

Increased expression of the chemokine CCL23 in eosinophilic chronic rhinosinusitis with nasal polyps

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Background: Chronic rhinosinusitis (CRS) is a heterogeneous chronic disease characterized by local inflammation of the sinonasal tissues. The pathogenesis of CRS remains controversial, but it has been associated with the accumulation of various immune and inflammatory cells in sinus tissue.

Objectives: The objective of this study was to investigate the expression of the chemokine CCL23, which is known to bind to CCR1 and recruit monocytes, macrophages, and dendritic cells, in patients with CRS.

Methods: We collected nasal tissue from patients with CRS and control subjects. We assayed mRNA for CCL23 by using real-time PCR and measured CCL23 protein by means of ELISA, immunohistochemistry, and immunofluorescence.

Results: CCL23 mRNA levels were significantly increased in nasal polyps (NPs) from patients with CRS with nasal polyps (CRSwNP; $P < .05$) compared with inferior turbinate and uncinate tissue from patients with CRS or control subjects. CCL23 protein levels were also increased in NPs, although these levels were not statistically significant. Immunohistochemical analysis revealed CCL23 expression in mucosal epithelial cells and inflammatory cells, but accumulation of CCL23⁺ inflammatory cells occurred only in NPs. Immunofluorescence data showed CCL23 colocalization with eosinophil cationic protein–positive eosinophils. The concentration of CCL23 in NPs positively correlated with the concentration of eosinophil cationic protein, suggesting that eosinophils are major CCL23-producing cells in NPs. Finally, we found that CCL23 protein levels were significantly increased in NPs from patients with CRSwNP with aspirin sensitivity.

Conclusion: Overproduction of CCL23 in NPs might contribute to the pathogenesis of eosinophilic CRSwNP through the recruitment of CCR1⁺ inflammatory cells, including monocytes

and macrophages, and the amplification of local inflammation. (*J Allergy Clin Immunol* 2011;■■■■:■■■-■■■.)

Key words: Chronic rhinosinusitis, nasal polyps, CCL23, CCR1, eosinophils, aspirin sensitivity

Chronic rhinosinusitis (CRS) is a heterogeneous disease characterized by local inflammation of the upper airways and sinuses that is unresponsive to antibiotic therapy and that persists for at least 12 weeks. CRS is one of the most common chronic diseases in adults in the United States, affecting more than 30 million Americans, and is responsible for more than 250,000 surgical interventions annually.¹⁻³ The cause and pathogenesis of CRS remain controversial, but both fungi and bacteria have been implicated as key infectious agents in inciting the intense host inflammatory responses.⁴ Primarily on the basis of physical examination, histology, and clinical course, CRS is frequently divided into 2 types: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). Histologic studies have demonstrated significant tissue eosinophilia in patient with CRSwNP, most prominently in western countries. T-cell numbers in the mucosa are increased in patients with both forms of CRS and are skewed to T_H2 cytokine expression in patients with CRSwNP.^{2,3,5} In addition, we and other groups have found that B cells, plasma cells, macrophages, and neutrophils are also accumulated in the nasal mucosa of patients with CRSwNP.⁵⁻⁹ Therefore it is now recognized that accumulation of inflammatory cells and upregulation of the factors that recruit them are important in the pathogenesis of CRS, especially CRSwNP.

Chemokines mainly participate in the selective recruitment of inflammatory cells into tissue sites. Chemokines are a large superfamily of low-molecular-mass, secreted, and heparin-binding molecules that can be classified into several groups based on their molecular structures.^{10,11} They are known to be involved in many inflammatory diseases, including asthma, atopic dermatitis, rheumatoid arthritis, Crohn disease, and inflammatory bowel disease.^{11,12} Several chemokines have also been reported to be involved in CRS. The regulation and production of chemokines involved in the recruitment of CCR3⁺ cells (eg, eosinophils), such as RANTES (CCL5) and eotaxins (CCL11 [eotaxin-1], CCL24 [eotaxin-2], and CCL26 [eotaxin-3]), in patients with CRSwNP have been studied by many groups.^{5,13-17} In addition, levels of several other chemokines, including CCL2 (monocyte chemoattractant protein 1), CCL20 (liver and activation regulated chemokine), CXCL8 (IL-8), CXCL12 (stromal cell–derived factor 1 α), and CXCL13 (B cell–attracting chemokine 1), have also been reported to be increased in patients with CRS.^{8,18-22}

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Abbreviations used

CRS:	Chronic rhinosinusitis
CRSsNP:	CRS without nasal polyps
CRSwNP:	CRS with nasal polyps
ECP:	Eosinophil cationic protein
GUSB:	β -Glucuronidase
hpf:	High-powered field
IT:	Inferior turbinate
MMR:	Macrophage mannose receptor
NMFF:	Northwestern Medical Faculty Foundation
NP:	Nasal polyp
UT:	Uncinate tissue

CCL23 (also known as myeloid progenitor inhibitory factor 1, macrophage inflammatory protein 3, and chemokine β 8) was originally identified from a human aortic endothelial cell library and from the human monocytic cell line THP-1.^{23,24} Because a homolog of CCL23 in rodents has not been discovered, the function and regulation of CCL23 is not well described. CCL23 has chemotactic activity for monocytes, dendritic cells, and lymphocytes through the chemokine receptor CCR1.²³⁻²⁵ In addition, it has been reported that CCL23 can induce endothelial cell migration, tube formation, and angiogenesis through CCR1.²⁶ A protease cleaved product of CCL23 has chemotactic activity for monocytes and neutrophils through formyl peptide receptor-like 1.²⁷ In contrast, CCL23 has been shown to suppress progenitor cells of granulocyte and monocyte lineages.^{23,24} CCL23 has been reported to be expressed in activated monocytes and dendritic cells and is a biomarker for some inflammatory diseases, including atopic dermatitis, rheumatoid arthritis, and systemic sclerosis.^{25,28-30} Expression of CCL23 in monocytes has been shown to be regulated by the T_H2 cytokines IL-4 and IL-13.²⁸ Although CRSwNP is classically a T_H2 -related sinus disease and inflammatory cells are recruited to nasal polyps (NPs), expression of CCL23 in patients with CRS has not been studied. In this study we examined expression of CCL23 in patients with CRS and found that CCL23 levels were increased in patients with eosinophilic CRSwNP.

METHODS**Patients**

Patients with CRS were recruited from the Allergy-Immunology Clinic and the Otolaryngology Clinic of the Northwestern Medical Faculty Foundation (NMFF), the group practice for physician faculty members of Northwestern University and the Northwestern Sinus Center at NMFF. Sinonasal and NP tissues were obtained from routine Functional Endoscopic Sinus Surgery in patients with CRS. All patients met the criteria for CRS, as defined by the American Academy of Otolaryngology–Head and Neck Surgery Chronic Rhinosinusitis Task Force.^{1,31} Patients with an established immunodeficiency, pregnancy, coagulation disorder, diagnosis of classic allergic fungal sinusitis, Churg-Strauss syndrome, or cystic fibrosis did not participate in the study. Details of subjects' characteristics are included in Table I and in the Methods section of 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

Total RNA from sinus tissue was extracted with QIAzol (Qiagen, Valencia, Calif), and the quality of total RNA from sinus tissue was

assessed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, Calif). Semiquantitative real-time RT-PCR was performed by using the TaqMan method, as described previously.³² The mRNA expression levels were normalized to the median expression of the housekeeping gene β -glucuronidase (GUSB). Details can be found in the Methods section of this article's Online Repository.

ELISA and eosinophil culture

The concentrations of CCL23 (R&D Systems, Minneapolis, Minn) and ECP (MBL, Woburn, Mass) in cell-free supernatants were determined with specific ELISA kits. The minimal detection limit for these kits is 15 pg/mL and 0.4 ng/mL, respectively. The concentration of total protein was measured with a Bradford method-based Bio-Rad protein assay kit (Bio-Rad, Hercules, Calif). Concentrations of CCL23 and ECP in the tissue homogenate were normalized to the concentration of total protein. Details of ELISA and eosinophil culture can be found in the Methods section in this article's Online Repository.

Immunohistochemistry

Immunohistochemistry was performed as described previously.⁷ Briefly, tissue sections were incubated with 1.5 μ g/mL biotinylated goat anti-human CCL23 antibody (R&D Systems) at 4°C overnight. After washing, sections were incubated in ABC reagent (Vector Laboratories, Burlingame, Calif) for 1 hour. Sections were rinsed and incubated in DAB reagent (Invitrogen, Carlsbad, Calif) and then counterstained with hematoxylin. Slides were blinded, and 10 pictures were randomly taken from each slide. The number of CCL23⁺ cells in the nasal mucosa was counted by 2 independent observers. Details of the methods for immunofluorescence and immunohistochemistry are described in the Methods section of this article's Online Repository.

Statistics

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

RESULTS**CCL23 expression in patients with CRS**

Sinonasal and polyp tissues were collected from 49 subjects with CRSsNP, 95 subjects with CRSwNP, and 42 control subjects to determine the presence of CCL23 expression in patients with CRS. Subjects' characteristics are shown in Table I.

We assessed the expression of CCL23 in inferior turbinate (IT) tissue and uncinate tissue (UT) from patients with CRSsNP, patients with CRSwNP, and control subjects, as well as in NP tissue from patients with CRSwNP. Expression of the housekeeping gene GUSB was not significantly different among the 7 groups (data not shown). CCL23 mRNA levels were only significantly increased in NP tissue from patients with CRSwNP ($P < .001$) in comparison with those seen in IT tissue and UT from either patients with CRS or control subjects (Fig 1, A). To confirm this observation at the protein level, we made detergent extracts from homogenates of IT and NP tissues and then measured the concentration of CCL23 with ELISA. Although not statistically significant, CCL23 protein levels were increased in NP tissue from patients with CRSwNP ($n = 44$; 218.0 ± 48.4 pg/mg; range, 10.9–1899 pg/mg) compared with those seen in IT tissue from patients with CRSsNP ($n = 20$; 80.9 ± 6.5 pg/mg; range, 27.0–139.3 pg/mg), patients with CRSwNP ($n = 21$; 175.5 ± 39.4 pg/mg; range, 16.3–636.4 pg/mg), and control

TABLE I. Subjects' characteristics

	Control subjects			Patients with CRSsNP			Patients with CRSwNP			Patients with CRSwNP and AS		
Total no. of subjects	42 (27 male)			49 (18 male)			83 (61 male)			12 (7 male)		
Age (y), median (range)	37 (16-77)			36 (23-64)			42 (21-74)			50 (26-61)		
	Y	N	U	Y	N	U	Y	N	U	Y	N	U
Atopy	5	31	6	19	20	10	38	28	17	7	2	3
Asthma	0	38	4	5	40	4	35	45	3	10	2	0
Methodologies used												
PCR	IT			IT			IT			NP		
No. of subjects	17 (14 male)			16 (7 male)			15 (8 male)			24 (18 male)		
Age (y), median (range)	36 (20-77)			41 (16-59)			40 (28-68)			46 (27-74)		
ELISA	IT			IT			IT			NP		
No. of subjects	12 (7 male)			20 (8 male)			21 (14 male)			44 (37 male)		
Age (y), median (range)	40 (17-63)			35 (24-60)			41 (26-63)			41 (21-74)		
IHC	UT			UT			UT			NP		
No. of subjects	11 (4 male)			12 (3 male)			10 (8 male)			10 (9 male)		
Age (y), median (range)	41 (16-77)			33 (26-55)			42 (26-67)			40 (26-70)		

AS, Aspirin sensitive; IHC, immunohistochemistry; M, male; N, no; Y, yes; U, unknown.

subjects ($n = 12$; 84.8 ± 12.8 pg/mg; range, 15.3-173.8 pg/mg; Fig 1, B). We thought the large variation in the concentration of CCL23 in NP tissue might be due to the asthmatic or atopic status of the patients. However, this did not turn out to be the case because mean levels of CCL23 were not affected by asthmatic or atopic status (Fig 1, C).

Localization of CCL23⁺ cells in nasal mucosa of patients with CRS

To examine the CCL23-producing cells in nasal mucosa, we used immunohistochemistry to detect CCL23⁺ cells in UT and NP tissue. As shown in Fig 2, CCL23 staining was observed mainly in submucosal inflammatory cells, although light CCL23 staining was also observed in some mucosal and glandular epithelium. In addition, we did not observe a significant difference in the number of CCL23⁺ cells in the epithelium and glands among the 3 groups of subjects (Fig 2 and data not shown). We therefore focused on inflammatory cells in this study. We found that CCL23⁺ inflammatory cells were highly increased in NP tissue from patients with CRSwNP (Fig 2, D). We counted the number of CCL23⁺ inflammatory cells using a semiquantitative method and found that CCL23⁺ inflammatory cell numbers were significantly increased in the submucosal region of NP tissues from patients with CRSwNP ($n = 10$; 10.6 ± 2.5 CCL23⁺ cells/high-powered field [hpf]) compared with those seen in UT from patients with CRSsNP ($n = 12$, 1.4 ± 1.7 CCL23⁺ cells/hpf, $P < .05$), patients with CRSwNP ($n = 10$, 3.2 ± 2.1 CCL23⁺ cells/hpf, $P < .05$), and control subjects ($n = 11$, 1.3 ± 1.8 CCL23⁺ cells/hpf, $P < .05$; Fig 2, F).

CCL23 is expressed by eosinophils in NPs

CCL23 is known to be produced by monocytes, macrophages, and dendritic cells.^{25,28,33} We therefore first examined whether macrophages and dendritic cells were major CCL23-producing cells in NP tissue. Immunofluorescence data showed that less than 5% of CCL23⁺ cells were costained with the macrophage marker CD68, and the majority of CD68⁺ cells lacked CCL23 staining in NP tissue (Fig 3, A, and see Fig E1 in this article's

Online Repository at www.jacionline.org). In addition, we could not find CCL23 staining in CD1c⁺ dendritic cells in NP tissue (see Fig E1). To identify the major CCL23-producing cells in submucosal tissue of NPs, we performed immunofluorescence analysis with anti-CCL23 and antibodies against several other cell-specific markers, including CD3 (T cells), CD20 (B cells), CD138 (plasma cells), eosinophil cationic protein (ECP; eosinophils), and tryptase (mast cells). Interestingly, we found CCL23 colocalization in ECP⁺ eosinophils but not in T cells, B cells, plasma cells, or mast cells (Fig 3, B, and see Fig E1).

NPs from patients with CRSwNP have long been known to be characterized by eosinophilic inflammation.¹⁵ Immunofluorescence staining showed that more than 90% of CCL23⁺ cells in NP tissue were eosinophils (Fig 3). However, to our knowledge, no one has reported production of CCL23 in eosinophils. We first examined the expression of CCL23 in eosinophils isolated from peripheral blood from control subjects. We found that CCL23 mRNA was constitutively expressed in isolated eosinophils and that levels were significantly higher than in PBMCs or neutrophils ($n = 3-4$; see Fig E2, A, in this article's Online Repository at www.jacionline.org). Purified blood eosinophils were stimulated with common eosinophil activators, IL-5, GM-CSF, phorbol 12-myristate 13-acetate/A23187, and TNF, and the known CCL23 inducers^{28,33} IL-4, IL-13, and IFN- γ to investigate the regulation of CCL23 in eosinophils. However, the concentration of CCL23 was less than the lowest standard in the ELISA (15.6 pg/mL) in most eosinophil supernatants (see Fig E2, C). We also measured the expression of CCL23 mRNA in eosinophils 24 hours after stimulation. Interestingly, CCL23 mRNA levels were decreased 24 hours after culture ($n = 3$, 0.02-fold; Fig E2, A and B). Decreased expression of CCL23 mRNA was rescued by GM-CSF, IL-5, and TNF (see Fig E2, B). Because the levels of CCL23 protein were at the limit of detectability in cultured eosinophils, we then assessed whether CCL23 was detected in eosinophils that were freshly isolated from nasal lavage fluid in patients with CRSwNP. We found that CCL23 was expressed in ECP⁺ eosinophils from nasal lavage fluid but not in elastase-positive neutrophils (Fig 4).

We next examined whether levels of CCL23 correlated with eosinophilic inflammation in NPs. We assayed the levels of ECP

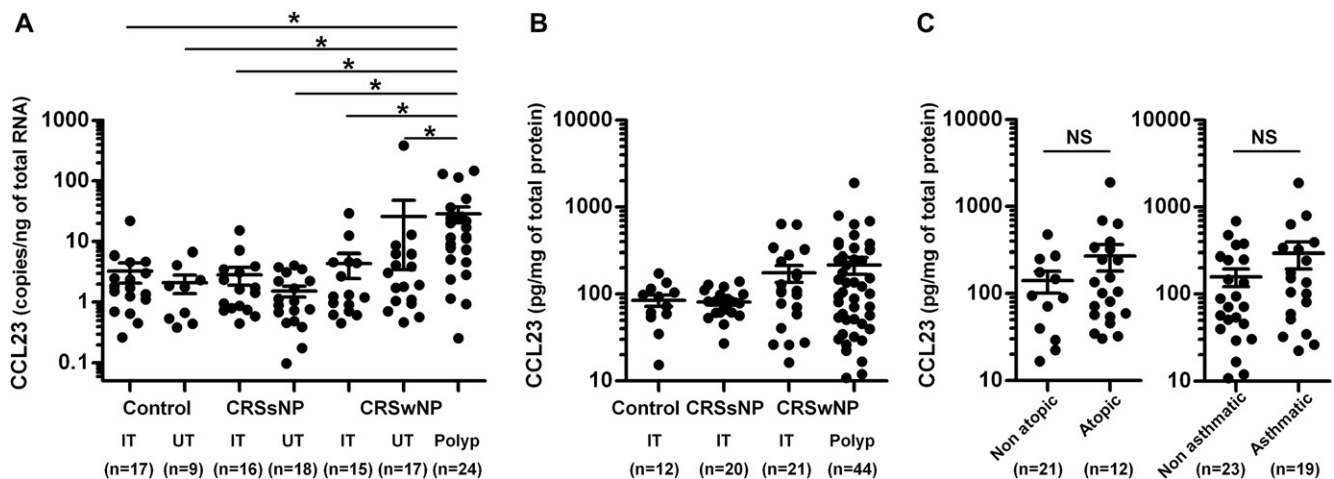


FIG 1. Increased expression of CCL23 in NP tissue. **A**, Total RNA was extracted from IT tissue, UT, and NPs (Polyp), and expression of CCL23 was analyzed by using real-time PCR. **B** and **C**, The concentration of CCL23 in tissue homogenates of IT tissue (**B**) and NPs (**C**) was measured by using ELISA. The CCL23 concentration was normalized to the concentration of total protein. * $P < .05$. NS, Not significant.

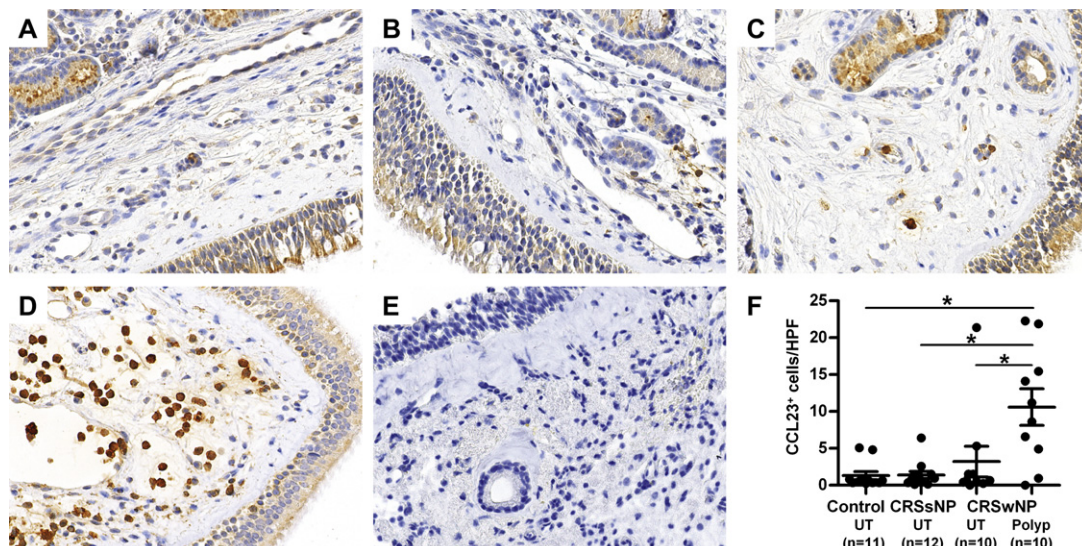


FIG 2. Immunohistochemistry of CCL23 was performed with an anti-human CCL23 antibody. Representative immunostaining for CCL23 in UT from a control subject (**A**), a patient with CRSsNP (**B**), a patient with CRSwNP (**C**), and NP tissue (**D**). Negative control antibody staining in NP tissue from a patient with CRSwNP (**E**) is shown. The number of CCL23⁺ cells in UT from control subjects ($n = 11$), patients with CRSsNP ($n = 12$), and patients with CRSwNP ($n = 10$) and NPs ($n = 10$) was counted with the National Institutes of Health-issued Image J software (**F**). Magnification: $\times 400$. * $P < .05$.

as a marker for the presence of eosinophils in NP tissue. We found that the concentration of CCL23 in NP tissue significantly correlated with the concentration of ECP ($r = 0.4952$, $P = .004$, $n = 32$; **Fig 5, A**). NPs in aspirin-sensitive patients are typically more eosinophilic than those in aspirin-tolerant patients.¹⁵ Although we initially excluded patients who had aspirin sensitivity in this study, we decided to determine levels of CCL23 in NPs from patients with CRSwNP/aspirin sensitivity. We found that CCL23 protein levels were significantly increased in NP tissue from patients with CRSwNP/aspirin sensitivity ($n = 12$; 499.1 ± 142.7 pg/mg; range, 25.7-1640 pg/mg) compared with those seen in IT tissue from control subjects ($n = 12$, 84.8 ± 12.8 pg/mg, $P = .040$), patients with CRSsNP ($n = 20$, $80.9 \pm$

6.5 pg/mg, $P = .023$), and patients with CRSwNP ($n = 21$, 175.5 ± 39.4 pg/mg, $P = .070$; **Figs 1, B, and 5, B**).

Correlation of CCL23 and CCR1 in NPs

CCL23 is a ligand of the chemokine receptor CCR1, which is expressed on monocytes, macrophages, and dendritic cells. We therefore measured the expression of CCR1 in sinus tissue. CCR1 mRNA levels were significantly upregulated in NP tissue from patients with CRSwNP ($P < .05$) in comparison with those seen in UT from either patients with CRS or control subjects (**Fig 6, A**). We next examined whether the expression of CCL23 correlated with the levels of CCR1 and markers of macrophages and dendritic cells

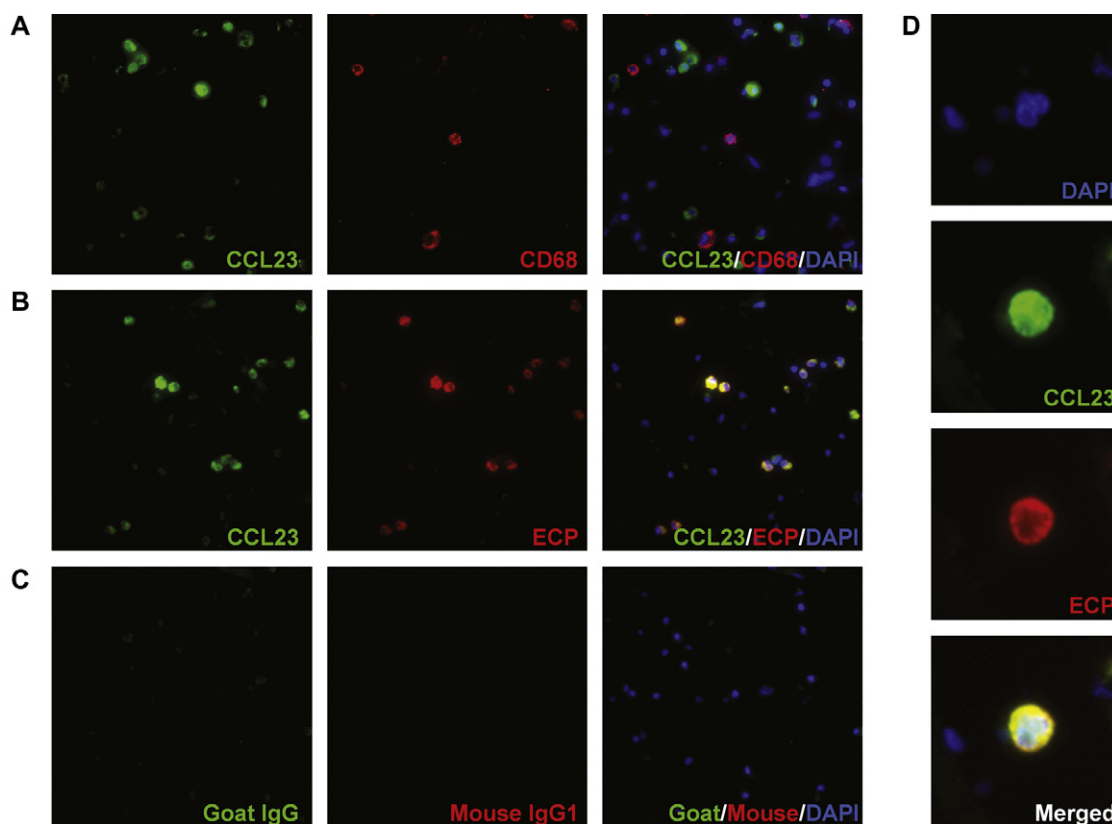


FIG 3. Immunofluorescence of CCL23 in NP tissue. Immunofluorescence assay was performed with anti-CCL23 (green fluorescence) and anti-CD68 mAb (red fluorescence) for macrophages (**A**), anti-ECP mAb (red fluorescence) for eosinophils (**B** and **D**), and control IgG (**C**). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue fluorescence). The results are representative of 3 to 6 separate patients.

in NP tissue. We found that CCL23 mRNA significantly correlated with mRNA levels for CCR1 ($r = 0.5591$, $P = .0045$), macrophage mannose receptor (MMR; $r = 0.5739$, $P = .0034$), and CD1c ($r = 0.4304$, $P = .0358$) in NP tissue ($n = 24$, Fig 6).

DISCUSSION

This study provides the first demonstration that CCL23 levels are increased in polypoid tissue from patients with CRSwNP (Figs 1 and 2). Significant levels of CCL23 protein were found in polypoid tissue obtained from patients with CRSwNP, especially in patients with aspirin sensitivity (Figs 1 and 5). CCL23⁺ cell numbers were increased in the submucosal tissue of NPs from patients with CRSwNP (Figs 2 and 3), and CCL23 was found to be colocalized predominantly with ECP, indicating that eosinophils are major CCL23-producing cells in NPs (Figs 3 and 4). We also demonstrate that levels of CCL23 significantly correlated with levels of ECP, MMR, and CCR1 in NPs (Figs 5 and 6).

It has been reported that lymphocytes, macrophages, dendritic cells, and neutrophils also accumulate in the nasal mucosa of patients with CRSwNP.⁵⁻⁹ In the current study we demonstrated that levels of mRNA encoding CCL23, which is a known ligand of CCR1 and is able to recruit monocytes, dendritic cells, lymphocytes, and endothelial cells, were significantly increased in NP tissue (Fig 1, A). Although increased CCL23 mRNA levels were detected in 70% to 80% of NP tissue, increased CCL23 protein levels were detected in only 50% of NP extracts (Fig 1,

B). It has been reported that CCL23 can be cleaved by proteases, including mast cell chymase, neutrophil elastase, and cathepsin G,^{27,34} suggesting that the stability of CCL23 protein in sinus mucosa might be compromised by the presence of activated immune cells. Alternatively, it might be that the extraction procedure we use does not adequately free the CCL23 for detection in our ELISA assay. Further studies will be required to generate an assay system for the cleaved product of CCL23 and test these possibilities. Importantly, the known CCL23 cleaved product does not activate CCR1 but has activity for another receptor, formyl peptide receptor-like 1, that is known to be expressed on monocytes and neutrophils.²⁷ Although neutrophils generally accumulate in NP tissue, the precise mechanisms for their recruitment in patients with CRSwNP are not well understood. NPs from patients with cystic fibrosis are characterized by neutrophilia, and IL-8 is known to be chemotactic for neutrophils in this disease.^{5,35} In contrast, the effect of IL-8 in the recruitment of neutrophils in patients with CRSwNP is still unclear. The relationship between the production and degradation of CCL23 and the recruitment of neutrophils in patients with CRSwNP will hence be worthy of investigation.

It has long been known that NPs from patients with CRSwNP are characterized by eosinophilic inflammation. Our immunofluorescence data clearly show that CCL23 was detected in ECP⁺ eosinophils in the submucosa of NPs (Fig 3). However, the production of CCL23 in eosinophils has not been reported. In addition, our understanding of the regulation of CCL23 is limited.

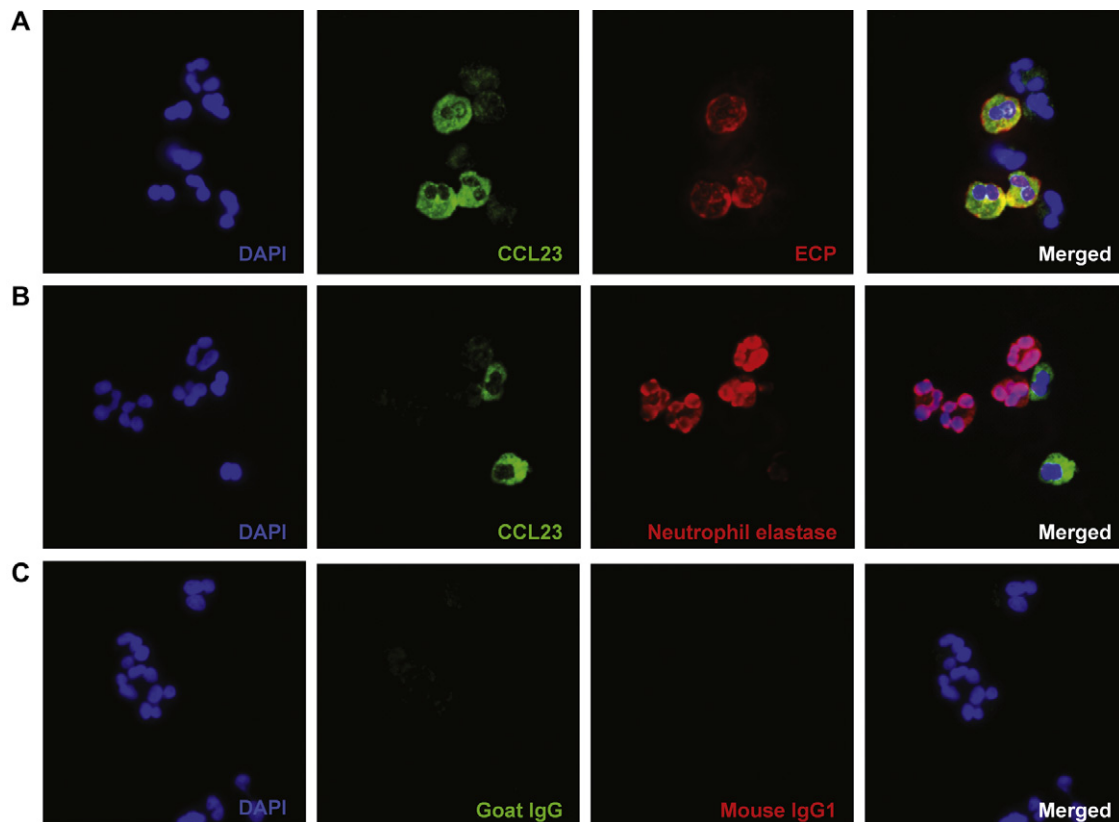


FIG 4. Detection of CCL23 in nasal lavage eosinophils from patients with CRSwNP. Immunofluorescence assay was performed with anti-CCL23 (green fluorescence) and anti-ECP mAb (red fluorescence) for eosinophils (**A**), anti-neutrophil elastase (red fluorescence) for neutrophils (**B**), and control IgG (**C**). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue fluorescence). The results are representative of 4 separate patients.

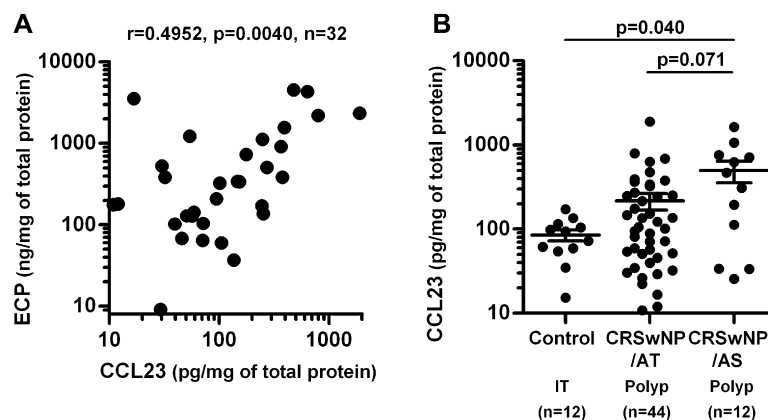


FIG 5. Increased expression of CCL23 in eosinophilic NPs. The relationship of CCL23 and ECP in NP tissue was evaluated by using ELISA. The correlations were assessed by using the Spearman rank correlation (**A**). The concentrations of CCL23 in NP tissue homogenates from aspirin-sensitive patients (CRSwNP/AS) and aspirin-tolerant patients (CRSwNP/AT) are shown (**B**). The concentration of CCL23 in IT tissue from control subjects and in NPs from CRSwNP/AT patients was used from Fig 1, B.

Forssmann et al³³ showed that IL-1 β and IFN- γ had the ability to induce CCL23 expression at low levels in monocytes. Novak et al²⁸ showed that the T_H2 cytokines IL-4 and IL-13 were potent inducers of CCL23 in monocytes and that the activity was dependent on signal transducer and activator of transcription 6. We therefore tested whether purified eosinophils from the peripheral

blood of healthy subjects were able to produce CCL23 when stimulated by known CCL23 inducers and the common eosinophil activators. Although CCL23 mRNA was constitutively expressed in eosinophils and levels were 100-fold higher than in PBMCs or neutrophils, we could not obtain significant evidence indicating that either resting or stimulated eosinophils purified from

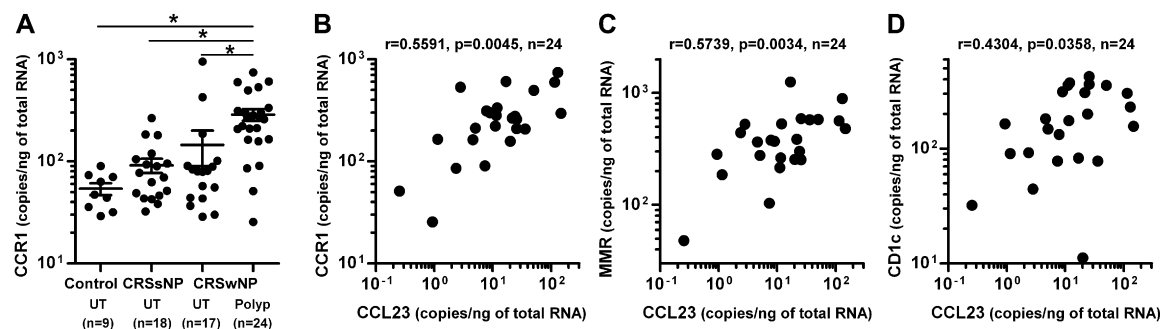


FIG 6. Correlation of CCL23 with CCR1 in NPs. **A-D**, Total RNA was extracted from UT and NP tissue, and the expression of CCL23 (Fig 6, B-D), CCR1 (Fig 6, A and B), and the cell-specific markers MMR (Fig 6, C) and CD1c (Fig 6, D) was analyzed by means of real-time PCR. The correlations were assessed by using Spearman rank correlation. * $P < .05$.

peripheral blood release CCL23 (see Fig E2). Interestingly, levels of mRNA for CCL23 decreased to 2% of basal expression in blood eosinophils after 24 hours of culture, and this downregulation was somewhat rescued by eosinophil activators. We also assessed the concentration of CCL23 in serum and found that levels of CCL23 in patients with CRSwNP (1.10 ± 0.12 ng/mL, $n = 15$) were not different compared with those seen in patients with CRSsNP (0.83 ± 0.19 ng/mL, $n = 15$) or control subjects (0.94 ± 0.22 ng/mL, $n = 9$). This suggests that overproduction of CCL23 in eosinophils occurs only locally in NPs. Previous studies have shown that eosinophils isolated from bronchoalveolar lavage are phenotypically distinct from peripheral blood eosinophils and *in vitro*-primed eosinophils.³⁶⁻³⁸ We therefore examined whether eosinophils that were freshly isolated from nasal lavage fluid in patients with CRSwNP expressed CCL23. We found that CCL23 was detected in nasal lavage eosinophils but not in neutrophils (Fig 4). Future studies will be required to identify the regulation and production of CCL23 in NP eosinophils.

Initially, we did not find a statistically significant increase in CCL23 protein levels in NP tissue. NPs are usually characterized by eosinophilic inflammation, but this is not observed in all patients. In fact, 20% to 30% of NPs do not have eosinophilia (ie, <5 eosinophils/hpf) in our clinic. In the current study we found that CCL23 was detected in eosinophils in NP tissue (Fig 3) and that CCL23 levels were significantly increased in NP tissue from patients with CRSwNP with aspirin sensitivity, who usually had more eosinophilic inflammation (Fig 5).¹⁵ Although we found that the concentration of CCL23 was significantly correlated with ECP levels in NP tissue (Fig 5), there were some patient samples in which ECP levels were high and CCL23 levels were low, suggesting that our ability to detect CCL23 might be compromised in some patients' samples because of the protease activity referred to above or another factor. On balance, these data suggest that CCL23 is produced by eosinophils in affected tissue and might contribute to the pathogenesis of eosinophilic CRSwNP.

We detected ECP⁺ eosinophils by using an EG2 antibody. EG2⁺ cells are classically defined as activated eosinophils. Importantly, it has been shown that EG2⁺ activated eosinophils are found in about 80% of NP tissue from patients with CRSwNP.³⁹ In contrast, no EG2⁺ eosinophils are detected in turbinate tissue from patients with CRSsNP and from control subjects, although some resting eosinophils are found.³⁹ In this study we found CCL23 was detected in EG2⁺ cells (Fig 3).

This suggests the possibility that activated eosinophils, but not resting eosinophils, produce CCL23 in NP tissue.

CCL23 is known to recruit monocytes and macrophages through CCR1,^{24,25} and we found that both CCL23 and CCR1 expression were significantly upregulated in NP tissue. In contrast to eosinophils, the role of monocytes and macrophages in the pathogenesis of CRS is poorly understood. CD68⁺ macrophage numbers were reported to be increased in patients with CRSwNP, but this did not achieve statistical significance in some small studies.^{5,40} Macrophages can be polarized by their microenvironment, especially by T_H cytokines and pathogens. Alternatively activated macrophages (also known as M2 macrophages) are primed by the T_H2 cytokines IL-4 and IL-13.^{41,42} Importantly, eosinophilic NPs contain an environment enriched for T_H2 cytokines.⁵ Interestingly, Claeys et al^{6,35} showed that mRNA levels for MMR (also known as mannose receptor C type 1 and CD206) and MMR⁺ macrophage numbers were significantly increased in NP tissue from patients with CRSwNP. MMR is now widely accepted to be a marker of M2 macrophages.^{41,42} We therefore examined the correlation of CCL23 and MMR levels in NP tissue and found that MMR levels were significantly increased in NP tissue and positively correlated with expression of CCL23 (Fig 6 and data not shown). Very recently, Krysko et al⁴³ showed that CD68⁺ macrophages and M2 macrophages (MMR⁺, HLADR⁺, CD14⁺, CD11c⁺, CD20⁻) were increased in NP tissue. One possibility is that CCL23 might be involved in the recruitment of monocytes and macrophages into NPs, followed by polarization of the recruited cells to the M2 phenotype by T_H2 cytokines. In addition, eotaxin levels are increased in NPs, and eotaxin is released from nasal epithelial cells and macrophages in the nasal mucosa of patients with CRS and allergic rhinitis.⁴⁴ M2 macrophages are now known to be a rich source of eotaxin/CCL11 in mice.⁴⁵ In human subjects high levels of CCL11 and eotaxin-3/CCL26 are expressed in *in vitro*-polarized M2 macrophages.^{41,42,46,47} Thus the production of CCL23 from eosinophils and the subsequent recruitment of macrophages, which in turn release eotaxins that are chemotactic for eosinophils, might represent a cycle through which inflammation in polyp tissue is perpetuated. This suggests that production of the chemokines, CCL23, CCL11, and CCL26; recruitment of macrophages and eosinophils; and the presence of T_H2 cytokines in NPs might be linked to each other and might be involved in the pathogenesis of eosinophilic CRSwNP. Future study will be required to

determine the relationships of these factors in eosinophilic NPs. It has been widely accepted that other allergic airway diseases, including asthma and allergic rhinitis, are characterized by T_H2 inflammation and eosinophilia. Thus future studies might be directed toward investigating the presence of CCL23 in these patients and might help explain their disease pathology.

Angiogenesis and microvascular remodeling are features of tissue remodeling in chronic inflammatory diseases. Recently, a number of studies have reported a role for angiogenesis based on upregulation of proangiogenic factors, including vascular endothelial growth factor angiopoietin, and metalloproteinase 33, in NPs.⁴⁸⁻⁵¹ CCL23 has been reported to induce the migration of endothelial cells, as well as tube formation,²⁶ and is known to induce the production of matrix metalloproteinase 2 in endothelial cells.⁵² In addition, increased expression of matrix metalloproteinase 2 in NPs has been reported.⁵³ These data suggest that there should be consideration of the possibility that CCL23 plays a role in the angiogenesis seen in patients with CRSwNP.

In summary, we report here that eosinophils produce the chemokine CCL23 and that patients with eosinophilic CRS have increased levels of CCL23 in polypoid tissue. Our findings indicate that CCL23 is a novel marker of patients with eosinophilic CRSwNP and that the overproduction of CCL23 in NPs might contribute to the pathogenesis of eosinophilic CRSwNP.

Clinical implications: Overexpression of CCL23 in NPs might have a pathogenic role in patients with eosinophilic 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, the group practice for physician faculty members of Northwestern University, and the Northwestern Sinus Center at NMFF. Sinonasal and NP tissues were obtained from 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.^{E1,E2} The presence of sinusitis or bilateral NPs was confirmed by means of office-based endoscopy and computed tomographic imaging.

All patients scheduled for surgery had previously undergone unsuccessful 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 did not participate in the study. Details of subjects' characteristics are included in Table 1. 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 NMFF.

Subjects were skin tested to pollens, dust mites, pets, molds, and cockroach by using Hollister-Stier Canada (Toronto, Ontario, Canada) 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.

Aspirin sensitivity was determined based on history. Patients were taking a variety of medications, including topical 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. A portion of each sample was transferred into RNeasy lysis buffer for isolation of RNA (Qiagen, Austin, Tex) and stored at -20°C .

Real-time PCR

Total RNA from sinus tissue, eosinophils, and neutrophils was extracted with QIAzol (Qiagen) and was cleaned and treated with DNase I by using RNeasy, according to the manufacturer's instructions. The quality of total RNA from sinus tissue was assessed by using a 2100 Bioanalyzer (Agilent Technologies) with 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 the TaqMan method by using an Applied Biosystems 7500 Sequence Detection System (Applied Biosystems, Foster City, Calif) in 15- μL reactions (7.5 μL 2x TaqMan Gene Expression Master mix [Applied Biosystems], 400 nmol/L of each primer, and 200 nmol/L of TaqMan probe plus cDNA). A primer and probe set for CCL23 (sense, 5'-TCCAAGCCGGGTGT-CATC-3'; antisense, 5'-TCAGCATTCTACGCAAACCT-3'; and FAM/black hole quencher 1 labeled probe, 5'-TTCTGTGCCAACCCAGTGA-TAAGCA-3') was synthesized by Integrated DNA Technologies (Coralville, Iowa). Primer and probe sets for GUSB (human β -glucuronidase endogenous control, PN; 4326320E) CCR1 (Hs00174298_m1), MMR (Hs00267207_m1), and CD1c (Hs00233509_m1) were purchased from 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 GUSB.

Measurement of CCL23 and ECP 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 CCL23 (R&D Systems) and ECP (MBL, Woburn, Mass) in cell-free supernatants were determined with specific ELISA kits. The minimal detection limit for these kits is 15 pg/mL and 0.4 ng/mL, respectively. The concentration of total protein was measured with a Bradford method-based Bio-Rad protein assay kit (Bio-Rad, Hercules, Calif). Concentrations of CCL23 and ECP in the tissue homogenate were normalized to the concentration of total protein.

Immunohistochemistry

Nasal tissue was dehydrated, infiltrated, and embedded with paraffin, and tissue was sectioned at 3 μm by using a Leica RM2240 Cryostat. Sections were rehydrated and blocked for endogenous peroxidase activity with 3% H_2O_2 /methanol. Tissue sections were then boiled in a citrate buffer (Dako, Carpinteria, Calif) for 15 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 1.5 $\mu\text{g}/\text{mL}$ biotinylated goat anti-human CCL23 antibody (R&D Systems) or 1.5 $\mu\text{g}/\text{mL}$ biotinylated control goat IgG (R&D Systems) at 4°C overnight. After washing, 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 DAB reagent (Invitrogen) for 5 minutes at room temperature. They were then rinsed in deionized H_2O , counterstained with hematoxylin, dehydrated, cleaned, mounted, and placed under cover slips with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, Mich) in preparation for microscopic analysis. Slides were blinded, and then 10 pictures were randomly taken from each slide. The number of CCL23⁺ cells in nasal mucosa was counted by using the National Institutes of Health–issued Image J software by 2 independent observers.

For the immunofluorescence assay, rehydrated sections were blocked with 5% donkey serum/0.3% Tween 20/PBS and then were incubated with 4 $\mu\text{g}/\text{mL}$ goat anti-human CCL23 antibody and 0.1 $\mu\text{g}/\text{mL}$ mouse anti-human ECP mAb (clone: EG2, IgG1, Diagnostics Development, Sweden) or 24 ng/mL mouse anti-human CD68 mAb (clone: PG-M1, IgG3, Thermo scientific/Lab Vision, Fremont, Calif) 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 4 $\mu\text{g}/\text{mL}$ Alexa Fluor 488–conjugated donkey anti-goat IgG (Invitrogen) and 4 $\mu\text{g}/\text{mL}$ Alexa Fluor 647–conjugated donkey anti-mouse IgG (Invitrogen) for 1 hour at room temperature in the dark. After a final washing, cover slips were mounted onto slides with SlowFade Gold antifade reagent with 4',6-diamidino-2-phenylindole (Invitrogen).

Nasal lavage was performed by instilling 5 mL of sterile PBS into each nostril, holding for 5 seconds, and then expelling into a sterile container. Cells in the nasal lavage fluid were collected by means of centrifugation. After making cytocentrifugation preparations with a Cytospin (Shandon), cells were fixed with 3.7% formaldehyde (Fisher Biotech, Waltham, Mass) in PBS for 20 minutes and were permeabilized by 0.3% Tween 20 in PBS for 10 minutes. After blocking, cells were incubated with 1.5 $\mu\text{g}/\text{mL}$ goat anti-human CCL23 antibody and 0.4 $\mu\text{g}/\text{mL}$ mouse anti-human ECP mAb or 1 $\mu\text{g}/\text{mL}$ mouse anti-human neutrophil elastase mAb (clone: NP57, IgG1, Dako) in blocking buffer at 4°C overnight. Images from immunofluorescence slides were obtained with an Olympus IX71 inverted research microscope by using a $\times 40$ objective lens, and images were collected by using SlideBook software (Olympus, Center Valley, Pa).

Cell culture

Buffy-coated human peripheral blood was obtained from the American Red Cross (St Paul, Minn). Human PBMCs were isolated by means of centrifugation on a Ficoll–sodium diatrizoate density gradient ($d = 1.077 \text{ g}/\text{mL}$, Ficoll-Paque PREMIUM; GE Healthcare, Piscataway, NJ), according to the manufacturer's directions. Eosinophils and neutrophils were purified from the granulocyte layer after the density gradient. After lysing erythrocytes, eosinophils and neutrophils were purified with the MACS system (Miltenyi

Biotec, Bergisch Gladbach, Germany) with the human eosinophil isolation kit (Miltenyi Biotec) and anti-CD16 MicroBeads (Miltenyi Biotec), respectively. The purity of the eosinophils and neutrophils was greater than 98% based on Diff-Quik (American Scientific Products, McGraw Park, Ill) staining.

Eosinophil cultures were described previously.^{E3} Briefly, purified eosinophils were suspended at a cell density of $1 \times 10^6/\text{mL}$ in Iscove's modified dulbecco's medium (Invitrogen) 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.

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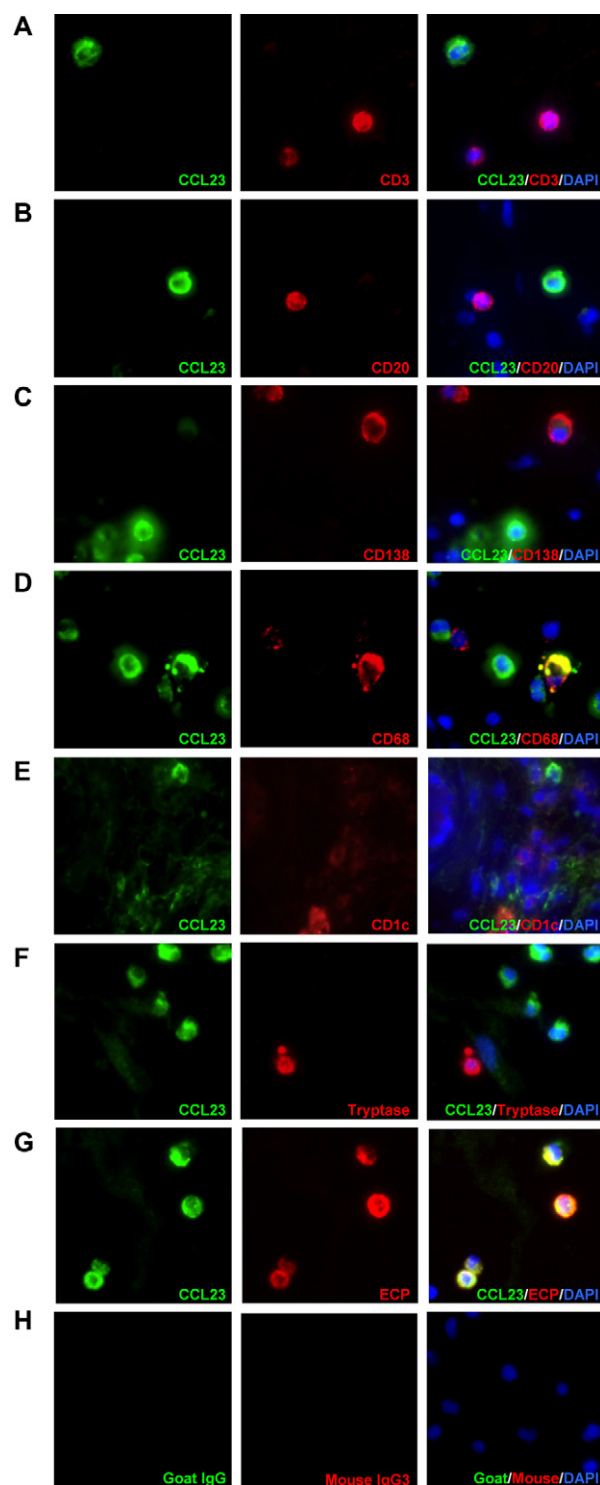


FIG E1. Immunofluorescence of CCL23 in NP tissue. Immunofluorescence assay was performed with anti-CCL23 (green fluorescence) and anti-CD3 mAb (red fluorescence) for T cells (**A**), anti-CD20 mAb (red fluorescence) for B cells (**B**), anti-CD138 mAb (red fluorescence) for plasma cells (**C**), anti-CD68 mAb (red fluorescence) for macrophages (**D**), anti-CD1c mAb (red fluorescence) for dendritic cells (**E**), anti-tryptase mAb (red fluorescence) for mast cells (**F**), anti-ECP mAb (red fluorescence) for eosinophils (**G**), and control IgG (**H**). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue fluorescence). The results are representative of 3 to 6 separate patients.

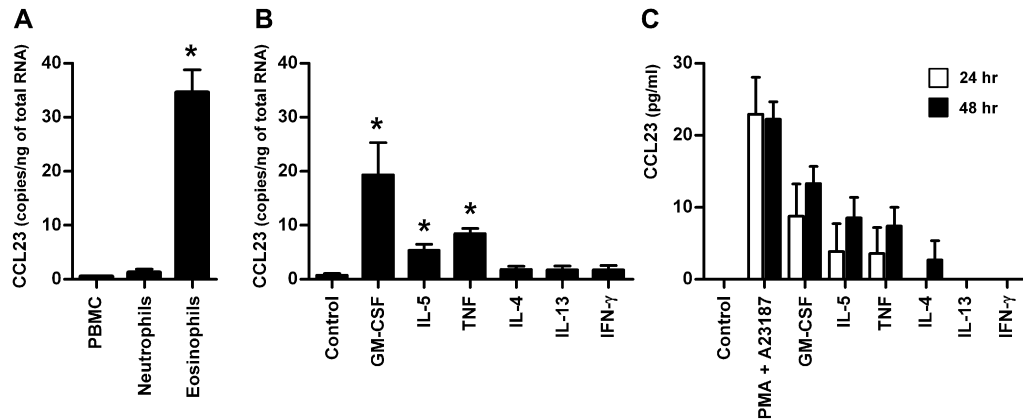


FIG E2. Detection of CCL23 in peripheral blood eosinophils. **A**, PBMCs, neutrophils, and eosinophils were isolated from the peripheral blood of control subjects, and expression of CCL23 in freshly isolated cells was analyzed by using real-time PCR. **B** and **C**, Purified eosinophils were incubated for 24 (**Fig E2, B** and **C**) or 48 (**Fig E2, C**) 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, and 10 ng/mL IFN- γ . The expression of mRNA for CCL23 was analyzed by means of real-time PCR (**Fig E2, B**), and protein expression of CCL23 in supernatants was analyzed by means of ELISA. Results shown are means $\pm$ SEMs of 3 to 4 separate subjects. * $P < .05$.