCL18 is regulated by T_H2 cytokines.^{22,28,29} CRSwNP is well known to be characterized by T_H2-mediated inflammation and eosinophilia.^{5,6} In the current study we demonstrated that CCL18 levels were significantly increased in NPs (Fig 1). There are very few studies comparing identical tissue taken from patients with CRSsNP and patients with CRSwNP, as well as from control subjects, and most of those studies show an increase or reduction in NPs compared with nonpolypoid tissue. In this study we present rare evidence that upregulation of mRNA and protein for CCL18 was also found in UT, which is one of the surrounding mucosal tissues in close proximity to NPs, from patients with CRSwNP compared with levels seen in UT from control subjects (Fig 1). This suggests that CCL18 might be involved in the initiation of the inflammatory process in patients with CRSwNP. Interestingly, patients with CRSwNP in China have been reported to have less eosinophilia and T_H2 inflammation than such patients in Western countries.⁴⁰ It would be of interest to determine whether the CCL18 increase exists in patients with this form of polypoid CRS.

Very recently, Plager et al⁴¹ published a microarray study of asthmatic patients with CRSwNP and showed that CCL18 mRNA levels were upregulated in NPs compared with those seen in control tissue. Our study unequivocally demonstrates an increase in CCL18 levels in patients with CRSwNP. Plager et al focused on the expression of CCL18 only in asthmatic patients with CRSwNP. When we evaluated whether overexpression of CCL18 in patients with CRSwNP was associated with asthmatic status, atopic status, or glucocorticoid treatment, we found no significant difference in CCL18 levels between atopic and non-atopic patients, between asthmatic and nonasthmatic patients and no difference based on the status of nasal steroid treatment (Fig E4). Because we might not have large enough subgroups of patients to make firm conclusions about the effect of oral steroid treatment, future studies will be required to test this in larger cohort. In contrast, we found that levels of CCL18 in NPs were higher in patients with Samter's triad compared with those seen in patients with aspirin-tolerant CRSwNP (Fig 1). In general, tissues from aspirin-sensitive patients are more inflamed than those from aspirin-tolerant patients.³² This suggests that levels of CCL18 in patients with CRSwNP might be related to the severity of inflammation rather than asthmatic or atopic status.

It is well established that CCL18 is produced by monocytes and macrophages. Our immunofluorescence data clearly show that CCL18 was detected in CD68⁺ macrophages, as well as tryptase-positive mast cells. This suggests that macrophages and mast cells might be major CCL18-producing cells in NPs. However, the regulation of CCL18 in mast cells is poorly understood. We showed previously that mast cells could release CCL18 on FcεRI cross-linking.²⁶ In our present study we investigated the effect of cytokines on the production of CCL18 in mast cells. We found that the

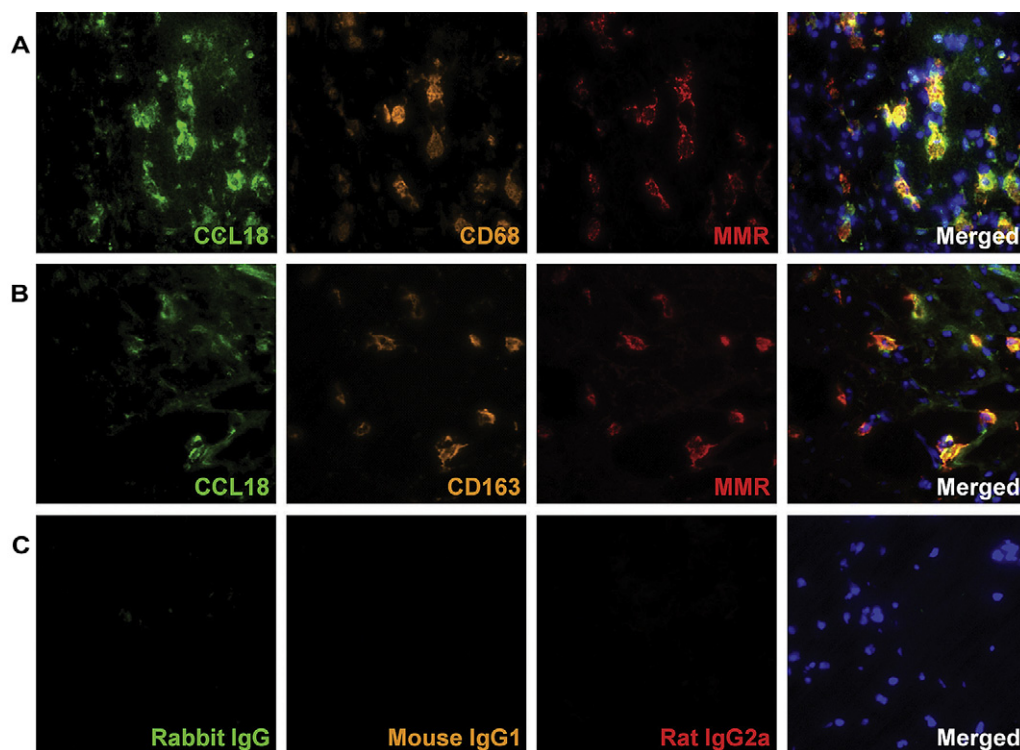


FIG 5. Detection of CCL18 in M2 macrophages in NPs. **A-C**, An immunofluorescence assay was performed with anti-CCL18 (Fig 5, A and B, green), anti-CD68 mAb (Fig 5, A, orange), anti-CD163 mAb (Fig 5, B, orange), anti-MMR mAb (Fig 5, A and B, red), and control IgG (Fig 5, C). Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (blue). The results are representative of 4 separate patients.

T_H1 cytokine IFN- γ and the T_H2 cytokines IL-4 and IL-13 significantly enhanced IgE-mediated CCL18 production in mast cells. This suggests that the effect of IL-4 and IL-13 on the induction of CCL18 is well conserved across cell types. In contrast, IFN- γ is known to inhibit CCL18 expression in monocytes, macrophages, and DCs, enhance it in keratinocytes and has no effect in PBMCs.^{21,25,29} These data suggest that the regulation of CCL18 by IFN- γ might be cell type specific. Although we detected CCL18 protein in mast cells (20–30 pg/10⁶ cells, see Fig E2), these levels were more than 50-fold lower than those found to be produced by monocytes/macrophages (1–15 ng/10⁶ cells).^{25,37,42–44} These results suggest that mast cells might be a less important source of CCL18 in NPs.

CCL18 was detected in stimulated mast cells only in the presence of protease inhibitors. This demonstrates that CCL18 is highly sensitive to mast cell proteases. In a separate clinical study we found that mast cells are increased in NPs, and some of them are degranulated (T. Takabayashi, A. Kato, and R. P. Schleimer, manuscript in preparation). Although CCL18 mRNA levels were 500-fold higher in NPs than in control UT, upregulation of CCL18 protein was only 9-fold. This suggests that local CCL18 production in NPs might be much more abundant but that a portion might be cleaved immediately by proteases from degranulated mast cells or other tissue cells. This could explain the variability of CCL18 protein levels in NPs. Future studies will be required to identify mast cell proteases that can cleave CCL18 and to investigate the effect of those proteases on the stability of CCL18 in NPs *in vitro* and *in vivo*.

Although CRSwNP is well known to be characterized by eosinophilia and T_H2 -related inflammation, the role of macrophages in the pathogenesis of CRS is poorly understood. Recently, Krysko et al¹² showed that numbers of MMR⁺ M2 macrophages, but not M1 macrophages, were increased in NPs. In our present study we also found that the M2 macrophage markers MMR, CD163, and STAB1 were significantly upregulated in NPs (Fig 4). This suggests that upregulation of M2 macrophages in NPs should be considered a feature of this disease. CCL18 is well known to be released from M2 macrophages.^{37–39} We found that CCL18 was detected in MMR⁺ and CD163⁺ macrophages but not in MMR[−] macrophages in NPs (Fig 5 and data not shown). In addition, we found that levels of M2 macrophage markers correlated with levels of CCL18 (Fig 4). As discussed above, mast cells were able to produce CCL18, but the levels were much weaker than what has been reported in macrophages. These results suggest that M2 macrophages are the major CCL18-producing cell type in NPs. We also found that CCL18 levels were increased in UT from patients with CRSwNP compared with that seen in UT from healthy subjects (Fig 1). In contrast to NPs, we could not find a significant increase in M2 macrophage or mast cell numbers in UT from patients with CRSwNP (Fig 4 and data not shown). Although CCL18-producing cell numbers were not increased in UT, as determined by means of immunofluorescence, the increased protein level suggests that local cells produce it. Future studies will be required to identify the CCL18-producing cells in UT from patients with CRSwNP and whether they are distinct from the CCL18-producing cells in NPs.

Although we found that M2 macrophages were major CCL18-producing cells, little is known about the regulation of macrophage recruitment in NPs. Recently, we showed that CCL23 was highly upregulated in patients with eosinophilic CRSwNP, and eosinophils were a major source of CCL23 in NPs.¹³ CCL23 is a known ligand of CCR1 and is able to recruit monocytes and macrophages.⁴⁵ Importantly, we showed that levels of CCL23 were significantly correlated with levels of CCR1 and MMR in NPs.¹³ Because NPs are usually characterized by eosinophilia and T_H2-related inflammation, eosinophil-derived CCL23 might be involved in the recruitment of monocytes and macrophages to NPs, followed by polarization of the recruited cells to the M2 phenotype by T_H2 cytokines. M2 macrophages are now known to be a rich source of eotaxins.³⁷⁻³⁹ We found here that CCL18 was mainly produced by M2 macrophages in NPs. These findings suggest that accumulation and activation of M2 macrophages in NPs might further enhance tissue eosinophilia through the production of eotaxins and might induce local adaptive immunity through the production of CCL18 and its subsequent recruitment of naive T cells and immature DCs. Importantly, both T-cell and DC numbers were increased in NPs.^{5,6,13} Future studies will be required to determine the relationship between these chemokines and inflammation in NPs.

We found that CLC, which is a marker of eosinophils, significantly and positively correlated with CCL18 in NPs. Because CCL18 has been described as an antagonist of CCR3, which is predominantly expressed on eosinophils,⁴⁶ we thought eosinophils might contribute to the production of CCL18. In this regard Schraufstatter et al⁴² showed that peripheral blood eosinophils were able to produce CCL18. We also found that peripheral blood eosinophils were able to release small amounts of CCL18 (40-50 pg/10⁶ cells), but these levels were not enhanced by eosinophil activators or T_H2 cytokines (see Fig E3). Furthermore, our immunofluorescence data demonstrated that CCL18 was not detected in eosinophils in NPs (Fig 3). Another possibility is that CCL18 might be involved in the recruitment of eosinophils through a CCR3-independent manner. However, Nibbs et al⁴⁶ showed that CCL18 inhibited CCL11-dependent peripheral blood eosinophil migration. It is known that there are phenotypic differences between peripheral blood eosinophils and tissue eosinophils.⁴⁷ Therefore it is still possible that CCL18 is involved in the recruitment of NP eosinophils. Future studies will be required to investigate whether CCL18 contributes to the recruitment of eosinophils and whether eosinophils are a source of CCL18 in NPs.

In addition to its chemotactic activity, CCL18 is known to activate fibroblasts. CCL18 activated Sp1 and protein kinase C α and induced collagen production in lung fibroblasts.^{48,49} Proliferation and activation of fibroblasts are considered to be important events leading to NP formation.³² Therefore the role of CCL18 in these processes is worthy of further investigation.

In summary, we report here that M2 macrophages and mast cells produce the chemokine CCL18 and that patients with CRSwNP have increased levels of CCL18 in polypoid tissue, as well as in surrounding tissues. Our findings indicate that CCL18 is a novel marker in patients with CRSwNP and that the overproduction of CCL18 in NPs might contribute to the pathogenesis of CRSwNP.

Clinical implications: Overexpression of CCL18 in NPs might have a pathogenic role in patients with CRSwNP.

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METHODS

Patients and biopsy specimens

Patients with CRS were recruited from the Allergy-Immunology Clinic and the Otolaryngology Clinic of the NMFF and the Northwestern Sinus Center at NMFF. Sinonasal and NP tissues were obtained during routine functional endoscopic sinus surgery in 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 NPs was confirmed by means of office endoscopy and computed tomographic imaging. All patients scheduled for surgery had previously experienced failure of 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 and Tables E1 to E3. Sinus tissues from disease-free control subjects were obtained during endoscopic skull base brain tumor excisions, as well as intranasal procedures for obstructive sleep apnea and facial fracture repairs in patients without a history of CRS, nasal inflammation, or asthma recruited from the Otolaryngology clinic at NMFF.

Subjects underwent skin tests to pollens, dust mites, pets, molds, and cockroach by using Hollister-Stier (Spokane, Wash) 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.

Patients were taking a variety of medications, including nasal corticosteroids, antihistamines, decongestants, and short-acting or long-acting β -agonists, among others. 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.

Real-time PCR

Total RNA from sinus tissue was extracted with QIAzol (Qiagen, Valencia, Calif) and was cleaned and treated by using DNase I with RNeasy, according to the manufacturer's instructions. The quality of total RNA from sinus tissue was assessed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, Calif) by using an RNA 6000 Nano LabChip (Agilent Technologies). Single-strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen) and 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 2 $\times$ TaqMan Gene Expression Master mix (Applied Biosystems), 400 nmol/L of each primer, and 200 nmol/L of TaqMan probe plus cDNA). Primer and probe sets for CCL18 (sense, 5'-CCAGCTCACTCTGACCACTTCTC-3'; antisense, 5'-GGTGCAGACGAGGACAAGGA-3'; FAM/black hole quencher 1-labeled probe, 5'-CTGCCAGCATCATGAAGGGCCTT-3'), CD3E (Hs99999153_m1), CD20 (sense, 5'-CACTCTTCAGGAGGATGTCTTCACT-3'; antisense, 5'-TCTGGACAGCCCCAAAGT-3'; FAM/MGB, 5'-TCTTCATGAGGGGAATCTA-3'), CD56 (Hs00941830_m1), CD138 (sense, 5'-GCCTGGTGGGTTTCATGCT-3'; antisense, 5'-TCCTGTTTGGTGGGCTCTG-3'; FAM/MGB, 5'-AGAAGGACGAAGGCAGC-3'), MMR (Hs00267207_m1), CD1c (Hs00233509_m1), CLC (Hs00171342_m1), tryptase (TPSAB1 [tryptase α/β 1], Hs02576518_gH), CXCR1 (Hs00174146_m1), CCR3 (Hs00266213_s1), CD163 (Hs00174705_m1), STAB1 (Hs01109068_m1), and β -glucuronidase (human β -glucuronidase endogenous control, PN; 4326320E) were purchased from Integrated DNA Technologies or Applied Biosystems. Quantified aliquots of purified PCR fragments of the target genes were serially diluted and used as standards in each experiment to determine the exact copy number of the target genes. 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 the housekeeping gene β -glucuronidase. Expression of β -glucuronidase was not significantly different among the 4 groups (data not shown).

Measurement of CCL18 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 CCL18 (R&D Systems) in cell-free supernatants were determined by using specific ELISA kits. The minimal detection limit for these kits is 3.9 pg/mL. The concentration of total protein was measured with a Bradford method-based Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, Calif). Concentrations of CCL18 in the tissue homogenate were normalized to the concentration of total protein.

Western blot analysis

Tissue extracts were dissolved in LDL sample buffer (Invitrogen). To detect CCL18, 40 μ g of reduced tissue extracts was resolved on a 4% to 12% NuPAGE Bis-Tris Gel (Invitrogen) in an MES buffer system, and then the proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories). 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 0.25 μ g/mL goat anti-CCL18 antibody (R&D Systems) and mouse anti-actin mAb (1:1000, clone C4, IgG₁; MP Biomedicals, Solon, Ohio). After primary antibody incubation, the membranes were washed repeatedly in PBS/0.1% Tween-20 and then labeled by 45 minutes of incubation at room temperature with infrared secondary antibody Alexa Fluor 680 conjugated donkey anti-goat IgG (1:10,000, Invitrogen) and IRDye 800DX-conjugated donkey anti-mouse IgG (1:10,000, Rockland). Protein was detected on an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, Neb).

Immunofluorescence assay

Nasal tissue was dehydrated, infiltrated, and embedded with paraffin, and tissue was sectioned at 3 μ m with a Leica RM2240 Cryostat (Leica Microsystems, Wetzlar, Germany). Rehydrated tissue sections, except those stained for ECP, were boiled in a citrate buffer (Dako, Carpinteria, Calif) for 10 minutes to induce antigen retrieval. After washing, tissue sections were blocked for nonspecific binding with 5% donkey serum/0.3% Tween-20/PBS. Tissue sections were then incubated with the same concentrations of control rabbit IgG (Thermo Fisher Scientific), control mouse IgG₁ (clone P3; eBiosciences, San Diego, Calif) and control mouse IgG₃ (Invitrogen). After washing, sections were incubated with 4 μ g/mL Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Invitrogen) and 4 μ g/mL Alexa Fluor 568-conjugated donkey anti-mouse IgG (Invitrogen) for 1 hour at room temperature in the dark. Sections were then washed and incubated with 0.1% Sudan Black solution for 10 minutes in the dark. After a final washing, cover slips were mounted onto slides by using SlowFade Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). In some experiments we used frozen tissue. After fixation in cold acetone, tissue sections were blocked for nonspecific binding with 5% goat serum/PBS. Tissue sections were then incubated with 7.5 μ g/mL rabbit anti-human CCL18, 0.5 μ g/mL rat anti-human MMR mAb (clone 309210, IgG_{2a}, R&D Systems), and 2 μ g/mL mouse anti-human CD68 mAb (clone KP1, IgG₁, Thermo Fisher Scientific) or 0.5 μ g/mL mouse anti-human CD163 mAb (clone 5C6 FAT, IgG₁; BMA Biomedicals, Augst, Switzerland) in blocking buffer for 2 hours. After washing,

sections were incubated with 4 $\mu\text{g/mL}$ Alexa Fluor 488–conjugated goat anti-rabbit IgG (Invitrogen), 4 $\mu\text{g/mL}$ Alexa Fluor 568–conjugated goat anti-mouse IgG₁ (Invitrogen) and 4 $\mu\text{g/mL}$ Alexa Fluor 647–conjugated goat anti-rat IgG (Invitrogen) for 1 hour at room temperature in the dark. Images from immunofluorescence slides were obtained with an Olympus IX71 inverted research microscope using $\times 40$ objective lens and images were collected with SlideBook software (Olympus, Center Valley, Pa). For the quantification of CCL18⁺ cells, slides were blinded, and then 10 pictures were randomly taken from each slide. The number of CCL18⁺ cells in nasal mucosa was counted by using the National Institutes of Health–issued Image J software by 2 independent observers.

Mast cell culture

Buffy coats from human peripheral blood were obtained from the American Red Cross (St Paul, Minn). Human PBMCs were isolated by means of centrifugation on a Ficoll–sodium diatrizoate density gradient ($d = 1.077 \text{ g/mL}$; Ficoll–Paque PREMIUM; GE Healthcare, Piscataway, NJ), according to the manufacturer's directions. CD34⁺ hematopoietic progenitor cells were separated from human PBMCs by using the MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-CD34 MicroBeads (Miltenyi Biotec), according to the manufacturer's instructions. The cells were suspended in serum-free Iscove methylcellulose medium (MethoCult SFBIT H4236, Stem-Cell Technologies, Vancouver, British Columbia, Canada) containing 200 ng/mL stem cell factor, 50 ng/mL IL-6, 5 ng/mL IL-3, 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin and then incubated at 37°C in 5% CO₂. After 2 weeks of culture, fresh methylcellulose medium containing 200 ng/mL SCF, 50 ng/mL IL-6, 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin was layered over the methylcellulose cultures. At 4 weeks, a 1-mL aliquot of Iscove modified Dulbecco medium (IMDM; Invitrogen) supplemented with 200 ng/mL SCF, 50 ng/mL IL-6, insulin-transferrin-selenium (Invitrogen), 55 $\mu\text{mol/L}$ 2-mercaptoethanol (Invitrogen), 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ strep-

tomycin was layered over the methylcellulose cultures. At 6 weeks, all cells were retrieved after dissolving the methylcellulose medium with PBS. The cells were then suspended and cultured in IMDM supplemented with 100 ng/mL SCF, 50 ng/mL IL-6, 0.1% BSA, insulin-transferrin-selenium, 55 $\mu\text{mol/L}$ 2-mercaptoethanol, 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin, and the culture medium was changed a week later. After an additional week of culture, the culture medium was switched to IMDM supplemented with 100 ng/mL SCF, 50 ng/mL IL-6, 5% FBS (Invitrogen), 55 $\mu\text{mol/L}$ 2-mercaptoethanol, 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin. The culture medium was changed weekly thereafter, and the cells were incubated for an additional 5 to 7 weeks. The final purity of the mast cells always exceeded 98% based on toluidine blue staining and the expression of Fc ϵ RI by means of flow cytometry. The mast cells were then sensitized with 1 $\mu\text{g/mL}$ human myeloma IgE (Calbiochem, San Diego, Calif) at 37°C for 48 hours. After washing the cells, they were stimulated with 1.5 $\mu\text{g/mL}$ anti-IgE antibody (Dako), 20 ng/mL IL-4, 20 ng/mL IL-13, 20 ng/mL IFN- γ , 20 ng/mL IL-17A, and their combinations in the presence of 0.1% dimethyl sulfoxide (vehicle control, Sigma-Aldrich) or 0.1% PIC (Sigma-Aldrich) for 48 hours.

Eosinophil culture

Eosinophils were purified from the granulocyte layer after density gradient separation of human peripheral blood. After lysing erythrocytes, eosinophils were purified by using the MACS system (Miltenyi Biotec) with the human eosinophil isolation kit (Miltenyi Biotec). The purity of the eosinophils was greater than 98% based on Diff-Quik (American Scientific Products, McGraw Park, Ill) staining. Purified eosinophils were suspended at a cell density of $1 \times 10^6/\text{mL}$ in IMDM supplemented with 10% FCS (Invitrogen). The eosinophils were then cultured with 1 $\mu\text{mol/L}$ phorbol 12-myristate 13-acetate/A23187, 10 ng/mL GM-CSF, 10 ng/mL IL-5, 10 ng/mL TNF, 10 ng/mL IL-4, 10 ng/mL IL-13, and 10 ng/mL IFN- γ for 24 and 48 hours.

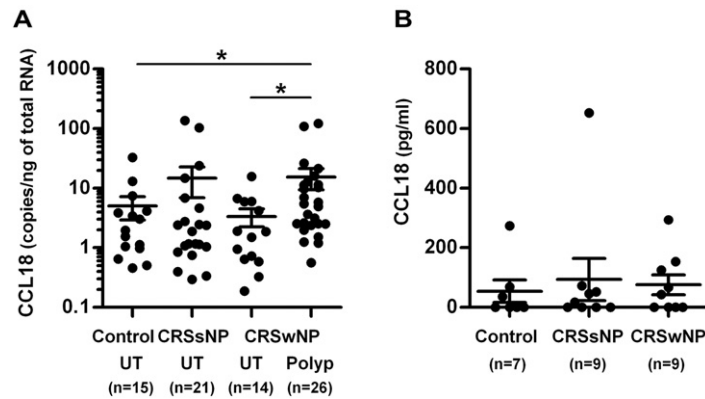


FIG E1. Expression of CCL18 in nasal epithelial cells. **A**, Total RNA was extracted from epithelial scraping cells from UT and NPs (*Polyp*), and expression of CCL18 mRNA was analyzed with real-time PCR. **B**, Expression of CCL18 protein in nasal lavage fluid was measured by using ELISA. * $P < .05$.

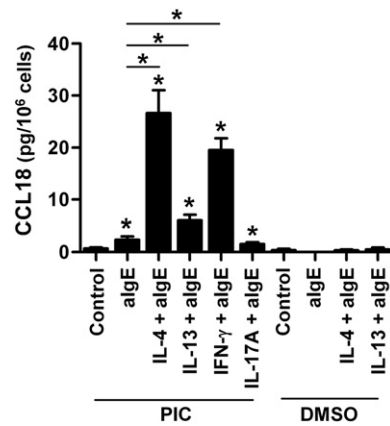


FIG E2. Detection of CCL18 protein in human mast cells. Human peripheral blood–derived mast cells were sensitized with 1 μ g/mL human myeloma IgE for 48 hours. After washing the cells, they were stimulated with 1.5 μ g/mL anti-IgE (*algE*) antibody, 20 ng/mL IL-4, 20 ng/mL IL-13, 20 ng/mL IFN- γ , 20 ng/mL IL-17A, or their combinations in the presence of 0.1% DMSO (vehicle control) or 0.1% PIC for 48 hours. Protein expression of CCL18 in supernatants was analyzed by means of ELISA. Results shown are means $\pm$ SEMs of 3 independent experiments with independent donors.

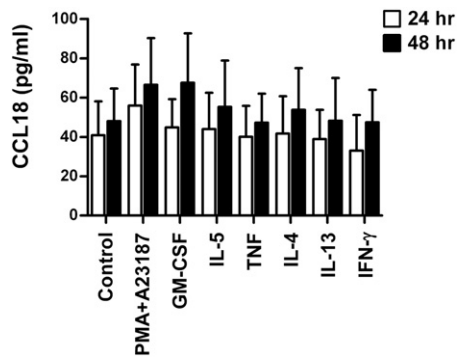


FIG E3. Detection of CCL18 in peripheral blood eosinophils. Purified peripheral blood eosinophils were incubated for 24 or 48 hours with 1 μ mol/L phorbol 12-myristate 13-acetate/A23187, 10 ng/mL GM-CSF, 10 ng/mL IL-5, 10 ng/mL TNF, 10 ng/mL IL-4, 10 ng/mL IL-13, or 10 ng/mL IFN- γ . The expression of CCL18 protein in supernatants was analyzed by using ELISA. Results shown are means $\pm$ SEMs of 3 separate subjects.

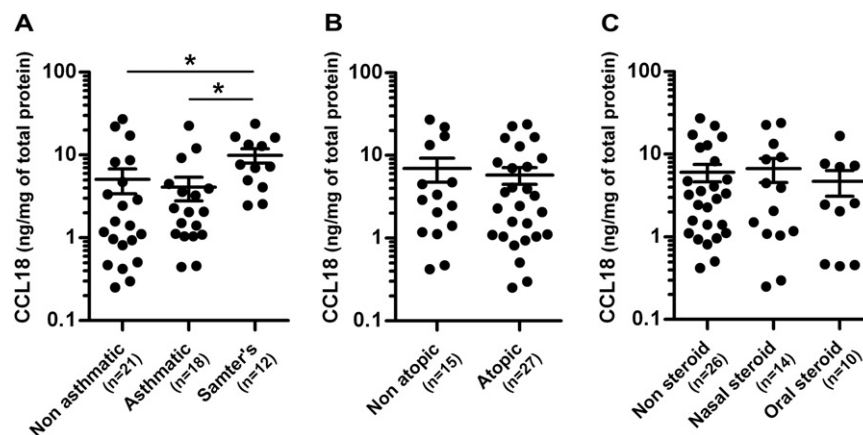


FIG E4. **A** and **B**, Asthmatic or atopic status did not affect the levels of CCL18 in NPs. Expression of CCL18 protein in tissue homogenates of NPs from patients with aspirin-tolerant CRSwNP and patients with Samter's triad was measured by using ELISA. **C**, We put the data from patients who took oral and nasal steroids into the oral steroid group. * $P < .05$.

TABLE E1. Subjects' characteristics for tissue RNA

	Control subjects, UT			Patients with CRSsNP, UT			Patients with CRSwNP, UT			Patients with CRSwNP, NPs		
Total no. of subjects (male subjects)	9 (3)			18 (8)			17 (14)			24 (18)		
Age (y), median (range)	41 (16-59)			36 (23-64)			41 (26-61)			44 (26-74)		
	Y	N	U	Y	N	U	Y	N	U	Y	N	U
Atopy	0	7	2	9	5	4	9	5	3	7	15	2
Asthma	0	8	1	2	12	4	7	10	0	8	15	1
Nasal steroid alone	0	9	0	0	2	16	1	16	0	0	24	0
Oral steroid alone	0	9	0	0	18	0	2	15	0	3	21	0
Oral and nasal steroid	0	9	0	0	18	0	1	16	0	0	24	0

Values are presented as medians (ranges), where shown.

N, No; *U*, unknown; *Y*, yes.

TABLE E2. Subjects' characteristics for tissue extracts

	Control subjects, UT			Patients with CRSsNP, UT			Patients with CRSwNP, UT			Patients with CRSwNP, NPs			Patients with Samter's triad, NPs		
Total no. of subjects (male subjects)	15 (9)			32 (15)			29 (20)			39 (28)			12 (6)		
Age (y), median (range)	49 (19-63)			37 (18-64)			41 (22-67)			41 (22-72)			47 (26-61)		
	Y	N	U	Y	N	U	Y	N	U	Y	N	U	Y	N	U
Atopy	0	14	1	13	13	6	15	12	3	20	13	6	7	2	3
Asthma	0	15	0	7	20	5	12	16	1	18	21	0	12	0	0
Nasal steroid alone	0	15	0	2	29	1	6	23	0	12	26	1	2	10	0
Oral steroid alone	0	15	0	1	30	1	2	27	0	4	34	1	2	10	0
Oral and nasal steroid	0	15	0	0	31	1	0	29	0	0	38	1	4	8	0

Values are presented as medians (ranges), where shown.

N, No; U, unknown; Y, yes.

TABLE E3. Subjects' characteristics for immunofluorescence analysis

	Control subjects, UT			Patients with CRSsNP, UT			Patients with CRSwNP, UT			Patients with CRSwNP, NPs		
Total no. of subjects (male subjects)	11 (8)			11 (6)			11 (9)			17 (11)		
Age (y), median (range)	53 (19-62)			38 (21-61)			48 (34-72)			52 (22-70)		
	Y	N	U	Y	N	U	Y	N	U	Y	N	U
Atopy	0	10	1	4	6	1	6	5	0	5	12	0
Asthma	0	11	0	4	5	2	3	8	0	11	6	0
Nasal steroid alone	0	12	0	3	8	0	2	9	0	3	13	1
Oral steroid alone	2	10	0	0	11	0	0	11	0	2	14	1
Oral and nasal steroid	0	12	0	1	10	0	3	8	0	3	13	1

Values are presented as medians (ranges), where shown.

N, No; U, unknown; Y, yes.