

Letter to the Editor

Tissue proteases convert CCL23 into potent monocyte chemoattractants in patients with chronic rhinosinusitis

To the Editor:

Although chronic rhinosinusitis with nasal polyps (CRSwNP) is known to be characterized by eosinophilia, other inflammatory cells, including macrophages and dendritic cells (DCs), are also widely accepted to have a pathogenic role in CRSwNP.¹⁻⁴ However, the mechanisms leading to accumulation of macrophages and DCs have yet to be identified. Recently, we found that the chemokine CCL23 is produced from eosinophils and upregulated in patients with eosinophilic CRSwNP and that mRNA expression of CCL23 positively correlates with expression of its receptor, CCR1, in nasal polyps (NPs).⁵ However, mature CCL23 (residues 22-120, referred to as CCL23) shows low affinity for CCR1, although it has chemotactic activity for monocytes and DCs at a high concentration.^{6,7} Therefore an increase in CCL23 protein levels alone cannot fully explain the positive correlation between CCL23 and CCR1 in NPs.

Recent publications suggest that CCL23 can be posttranslationally modified by several proteases and that truncated forms of CCL23 (residues 46-120 and 47-120, referred to as CCL23

[46-120] and CCL23 [47-120]) show 10 to 100 times higher CCR1 binding activity than CCL23.^{8,9} However, whether posttranslational modification of CCL23 can occur in NPs and whether this modification generates active forms of CCL23 has not been investigated.

To determine whether CCL23 protein can be posttranslationally modified in NPs, we incubated recombinant CCL23 with NP extracts and detected CCL23 protein by means of Western blotting. Details are provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org. We found that CCL23 was cleaved by NP extracts (Fig 1, A). Importantly, cleaved CCL23 was detected even after only 1 hour of exposure to NP extracts, and the molecular weight was similar to that of the known active form CCL23 (46-120; Fig 1, A and B). We also found that truncation of CCL23 by NP extracts was inhibited by a protease inhibitor cocktail (PIC), nafamostat, leupeptin, and tosyllysine chloromethyl ketone hydrochloride (TLCK) but not by various other inhibitors (Fig 1, C, and see Fig E1, A, in this article's Online Repository at www.jacionline.org), suggesting that truncation of CCL23 in NPs occurs in a trypsin-like serine protease-dependent manner.

We next examined the potential role of proteases released from inflammatory cells on the truncation of CCL23 in NPs. We found

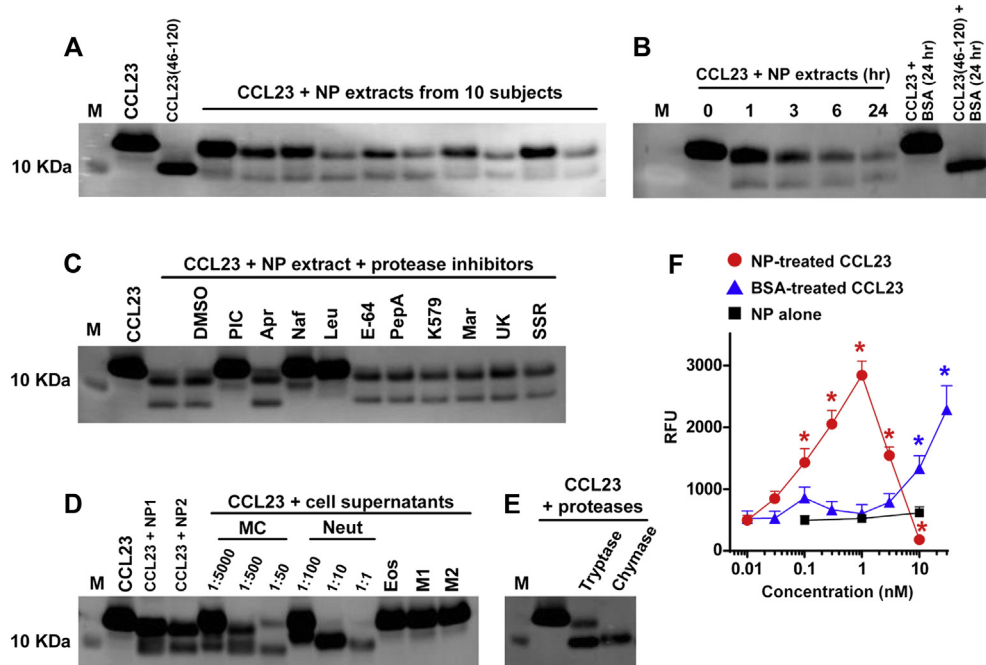


FIG 1. Posttranslational modification by NP trypsin-like serine proteases controls CCL23 activity. **A-E.** Recombinant CCL23 was incubated with 1 mg/mL NP extracts, 1 mg/mL BSA (control), supernatants from activated mast cells (MC), neutrophils (Neut), eosinophils (Eos), M1 macrophages (M1) and M2 macrophages (M2), trypsin β II, or skin chymase for 6 (Fig 1, A and C-E) or 0 to 24 (Fig 1, B) hours. CCL23 was incubated with NP extract in the presence of DMSO, PIC, aprotinin (Apr), nafamostat mesylate (Naf), leupeptin (Leu), E-64, pepstatin A (PepA), K579, marimastat (Mar), UK370106 (UK), SSR69071 (SSR), TLCK, or tosyl phenylalanyl chloromethyl ketone (TPCK) for 6 hours (Fig 1, C). CCL23, cleaved CCL23, and CCL23 (46-120) were detected by means of Western blotting with an anti-CCL23 antibody. Results are representative of 3 separate experiments with separate donors (Fig 1, B-D). **F.** THP-1 cells were incubated with NP-treated CCL23 (solid circles), the same concentration of NP extracts without CCL23 (solid squares), or BSA-treated CCL23 (solid triangles) for 2 hours by using a microchemotaxis plate ($n = 4$). RFU, Relative fluorescence units. * $P < .05$, 1-way ANOVA, compared with medium control (Fig 1, F).

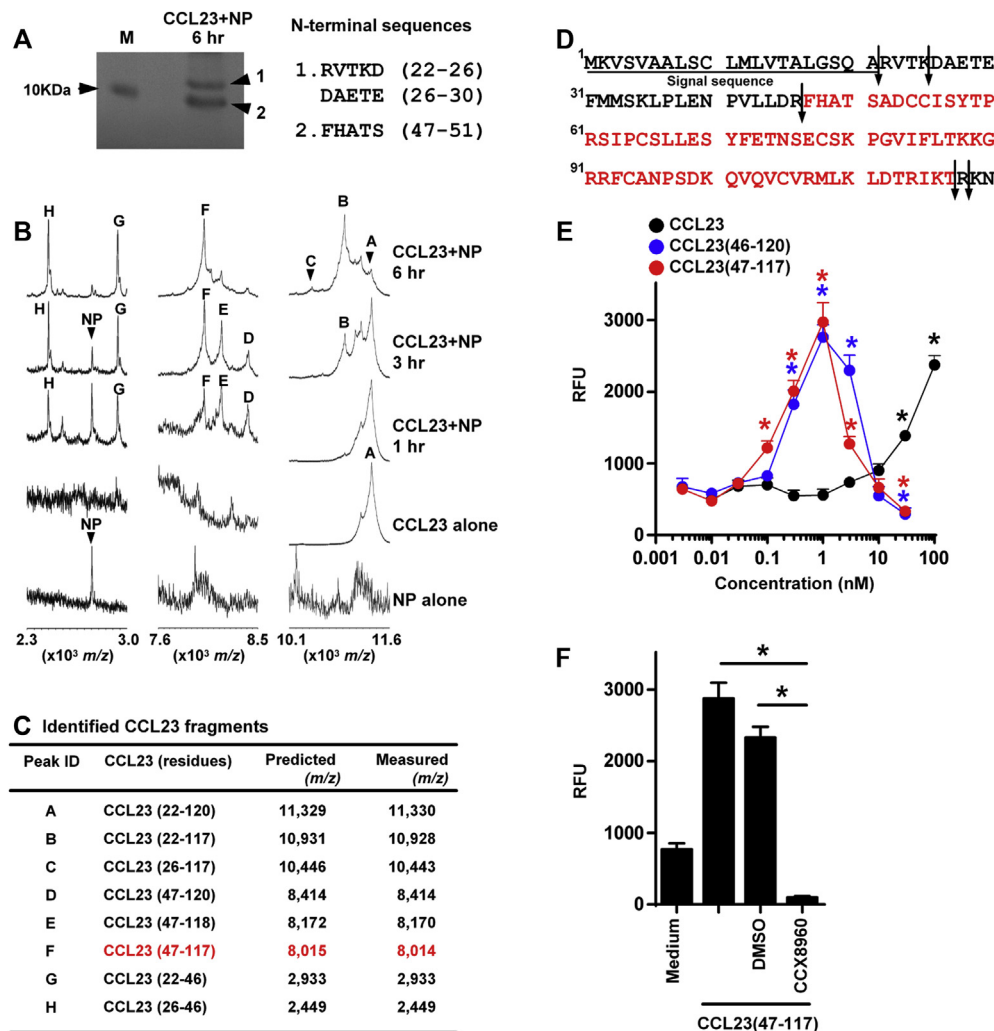


FIG 2. CCL23 (47-117) is an active CCL23 metabolite in NP extracts. NP extract-treated CCL23 proteins were separated by using SDS-PAGE. **A**, N-terminal protein sequences of each product were detected by using an Edman-based sequencer. **B**, CCL23 was incubated with NP extracts for 1 to 6 hours. Truncated products were detected by using MALDI-TOF mass spectrometry. **C**, Summary table of identified truncated products assigned by comparison of measured with predicted m/z values by using MALDI-TOF mass spectrometry and N-terminal sequencing. **D**, Protein sequence of CCL23. Arrows indicate identified cleavage sites. Red color indicates the sequence of the potential major active metabolite CCL23 (47-117) in NPs. Results are representative of 3 separate experiments with separate donors (Fig 2, A-C). **E**, THP-1 cells were incubated with CCL23, CCL23 (46-120), or CCL23 (47-117) for 2 hours by using a microchemotaxis plate (n = 4). **F**, THP-1 cells were incubated with 1 nmol/L CCL23 (47-117) for 2 hours by using a microchemotaxis plate in the presence of 0.02% DMSO (vehicle control) or 100 nmol/L CCX8960 (n = 3). RFU, Relative fluorescence units. *P < .05, 1-way ANOVA, compared with medium control (Fig 2, E).

that CCL23 was truncated by supernatants from mast cells and neutrophils but not by supernatants from eosinophils or macrophages (Fig 1, D). Because trypsin-like serine proteases have a major role in the truncation of CCL23 in NPs and inhibitors of neutrophil elastase (SSR69071) and matrix metalloproteinases (MMPs; marimastat), which are known to cleave CCL23,^{8,9} did not block the truncation (Fig 1, C), we focused on the mast cell trypsin-like serine protease tryptase. We found that CCL23 was truncated by tryptase and chymase (a positive control)⁸ and that tryptase-dependent truncation was inhibited by TLCK (Fig 1, E, and see Fig E1, B). This suggests that mast cell tryptase might be involved in the truncation of CCL23 in NPs.

To examine whether NP-treated CCL23 manifests chemotactic activity, we initially used PBMCs to determine the time course of

migration (see Fig E2 in this article's Online Repository at www.jacionline.org). We then used THP-1 cells, a human monocytic cell line that expresses CCR1, to develop a more sensitive assay. We found that NP-treated CCL23 displayed typical bell-shaped migration responses and that 1 nmol/L NP-treated CCL23 had the highest migration activity on THP-1 cells, although the same concentration of NP extract without CCL23 and BSA-treated CCL23 did not show a response (Fig 1, F). We also found that 1-hour NP-treated CCL23 had significantly higher chemotactic activity for THP-1 cells than mature CCL23 (see Fig E3, A, in this article's Online Repository at www.jacionline.org), indicating that migration activity was controlled by truncation of CCL23. Importantly, the CCR1 antagonist CCX8960 completely blocked NP-treated CCL23-dependent but not CCL2 (a CCR2

ligand)-dependent migration of THP-1 cells, indicating that truncation of CCL23 by NP extracts results in increased CCR1-dependent migration of THP-1 cells (see Fig E3, B).

Because previously known active truncated forms of CCL23 are formed by N-terminal truncations, we initially assessed N-terminal protein sequences. The N-terminal sequence of the truncated form was Phe-His-Ala-Thr-Ser, indicating that the N-terminal cleavage site of CCL23 by NP extracts was between residues 46 and 47 (Fig 2, A and D). We also used matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry to assess the truncation products. We found that 2 N-terminal truncation products with spectral peaks having mass to charge ratio (m/z) values of 2933 and 2449 were time dependently increased by NP extracts (Fig 2, B and C), suggesting that a 4-amino-acid truncation also occurred at the N-terminus. Because we could not detect migration activity of THP-1 cells induced by CCL23 (22-46) (data not shown), we focused on C-terminal end-truncation products. We detected 3 peaks (m/z 8414, 8170, and 8014) representing CCL23 (47-120), CCL23 (47-118), and CCL23 (47-117) and appearing after 1 hour of incubation with NP extracts, and CCL23 (47-117) was a major product at 6 hours of incubation (Fig 2, B and C). We also detected CCL23 (22-117) and CCL23 (26-117). Synthesis of all truncation products by NP extracts was blocked by the serine protease inhibitor nafamostat (Fig 2, B and C, and see Fig E4 in this article's Online Repository at www.jacionline.org). These results suggest that truncation of CCL23 in NPs can occur from both the N- and C-terminus by serine proteases and that the novel truncated form CCL23 (47-117) might be the major active product in NPs (Fig 2, D).

Although CCL23 (46-120) and CCL23 (47-120) have potent chemoattractant activity,^{8,9} there are no reports of the existence and activity of CCL23 (47-117). Therefore we synthesized recombinant CCL23 (47-117; see Fig E5 in this article's Online Repository at www.jacionline.org) and examined its activity by using THP-1 cells. We found that 1 nmol/L CCL23 (47-117) had the highest migration activity on THP-1 cells and that the activity of CCL23 (47-117) was significantly higher than that of the known active metabolite CCL23 (46-120) at 0.1 nmol/L and more than 100-fold higher than that of mature CCL23 (Fig 2, E).

CCL23 (47-117)-dependent migration was completely blocked by CCX8960 (Fig 2, F), suggesting a CCR1-dependent mechanism. The presence of CCR1 on macrophage and DC subsets had not yet been established in NPs. We found that CCR1 was expressed on both M1 and M2 macrophages (see Fig E6 in this article's Online Repository at www.jacionline.org). We also found that CCR1 was expressed on myeloid dendritic cells (mDCs), both type 1 (mDC1) and type 2 (mDC2), numbers of which are increased in NPs³ but not on plasmacytoid DCs or B cells (negative control cells) in NPs (see Fig E7 in this article's Online Repository at www.jacionline.org). This indicates that active CCL23 metabolites might be involved in the recruitment of macrophages and mDCs in NPs.

In conclusion, we report here that CCL23 is posttranslationally modified by trypsin-like serine proteases and that CCL23 (47-117) might be a major active form of CCL23 in NPs. Our findings indicate that overproduction and cleavage of CCL23 might play important roles in the inflammation seen in patients with CRSwNP through recruitment of macrophages and mDCs.

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REFERENCES

1. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. European Position Paper on Rhinosinusitis and Nasal Polyps 2012. *Rhinol Suppl* 2012;3 p preceding table of contents, 1-298.
2. Krysko O, Holtappels G, Zhang N, Kubica M, Deswarte K, Derycke L, et al. Alternatively activated macrophages and impaired phagocytosis of *S. aureus* in chronic rhinosinusitis. *Allergy* 2011;66:396-403.
3. Poposki JA, Peterson S, Welch K, Schleimer RP, Hulse KE, Peters AT, et al. Elevated presence of myeloid dendritic cells in nasal polyps of patients with chronic rhinosinusitis. *Clin Exp Allergy* 2015;45:384-93.
4. Kato A. Immunopathology of chronic rhinosinusitis. *Allergol Int* 2015;64:121-30.
5. Poposki JA, Uzzaman A, Nagarkar DR, Chustz RT, Peters AT, Suh LA, et al. Increased expression of the chemokine CCL23 in eosinophilic chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol* 2011;128:73-81.e4.
6. Patel VP, Kreider BL, Li Y, Li H, Leung K, Salcedo T, et al. Molecular and functional characterization of two novel human C-C chemokines as inhibitors of two distinct classes of myeloid progenitors. *J Exp Med* 1997;185:1163-72.
7. Berkhout TA, Gohil J, Gonzalez P, Nicols CL, Moores KE, Macphee CH, et al. Selective binding of the truncated form of the chemokine CXCL12 (25-99) to CC chemokine receptor 1 (CCR1). *Biochem Pharmacol* 2000;59:591-6.
8. Berahovich RD, Miao Z, Wang Y, Premack B, Howard MC, Schall TJ. Proteolytic activation of alternative CCR1 ligands in inflammation. *J Immunol* 2005;174:7341-51.
9. Starr AE, Dufour A, Maier J, Overall CM. Biochemical analysis of matrix metalloproteinase activation of chemokines CCL15 and CCL23 and increased glycosaminoglycan binding of CCL16. *J Biol Chem* 2012;287:5848-60.

METHODS

Patients and biopsy specimens

Patients with chronic rhinosinusitis (CRS) were recruited from the Otolaryngology Clinic and the Northwestern Sinus Center of Northwestern Medicine. 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.^{E1} Patients with an established immunodeficiency, pregnancy, coagulation disorder or diagnosis of classic allergic fungal sinusitis, Churg-Strauss syndrome, or cystic fibrosis were excluded from the study. Subjects were skin tested for pollens, dust mites, pets, molds, and cockroach by using Hollister-Stier (Spokane, Wash) extracts. Patients were taking a variety of medications, including nasal corticosteroids, antihistamines, decongestants, and short- or long-acting β -agonists among others. All subjects signed informed consent forms, and the protocol governing procedures for this study was approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine (IRB Project no. CR2_STU00072251).

Reagents and recombinant proteins

BSA, Tween-20, dimethyl sulfoxide (DMSO), PIC (PN; P8340), aprotinin, leupeptin, E-64, pepstatin A, TLCK, TPCK, phorbol 12-myristate 13-acetate, and A23487 were purchased from Sigma-Aldrich (St Louis, Mo); nafamostat mesylate, K579, marimastat, UK370106, SSR69071, recombinant IL-4, IFN- γ , macrophage colony-stimulating factor, GM-CSF, and CCL2 were purchased from R&D Systems (Minneapolis, Minn); and recombinant CCL23 (22-120) and CCL23 (46-120) were purchased from BioLegend (San Diego, Calif). Recombinant CCL23 (47-117) was synthesized at BioLegend. CCX8960 was obtained from ChemoCentryx (Mountain View, Calif).

Tissue homogenates

Freshly obtained tissue specimens were weighed, and 1 mL of PBS 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 . Before analysis, tissue homogenates were centrifuged at 16,000g for 15 minutes at 4°C, and we used those supernatants for each assay.

CCL23 cleavage assay and Western blot analysis

Recombinant CCL23 (22-120) was preincubated with 1 mg/mL tissue extracts or 1 mg/mL BSA (control) for 1 to 24 hours. In some experiments CCL23 was incubated with NP extract in the presence of 1% DMSO (vehicle control), 1% PIC, 1 $\mu\text{mol/L}$ aprotinin (serine protease inhibitor), 10 $\mu\text{mol/L}$ nafamostat mesylate (serine protease inhibitor), 10 $\mu\text{mol/L}$ leupeptin (serine/cysteine protease inhibitor), 10 $\mu\text{mol/L}$ E-64 (cysteine protease inhibitor), 10 $\mu\text{mol/L}$ pepstatin A (aspartyl protease inhibitor), 10 $\mu\text{mol/L}$ K579 (DPP-IV inhibitor), 20 $\mu\text{mol/L}$ marimastat (broad-spectrum MMP inhibitor), 10 $\mu\text{mol/L}$ UK370106 (MMP3/12 inhibitor), 1 $\mu\text{mol/L}$ SSR69071 (elastase inhibitor), 200 $\mu\text{mol/L}$ TLCK (trypsin-like protease inhibitor), or 200 $\mu\text{mol/L}$ TPCK (chymotrypsin-like protease inhibitor) for 6 hours. In some experiments CCL23 was incubated with 15 $\mu\text{g/mL}$ recombinant human trypsin βII (Enzo Life Sciences, Farmingdale, NY), 65 ng/mL purified human skin chymase (Enzo Life Sciences), and cell-free supernatants from activated mast cells, eosinophils, neutrophils, M1 macrophages, and M2 macrophages for 3 or 6 hours. Eosinophils and neutrophils were purified from human peripheral blood.^{E2} Mast cells were generated from human CD34⁺ hematopoietic progenitor cells, as described previously.^{E3} M1 macrophages were differentiated from monocytes by culturing in the presence of 10 ng/mL GM-CSF for 6 days and then in the presence of 100 ng/mL LPS and 20 ng/mL IFN- γ for 24 hours. M2 macrophages were differentiated from monocytes by culturing in the presence of 25 ng/mL macrophage

colony-stimulating factor for 6 days and then in the presence of 20 ng/mL IL-4 for 24 hours. Eosinophils, neutrophils, mast cells, and macrophages were stimulated with 1 $\mu\text{mol/L}$ phorbol 12-myristate 13-acetate and 1 $\mu\text{mol/L}$ A23487 for 6 hours, and we used cell-free supernatants in the assay.

Samples for Western blotting were dissolved in LDL sample buffer (Invitrogen, Carlsbad, Calif). To detect CCL23, reduced samples were resolved on a 12% NuPAGE Bis-Tris Gel (Invitrogen) in an MES buffer system, and then proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, Calif). The membranes were blocked in blocking buffer for fluorescent Western blotting (Rockland Immunochemicals, Gilbertsville, Pa) for more than 2 hours and subsequently incubated overnight at 4°C with 50 ng/mL biotinylated goat anti-human CCL23 polyclonal antibody (R&D Systems) or 1 $\mu\text{g/mL}$ rabbit anti-CCL23 polyclonal antibody (PeproTech, Rocky Hill, NJ). After primary antibody incubation, the membranes were washed repeatedly in 0.1% Tween 20/PBS and then labeled by means of 45 minutes of incubation at room temperature with IRDye 700DX-conjugated streptavidin (1:50,000, Rockland Immunochemicals) or IRDye 700-conjugated donkey anti-rabbit IgG antibody (1:10,000, Rockland Immunochemicals). Protein was detected on an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, Neb).

Migration assay

The migration assay was performed in a 96-well microplate format ChemoTx microchemotaxis system (Neuro Probe, Gaithersburg, Md). The THP-1 cells (ATCC, TIB-202) were resuspended in phenol red-free RPMI-1640 containing 0.1% BSA. Thirty microliters of chemokines was added to the lower wells, and 20 μL of THP-1 cells (4×10^6 cells/mL) was placed on the polycarbonate filter membranes (5- μm pore), after which the plates were incubated for 2 hours. After incubation, unigrated cells from the top side of the filter were rinsed away with water, and migrated cells were pelleted by means of centrifugation of the plate. Migrated cells were quantified by using CyQuant Reagent (Molecular Probes, Eugene, Ore) and analyzed with a SpectraMAX Gemini EM microplate reader (Molecular Devices, Sunnyvale, Calif; excitation at 485 nm and emission at 530 nm).

Protein sequencing and mass spectrometry

The cleaved products were separated by means of SDS-PAGE, and then proteins were transferred onto a polyvinylidene difluoride membrane. The N-terminal protein sequences of each product were determined by using an Edman-based ABI 494 Procise protein sequencer at the UC Davis Genome Center Proteomics Core Facility. Mass spectrometry was performed on a Shimadzu Axima CFR MALDI-TOF MS (Shimadzu, Kyoto, Japan) at the Texas A&M University, within the Protein Chemistry Laboratory.

Flow cytometric analysis

Tissue samples were obtained during surgery, and single-cell suspensions were prepared, as described previously.^{E4} Cells were first treated with Aqua LIVE/DEAD fixable dead cell staining reagent (Invitrogen) as a live/dead discriminator. Cells were then incubated with an Fc Block reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) for 5 minutes at room temperature. All antibodies were obtained from BioLegend, unless otherwise stated. The following antibodies and dilutions were used to stain cell surfaces: 0.6 $\mu\text{g/mL}$ Brilliant Violet 421 anti-CD45 (HI30), 2.5 $\mu\text{g/mL}$ phycoerythrin (PE)-Cy7 anti-CD19 (HIB19), 10 $\mu\text{g/mL}$ peridinin-chlorophyll-protein complex (PerCP)-Cy5.5 anti-CD163 (GHI/61), 2.5 $\mu\text{g/mL}$ PE anti-CD206 (15-2), 5 $\mu\text{g/mL}$ Alexa Fluor 488 anti-CD1c (L161), 2 $\mu\text{g/mL}$ allophycocyanin-Cy7 anti-CD11c (Bu15), PE anti-CD303 (AC144, 1:20; Miltenyi Biotec), 5 $\mu\text{g/mL}$ PerCP-Cy5.5 anti-CD141 (M80), and 10 $\mu\text{g/mL}$ Alexa Fluor 647 anti-CCR1 (TG4/CCR1). Cells were stained for 30 minutes at 4°C in the dark and washed with FACS buffer (BD Biosciences, San Jose, Calif). Cells were fixed with a BD Cytofix/Cytoperm Kit, resuspended in FACS buffer, and stored at 4°C in the dark before analysis on an LSRII (BD Biosciences). In the macrophage experiments cells were stained with

2.5 µg/mL fluorescein isothiocyanate anti-CD68 (Y1/82A) after fixing and permeabilization. All analysis and compensation were performed with FlowJo software, version 7.6.5 (TreeStar, Ashland, Ore), and each experiment contained the proper single-stained control beads (BD Biosciences) and fluorescence minus one negative controls.

Statistics

All data are reported as means $\pm$ SEMs. Differences between groups were analyzed by using the repeated-measures 1-way ANOVA Kruskal-Wallis Newman-Keuls multiple comparison test. All statistical analyses were performed with GraphPad prism 6.04 software (GraphPad Software, La Jolla, Calif). A *P* value of less than .05 was considered significant.

REFERENCES

- E1. Meltzer EO, Hamilos DL, Hadley JA, Lanza DC, Marple BF, Nicklas RA, et al. Rhinosinusitis: establishing definitions for clinical research and patient care. *J Allergy Clin Immunol* 2004;114:155-212.
- E2. Poposki JA, Uzzaman A, Nagarkar DR, Chustz RT, Peters AT, Suh LA, et al. Increased expression of the chemokine CCL23 in eosinophilic chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol* 2011;128:73-81.e4.
- E3. Nagarkar DR, Poposki JA, Tan BK, Comeau MR, Peters AT, Hulse KE, et al. Thymic stromal lymphopoietin activity is increased in nasal polyps of patients with chronic rhinosinusitis. *J Allergy Clin Immunol* 2013;132:593-600.e12.
- E4. Poposki JA, Peterson S, Welch K, Schleimer RP, Hulse KE, Peters AT, et al. Elevated presence of myeloid dendritic cells in nasal polyps of patients with chronic rhinosinusitis. *Clin Exp Allergy* 2015;45:384-93.

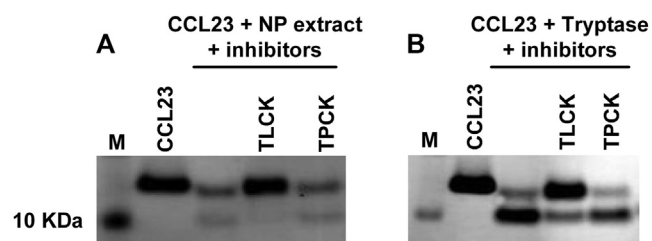


FIG E1. Trypsin-like serine protease inhibitor blocks truncation of CCL23 by NP extracts and trypsin. Recombinant CCL23 (2 μ g/mL) was preincubated with 1 mg/mL NP extracts (**A**) or 15 μ g/mL recombinant trypsin β II (**B**) in the presence or absence of 200 μ mol/L TLCK or 200 μ mol/L TPCK for 3 hours. CCL23 and cleaved CCL23 were detected by means of Western blotting with an anti-CCL23 antibody. Results are representative of 3 separate experiments.

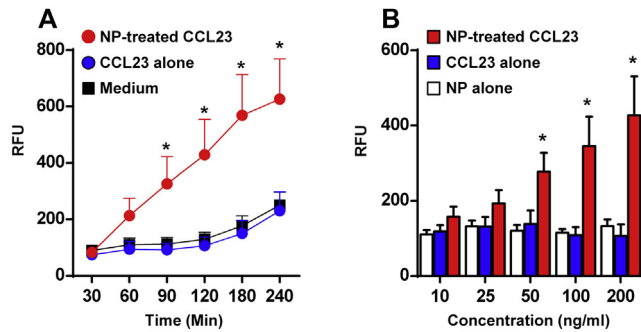


FIG E2. NP-treated CCL23 has potent chemoattractant activity for PBMCs. CCL23 (50 μ g/mL) was preincubated with 1 mg/mL NP extracts for 6 hours. The migration assay was performed in a 96-well microplate format FluoroBlok microchemotaxis system (3- μ m pore, BD Biosciences). PBMCs were labeled with Calcein AM dye and resuspended in phenol red-free RPMI-1640 containing 0.1% BSA. Labeled PBMCs were incubated with 200 ng/mL **(A)** or 10 to 200 ng/mL **(B)** CCL23 (blue symbols), NP-treated CCL23 (red symbols), the same concentration of NP extracts without CCL23 (open symbols), or medium alone (black symbols) for 0.5 to 4 (Fig E2, A) or 2 (Fig E2, B) hours by using a microchemotaxis plate ($n = 5$). Migrated cells were analyzed with a SpectraMAX Gemini EM microplate reader (excitation at 494 nm and emission at 517 nm). * $P < .05$, 1-way ANOVA, compared with medium control. RFU, Relative fluorescence units.

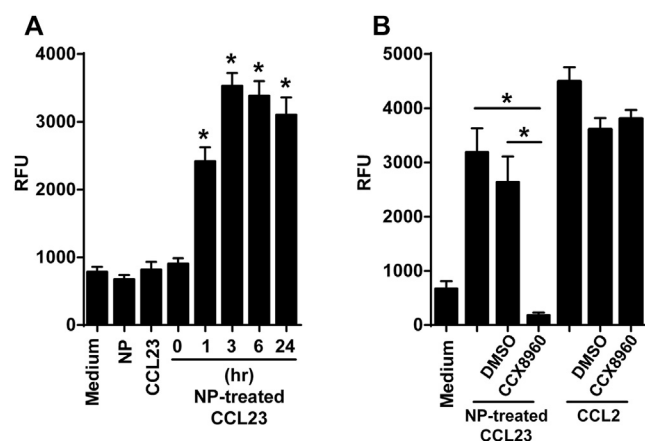


FIG E3. NP-pretreated CCL23 has potent chemoattractant activity for THP-1 cells through CCR1. **A**, Recombinant CCL23 (5 μ mol/L) was preincubated with 1 mg/mL NP extracts for 0 to 24 hours, and then THP-1 cells were incubated with 1 nmol/L NP-treated CCL23, the same concentration of NP extracts without CCL23, and 1 nmol/L untreated CCL23 for 2 hours by using a microchemotaxis plate ($n = 5$). **B**, THP-1 cells were incubated with 1 nmol/L NP-treated CCL23 and 1 nmol/L CCL2 for 2 hours by using a microchemotaxis plate in the presence of 0.02% DMSO (vehicle control) or 100 nmol/L CCX8960 (a CCR1-specific antagonist, $n = 3$). * $P < .05$, 1-way ANOVA, compared with medium control. RFU, Relative fluorescence units.

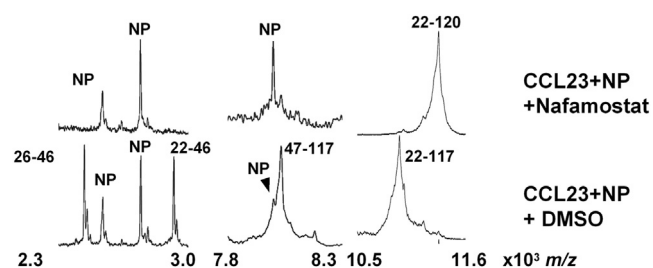


FIG E4. Serine protease inhibitor blocks truncation of CCL23 by NP extracts. CCL23 was incubated with NP extract in the presence of 1% DMSO (vehicle control) or 10 μ mol/L nafamostat for 6 hours. Truncated products were detected by using MALDI-TOF mass spectrometry. The *x-axis* of the mass spectra represents *m/z*. NP indicates proteins contained in the NP extracts. Numbered peaks refer to corresponding amino acid residues of CCL23.

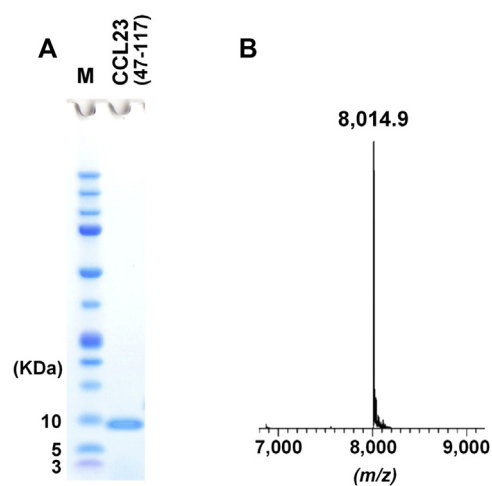


FIG E5. Synthesis of recombinant CCL23 (47-117). Recombinant CCL23 (47-117) was synthesized at BioLegend. Purity of recombinant CCL23 (47-117) was greater than 98% analyzed by using SDS-PAGE (**A**) and HPLC (not shown). **B**, Molecular mass was detected as 8014.9 by using ESI-TOF mass spectrometry.

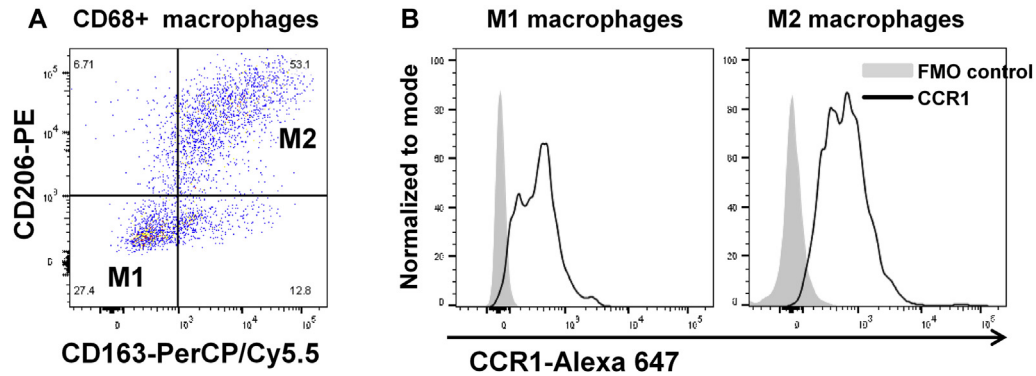


FIG E6. CCR1 is expressed on macrophages in NPs. We identified the population of macrophage subsets in NPs with the following steps. We first selected singlets (by using forward scatter [FSC]-A/FSC-W and side scatter [SSC]-A/SSC-W), excluded dead cells (Aqua+), selected the CD45⁺ population, excluded granulocytes (SSC^{high}), and then selected CD68⁺ macrophages. **A**, Representative flow cytometric plots for M1 macrophages (CD163⁻CD206⁻) and M2 macrophages (CD163⁺CD206⁺, n = 3). **B**, Expression of CCR1 was determined on M1 and M2 macrophages. *FMO*, Fluorescence minus one.

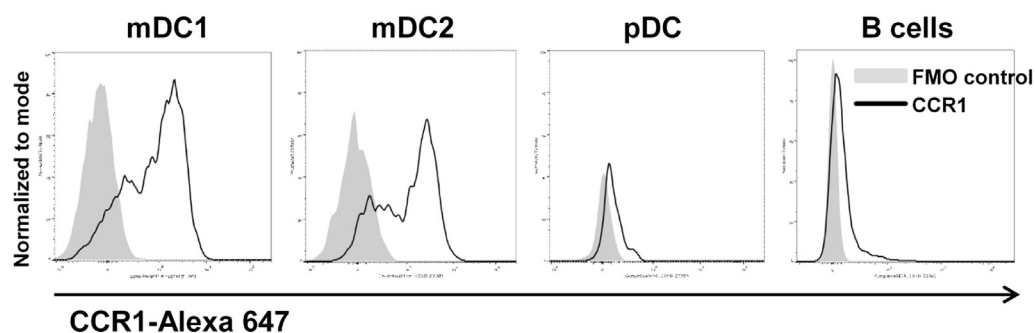


FIG E7. CCR1 is expressed on mDCs in NPs. We identified the population of DC subsets in NPs with the following steps. We first selected singlets (by using forward scatter [FSC]-A/FSC-W and side scatter [SSC]-A/SSC-W), excluded dead cells (Aqua+), selected the CD45⁺ population, and then removed granulocytes (SSC^{high}) and B cells (CD19⁺). We then identified DC subsets by the following markers: mDC1 (CD1c⁺CD11c⁺), mDC2 (CD141^{high}CD11c⁺), and plasmacytoid dendritic cells (pDC; CD303⁺). CCR1 expression was determined on mDC1s, mDC2s, pDCs, and CD19⁺ B cells (negative control). FMO, Fluorescence minus one.