

Neutrophils are a major source of the epithelial barrier disrupting cytokine oncostatin M in patients with mucosal airways disease

Kathryn L. Pothoven, PhD,^a James E. Norton, MSc,^a Lydia A. Suh, BSc,^a Roderick G. Carter, BSc,^a Kathleen E. Harris, BSc,^a Assel Biyasheva, PhD,^a Kevin Welch, MD,^b Stephanie Shintani-Smith, MD,^b David B. Conley, MD,^b Mark C. Liu, MD,^c Atsushi Kato, PhD,^a Pedro C. Avila, MD,^a Qutayba Hamid, MD, PhD,^d Leslie C. Grammer III, MD,^a Anju T. Peters, MD,^a Robert C. Kern, MD,^a Bruce K. Tan, MD,^b and Robert P. Schleimer, PhD^{a,b} *Chicago, Ill; Baltimore, Md; and Montreal, Quebec, Canada*

Background: We have previously shown that oncostatin M (OSM) levels are increased in nasal polyps (NPs) of patients with chronic rhinosinusitis (CRS), as well as in bronchoalveolar lavage fluid, after segmental allergen challenge in allergic asthmatic patients. We also showed *in vitro* that physiologic levels of OSM impair barrier function in differentiated airway epithelium.

Objective: We sought to determine which hematopoietic or resident cell type or types were the source of the OSM expressed in patients with mucosal airways disease.

Methods: Paraffin-embedded NP sections were stained with fluorescence-labeled specific antibodies against OSM, GM-CSF, and hematopoietic cell-specific markers. Live cells were isolated from NPs and matched blood samples for flow cytometric analysis. Neutrophils were isolated from whole blood and

cultured with the known OSM inducers GM-CSF and follistatin-like 1, and OSM levels were measured in the supernatants. Bronchial biopsy sections from control subjects, patients with moderate asthma, and patients with severe asthma were stained for OSM and neutrophil elastase.

Results: OSM staining was observed in NPs, showed colocalization with neutrophil elastase ($n = 10$), and did not colocalize with markers for eosinophils, macrophages, T cells, or B cells ($n = 3-5$). Flow cytometric analysis of NPs ($n = 9$) showed that $5.1\% \pm 2\%$ of $CD45^+$ cells were OSM^+ , and of the OSM^+ cells, $56\% \pm 7\%$ were $CD16^+Siglec-8^-$, indicating neutrophil lineage. Only $0.6 \pm 0.4\%$ of $CD45^+$ events from matched blood samples ($n = 5$) were OSM^+ , suggesting that increased OSM levels in patients with CRS was locally stimulated and produced. A majority of OSM^+ neutrophils expressed arginase 1 ($72.5\% \pm 12\%$), suggesting an N2 phenotype. GM-CSF levels were increased in NPs compared with those in control tissue and were sufficient to induce OSM production ($P < .001$) in peripheral blood neutrophils *in vitro*. OSM^+ neutrophils were also observed at increased levels in biopsy specimens from patients with severe asthma. Additionally, OSM protein levels were increased in induced sputum from asthmatic patients compared with that from control subjects ($P < .05$).

Conclusions: Neutrophils are a major source of OSM-producing cells in patients with CRS and severe asthma. (*J Allergy Clin Immunol* 2017;■■■■:■■■-■■■.)

Key words: Oncostatin M, epithelial barrier, neutrophils, chronic rhinosinusitis, atopic asthma, granulocyte-monocyte colony-stimulating factor

From ^athe Division of Allergy-Immunology, Department of Medicine and ^bthe Department of Otolaryngology, Northwestern University Feinberg School of Medicine, Chicago; ^cthe Divisions of Allergy and Clinical Immunology, Pulmonary and Critical Care Medicine, Johns Hopkins Asthma and Allergy Center, Baltimore; and ^dMeakins-Christie Laboratories of McGill University and McGill University Health Center Research Institute, Montreal.

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Corresponding author: Robert P. Schleimer, PhD, Northwestern University Feinberg School of Medicine, MED/Allergy-Immunology, McGaw Rm M-318, 240 E Huron St, Chicago, IL 60611. E-mail: rpschleimer@northwestern.edu. 0091-6749/\$36.00

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Epithelial barrier dysfunction has been shown to be important in the pathogenesis of many diseases, including asthma, atopic dermatitis, chronic rhinosinusitis (CRS), and eosinophilic esophagitis.¹⁻⁷ Our laboratory has previously shown that levels of the cytokine oncostatin M (OSM) were increased in nasal polyps (NPs) of patients with CRS, in esophageal biopsy specimens from patients with eosinophilic esophagitis, and in bronchoalveolar lavage (BAL) fluid after allergen challenge in allergic asthmatic patients.⁸ Physiologic levels of OSM were sufficient to induce barrier dysfunction in *ex vivo*-cultured airway epithelium, as measured based on decreased epithelial resistance, increased epithelial permeability, and loss of tight junction structure. Additionally, OSM levels in both nasal tissue from patients with CRS and BAL fluid from allergen-challenged patients with allergic

Abbreviations used

ALI:	Air-liquid interface
ARG1:	Arginase 1
BAL:	Bronchoalveolar lavage
CRS:	Chronic rhinosinusitis
CRSSNP:	Chronic rhinosinusitis without nasal polyps
CRSwNP:	Chronic rhinosinusitis with nasal polyps
EMR1:	EGF-like module-containing mucin-like hormone receptor-like-1
ECP:	Eosinophil cationic protein
FMO:	Fluorescence minus one
FSTL1:	Follistatin-like 1
HKSA:	Heat-killed <i>Staphylococcus aureus</i>
ICAM:	Intercellular adhesion molecule
IL-5R:	IL-5 receptor
MPO:	Myeloperoxidase
MRC1:	Mannose receptor C-type 1
NHBE:	Normal human bronchial epithelial
OSM:	Oncostatin M
RT:	Room temperature
UT:	Uncinate tissue

asthma correlated with markers of epithelial leak, suggesting that OSM might mediate barrier dysfunction *in vivo* in patients with mucosal disease.⁸ We hypothesized that OSM-induced loss of barrier function could allow the entry of various allergens, pathogens, and environmental factors into the tissue, where they could elicit a chronic immune response. It is important now to identify the cell type responsible for OSM production in patients with mucosal disease to understand the mechanism of barrier disruption in the setting of eosinophilic mucosal disease.

Neutrophil-derived OSM has been shown to contribute to the pathogenesis of many conditions, including acute lung injury, asthma, breast cancer, and rheumatoid arthritis.⁹⁻¹² Although osteopontin, prostaglandin E₂, follistatin-like 1 (FSTL1), complement factor 5a, and thrombin have all been shown to induce OSM expression in various cell types,¹³⁻¹⁷ only GM-CSF was sufficient to induce OSM in neutrophils.^{11,18} Elbjerrami et al¹⁸ showed that OSM was induced during neutrophil transendothelial migration in response to endothelial production of GM-CSF. Queen et al¹¹ showed that coculture of neutrophils with the breast cancer cell lines MDA-MB-231 and T-47D induced OSM production that was lost when GM-CSF was blocked. Additionally, Miller et al¹⁶ have shown that FSTL1 was necessary for OSM induction in a mouse model of chronic asthma. Levels of both GM-CSF and FSTL1 have been shown to be increased in patients with airways disease,¹⁹⁻²² and we hypothesized that one or both of these cytokines can induce neutrophil-derived OSM in patients with CRS.

Recent studies have described subtypes of polarized neutrophils: the classical proinflammatory N1 neutrophil and the anti-inflammatory or tumorigenic N2 neutrophil.²³ These N1 and N2 neutrophils have been shown to be very similar in function to their M1 and M2 macrophage counterparts.²³ N2 neutrophils have been shown specifically to promote tumor growth and metastasis, as well as to play an important role in repair processes and the resolution of inflammation.²⁴⁻²⁶ N1 and N2 neutrophil polarization has been best studied in mice, and N2 neutrophils have been shown to express the mannose receptor C-type 1 (MRC1), arginase 1 (ARG1), chitinase-like 3 (Ym1), IL-10, and TGF- β , which

are also characteristic markers of M2 macrophages. Both M2 macrophages and N2 neutrophils have been associated with tissue repair mechanisms.²⁵ Compared with N2 neutrophils, N1 neutrophils have been shown to express more proinflammatory cytokines and chemokines, such as TNF, IL-1 β , CCL3, CCL5, IL-6, and IL-12.²⁵ Although progress is being made, N2 neutrophils in human subjects are much less extensively studied, and the markers used to define N2 neutrophils in mice might not all be relevant as markers for human N2 neutrophils.²⁷ Because N2 neutrophils and OSM have been shown to be important for repair processes, we wanted to test the hypothesis that N2 neutrophils were responsible for OSM production in patients with CRS. In this article we show that neutrophils are a major source of OSM-producing cells in NPs and that neutrophils also express OSM in patients with severe asthma. We also show that levels of the OSM inducer GM-CSF were increased in NPs at levels sufficient to induce OSM production in cultured neutrophils.

METHODS**Patients and tissue sample collection**

Control subjects and patients with CRS were recruited from the Allergy-Immunology and Otolaryngology Clinics of Northwestern Medicine and Northwestern Sinus Center at Northwestern Medicine. NPs and peripheral blood were obtained during routine functional endoscopic sinus surgery from patients with CRS, as defined by the American Academy of Otolaryngology—Head and Neck Surgery Chronic Rhinosinusitis Task Force.²⁸ Patients with established immunodeficiency, pregnancy, coagulation disorder, or cystic fibrosis were excluded from this study. Characteristics of subjects in the study are shown in Table E1 in this article's Online Repository at www.jacionline.org. Control subjects did not have a history of sinonasal inflammation, and tissue was collected during endoscopic skull-base tumor excisions and benign skull-base procedures, including cerebrospinal fluid leak, encephalocele, and petrous apex cholesterol granuloma. The tumors excised included pituitary adenoma (n = 5), chordoma (n = 4), meningioma (n = 2), esthesioneuroblastoma (n = 1), clival metastatic carcinoma (n = 1), inverted Schneiderian papilloma (n = 1), and osteoma (n = 1). Asthma and atopic status were determined by the surgeon treating the patient. Segmental allergen challenge and BAL were performed in a patient cohort of allergic asthmatic patients, as previously described.^{29,30} Bronchial biopsy specimens were obtained from asthmatic patients during bronchoscopy at the Meakins-Christie Laboratories at McGill University Montreal (Montreal, Quebec, Canada), with the approval of the respective institutional review boards, which has been previously described.³¹ Induced sputum was obtained in the Allergy-Immunology Clinic of Northwestern Medicine, as previously described.^{32,33}

Immunofluorescent staining

NP specimens were processed for histology, as previously described.³⁴ Sections were blocked with PBS containing 5% goat serum (Vector Laboratories, Burlingame, Calif) and 0.5% Triton X-100 (Sigma-Aldrich, St Louis, Mo) for 1 hour at room temperature (RT) and then incubated with either rabbit anti-OSM (N-1, 1:400; Santa Cruz Biotechnology, Dallas, Tex), mouse anti-neutrophil elastase (NP57, 1:100; Dako North America, Carpinteria, Calif), mouse anti-CD68 (PG-M1, 1:50; Thermo Fisher, Waltham, Mass), mouse anti-eosinophil cationic protein (ECP; EG2, 1:1000; Diagnostics Development, Uppsala, Sweden), mouse anti-tryptase (AA1, 1:10000; Thermo Fisher), mouse anti-CD3 (SP7, 1:1; Thermo Fisher), mouse anti-CD20 (L26, 1:250; Thermo Fisher), or rat anti-GM-CSF (BVD2-21C11, 1:100; Abcam, Cambridge, Mass) for 1 hour at RT. The sections were then incubated with secondary antibody for 1 hour at RT with Alexa Fluor 488 goat anti-rabbit antibody, Alexa Fluor goat anti-mouse 568, Alexa Fluor goat anti-rat 568, Alexa Fluor goat anti-rabbit 647, or Alexa Fluor goat anti-mouse 647 (Thermo Fisher). Slides were mounted with SlowFade Gold Antifade reagent with 4'-6-diamidino-2-phenylindole dihydrochloride counterstain (Thermo

Fisher). Additional slides were stained with the secondary antibody only to control for background staining. Imaging was performed with a Nikon A1R confocal microscope (Nikon, Tokyo, Japan) by using the $\times 20$ objective. Images were processed with ImageJ software (National Institutes of Health, Bethesda, Md).

Flow cytometric analysis

Live cells were isolated from NP tissues obtained during endoscopic sinus surgery. NPs were incubated overnight on an oscillator in 1 mg/mL collagenase I (Worthington Biochemical, Lakewood, NJ) and 30 μ g/mL DNase (Worthington Biochemical) in RPMI at 4°C in a gentleMACS tube. Cells were then dissociated with a gentleMACS cell dissociator (Miltenyi Biotec, San Diego, Calif) and then passed through a 70- μ m cell strainer (BD Biosciences, San Jose, Calif). Live blood cells were isolated from the buffy coat of centrifuged peripheral blood to ensure that the granulocyte population would be present for analysis. For flow cytometry, cells were first stained with the LIVE/DEAD Aqua Dead Cell Staining Kit (Thermo Fisher) to facilitate gating only on live cells. Cells were blocked with 10 μ L of Fc blocking reagent for 15 minutes (Miltenyi Biotec, San Diego, Calif), and then cells were stained at RT for 15 minutes with one of 3 panels of antibodies listed in Table E2 in this article's Online Repository at www.jacionline.org. After cell-surface staining, cells were then fixed and permeabilized with the BD Cytofix/Cytoperm Kit for intracellular staining, according to the manufacturer's protocols (BD Biosciences), and cells were stained intracellularly for 30 minutes at 4°C. Cells were analyzed on an LSRII flow cytometer with FACSDiva software. All analysis and compensation were done with FlowJo software (TreeStar, Ashland, Ore). All flow cytometric studies were prepared by using the appropriate single-stained control beads (BD Biosciences and eBioscience, San Diego, Calif) and fluorescence minus one (FMO) controls.

Neutrophil isolation and cell culture

Neutrophils were isolated from peripheral blood by using an EasySep Direct Human Neutrophil Isolation Kit (STEMCELL Technologies, Vancouver, British Columbia, Canada). Cells were then counted and cultured at 10^7 cells/mL in RPMI supplemented with 5% FBS and 1% penicillin/streptomycin. Neutrophil cultures were treated with GM-CSF (1–25 ng/mL; R&D Systems, Minneapolis, Minn), FSTL1 (100–1000 ng/mL; R&D Systems), IFN- γ (10 ng/mL; R&D Systems), LPS (1 μ g/mL; Enzo Life Sciences, Farmingdale, NY), IL-4 (20 ng/mL; R&D Systems), IL-13 (20 ng/mL; R&D Systems), IL-25 (10–100 ng/mL; R&D Systems), IL-33 (10–100 ng/mL; R&D Systems), thymic stromal lymphopoietin (10–100 ng/mL; R&D Systems), or leukotriene C₄ (10^{-6} – 10^{-7} M; Cayman Chemical, Ann Arbor, Mich) for 20 hours; cell-culture supernatants were collected for protein analysis; and cell lysates were collected to isolate RNA. Plates were coated with E-selectin, intercellular adhesion molecule (ICAM; 50 ng/well; PeproTech, Rocky Hill, NJ), or BSA (50 ng/well; Sigma-Aldrich) in Tris-buffered saline (pH 8) overnight at 4°C. Neutrophils were seeded into the wells, and culture supernatants were harvested at 20 hours.

Normal human bronchial epithelial cell culture and injury

Normal human bronchial epithelial (NHBE) cells were cultured at the air-liquid interface (ALI), as previously described.⁸ As a model of injury, fully differentiated NHBE cells were treated apically with 0.5 mL of either PBS or PBS containing 5×10^8 particles/mL of heat-killed *Staphylococcus aureus* (HKSA). After culture for 48 hours, cell supernatants were collected for protein analysis.

Protein quantification

OSM protein was detected in neutrophil cell-culture supernatants by using a DuoSet ELISA Kit (R&D Systems). FSTL1 and GM-CSF proteins were detected in neutrophil cell-culture supernatants, and FSTL1 was detected in sinus tissue extracts by using DuoSet ELISA Kits (R&D Systems). GM-CSF

protein was detected in sinus tissue lysates by using a Human Magnetic Protein Luminex Assay (R&D Systems). OSM protein was detected in sinus tissue lysates, as previously described.⁸ Neutrophil elastase protein (Hycult Biotech, Plymouth Meeting, Pa) and eosinophilic cationic protein (MBL International, Woburn, Mass) were detected with an ELISA, and sinus tissue extracts were prepared, as previously described.³⁵

Quantitative RT-PCR and microarray analysis

RNA isolation from tissue samples was prepared, as previously described.³⁴ Single-strand cDNA was synthesized from 0.5 μ g of total RNA with SuperScript II reverse transcriptase and random primers (Invitrogen, Carlsbad, Calif). Quantitative real-time RT-PCR was performed with an Applied Biosystems 7500 Sequence Detection System (Applied Biosystems, Foster City, Calif) in 20- μ L reactions (10 μ L of 2 $\times$ TaqMan Master Mix [Applied Biosystems], 400 nmol/L of each primer, and 200 nmol/L of TaqMan probe plus 10 μ g of cDNA). Primer and probe sets for *OSM* (Hs00968200_g1), *ARG1* (Hs00968300_g1), and *MRC1* (Hs00267207_m1) were purchased from Applied Biosystems. Primers (forward: GAAGGTGAAGGTCCGAGTC, reverse: GAAGATGGTGATGGGATTTC) and probe (6-VIC-CAAGCTTCCCGTTCTCAGCC-MGB) for glyceraldehyde-3-phosphate dehydrogenase (*GUSB*) were synthesized by IDT Technologies (Coralville, Iowa) and used to detect the housekeeping gene in all RT-PCR studies. Microarray analysis was performed, as previously described.^{36,37} All microarray data have been deposited to the Gene Expression Omnibus (GSE36830).

Statistical analysis

All data were analyzed with GraphPad Prism 6 software (GraphPad Software, La Jolla, Calif). All data are reported as means $\pm$ SEMs. Differences between groups were analyzed by using a nonparametric Kruskal-Wallis ANOVA or Mann-Whitney *U* test. Correlations were assessed by using Spearman correlation.

RESULTS

Neutrophils are a major source of OSM in NPs

To determine which cell type was making OSM in patients with CRS, we first obtained sections from NPs and used immunofluorescence and specific antibodies to stain for OSM and markers of various cell types, including CD3, CD20, tryptase, elastase, ECP, and CD68. We did not observe colocalization of OSM with markers for macrophages (Fig 1, A and B) or eosinophils (Fig 1, C and D), whereas we observed some colocalization with a minor population of mast cells (Fig 1, E and F). We also did not observe colocalization of OSM with T or B cells (data not shown). Elastase-positive cells made up the majority of OSM⁺ cells, suggesting that neutrophils are an important source of OSM (Fig 1, G and H). Control images with no primary antibody for CD68, ECP, tryptase, and elastase staining strategies are shown in Fig E1, A to D, in this article's Online Repository at www.jacionline.org. Further analysis of our previously published microarray database (Gene Expression Omnibus: GSE36830) showed that *OSM* mRNA expression correlated with expression of the granulocyte marker *CD16* (*FCGR3*; $r = .61$, $P < .01$; see Fig E1, E) and the neutrophil-specific protease cathepsin G ($r = .56$, $P < .01$; see Fig E1, F) in NPs and uncinate tissue (UT) from control subjects, patients with chronic rhinosinusitis without nasal polyps (CRSsNP), and patients with chronic rhinosinusitis with nasal polyps (CRSwNP), suggesting a potential relationship between *OSM* expression and neutrophils.³⁶ Although *FCGR3* is not neutrophil specific, *FCGR3* and cathepsin G correlated with each other in the database ($r = .56$, $P < .01$; see Fig E1, G),

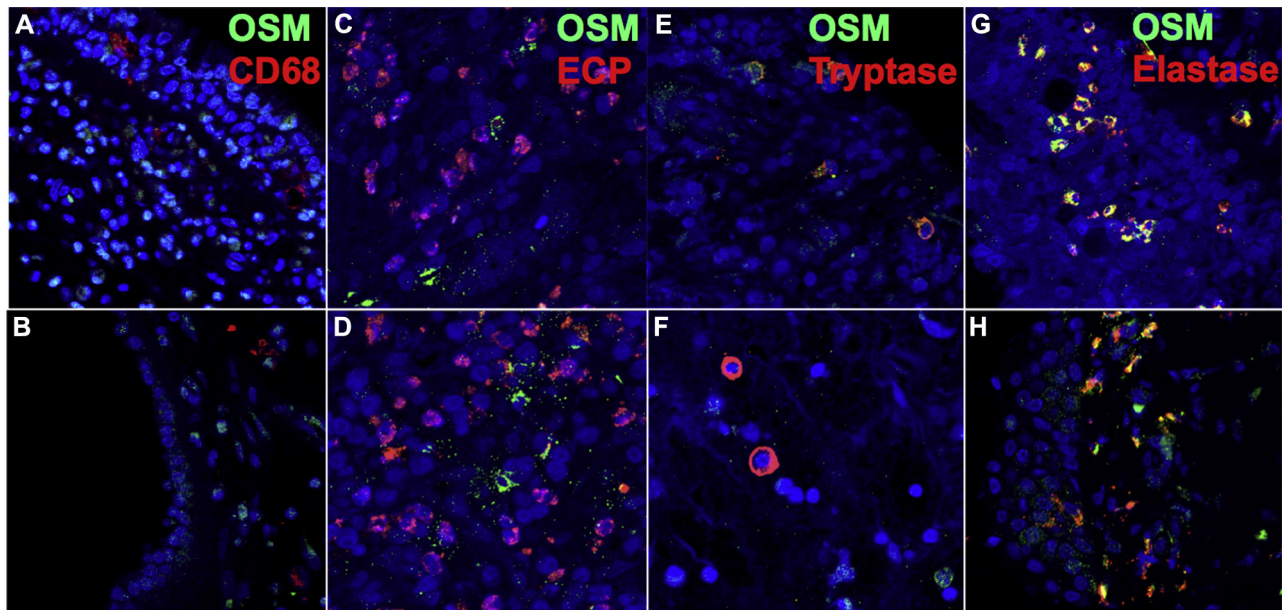


FIG 1. OSM was expressed in neutrophils. NP sections were stained for OSM in green, and various cell type-specific markers are indicated in red; 2 representative examples from separate patients are shown for each staining strategy. **A-F**, OSM did not colocalize with macrophages (Fig 1, **A** and **B**; $n = 3$) or eosinophils (Fig 1, **C** and **D**; $n = 5$) and had minimal colocalization with mast cells (Fig 1, **E** and **F**; $n = 6$). **G** and **H** OSM colocalized with neutrophils ($n = 10$).

suggesting that neutrophils are responsible for much of the *FCGR3* expression in sinonasal tissue. We also observed a positive correlation between elastase and OSM levels by using lysates from both NPs and UT from control subjects, patients with CRSsNP, and patients with CRSwNP ($r = .65$, $P < .01$; see Fig E1, **H**).

Flow cytometric analysis of OSM-producing cells in NPs

To further validate the observation that neutrophils are a major source of OSM producers in NPs, we used flow cytometry. Live cells were isolated from NPs and stained with CD45, OSM, CD16, and Siglec-8. Within the CD45 gate, $5.1\% \pm 2\%$ of the cells were OSM⁺, and OSM⁺ gate was determined by using the appropriate FMO control. Of the OSM⁺ cells, $29.8\% \pm 8\%$ were excluded during analysis of the data because the wavelengths of the fluorophores used to stain for CD16–allophycocyanin-H7 and Siglec-8–Alexa Fluor 647 were near each other, and the rigorous compensation algorithm that we used excluded them to ensure the accuracy of the data. The majority of the cells that were excluded were single positive for either CD16 or Siglec-8, suggesting they were granulocytes. Within the OSM⁺ gate, we observed 2 main populations; the major population was CD16⁺Siglec-8[−] ($80.4\% \pm 5\%$), which were neutrophils, and the minor population was CD16⁺Siglec-8⁺ ($14.1\% \pm 5\%$), which were either mast cells, basophils, or eosinophils. We observed a small CD16[−]Siglec-8[−] population ($5.5\% \pm 3\%$) that was present in less than half of the polyps we analyzed (Fig 2, **A-C**). Additionally, neutrophils were isolated from peripheral blood and NP tissue of 5 matched patients with CRSwNP, and OSM expression in blood neutrophils was only minimally observed in 1 patient ($0.6 \pm 0.4\%$), indicating that *in vivo* OSM induction was likely a locally induced event (Fig 2, **D**). The full gating strategy for this antibody panel, including the OSM

FMO control, is available in Fig E2 in this article's Online Repository at www.jacionline.org.

OSM was expressed in a minor population of mast cells

To further identify the cells within the minor CD16⁺Siglec-8⁺ population of OSM producers, we stained live cells isolated from NPs with CD45, OSM, CD16, FcεRI, c-kit, and either EGF-like module-containing mucin-like hormone receptor-like-1 (EMR1) or Siglec-8. In the CD45⁺ gate $4.6\% \pm 2\%$ events were OSM⁺. Within the OSM⁺ events, $15.6\% \pm 10\%$ were FcεRI⁺c-kit⁺, indicating mast cells; $3.7\% \pm 1\%$ were FcεRI⁺c-kit[−], indicating basophils; and of the FcεRI[−]c-kit[−] cells, only $2.3\% \pm 1\%$ were EMR1⁺ or Siglec-8⁺, which would indicate eosinophils, leaving the remaining $73.3\% \pm 11\%$ of the cells neutrophils (Fig 3). The full gating scheme for this antibody panel is available in Fig E3 in this article's Online Repository at www.jacionline.org, and the sample chosen as the representative example was selected to best show the gating strategy for basophils and mast cells.

GM-CSF levels were increased in NPs and induces neutrophil-derived OSM production *in vitro*

Because GM-CSF and FSTL1 have been shown to be important inducers of OSM, our next aim was to determine whether GM-CSF, FSTL1, or both might play a role in *in vivo* induction of neutrophil-derived OSM. We first wanted to determine whether GM-CSF or FSTL1 were sufficient to induce OSM in neutrophils. We treated neutrophils isolated from whole blood *in vitro* for 20 hours with GM-CSF, FSTL1, or both.^{11,16,18} GM-CSF alone induced OSM protein release into cell-culture supernatants at 1, 5, and 25 ng/mL, whereas FSTL1 alone at 100 or 1000 ng/mL did not induce OSM in purified neutrophils; the combination of 1 or 25 ng/mL GM-CSF with 1000 ng/mL FSTL1 was not more

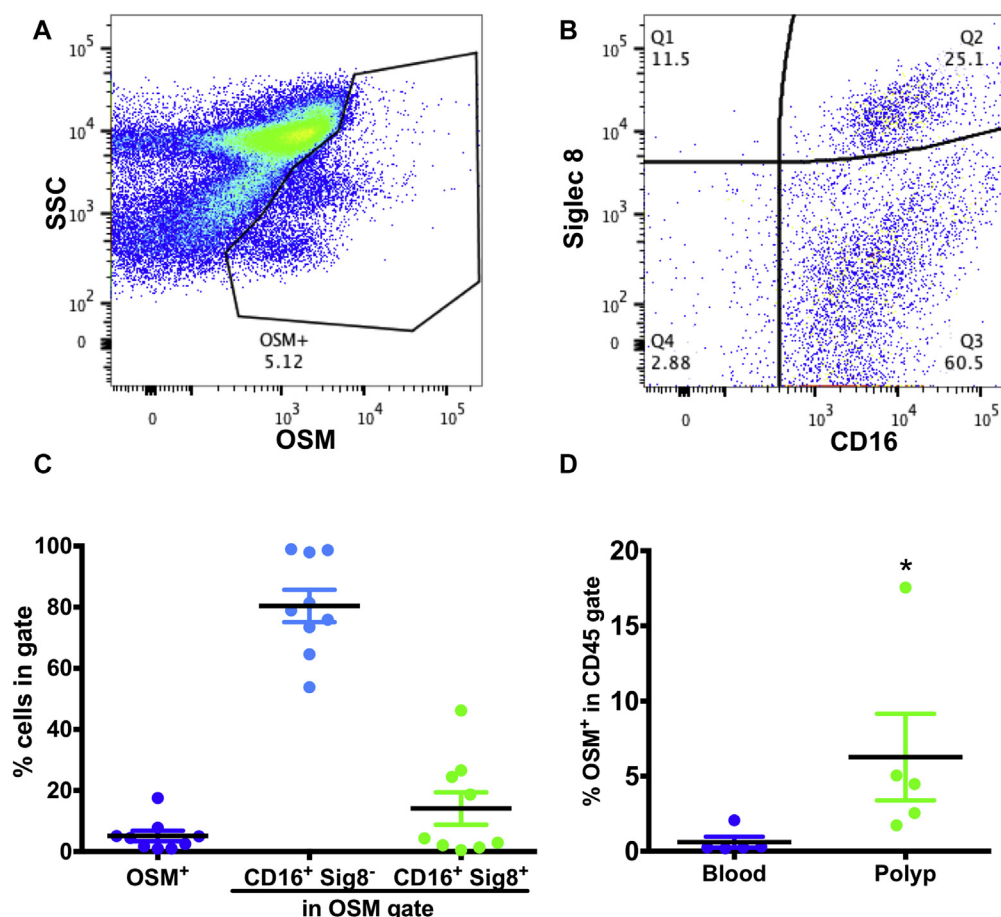


FIG 2. Flow cytometric analysis showed that neutrophils are a major source of OSM producing-cells in NPs. **A**, Within the CD45 gate, $5.1\% \pm 1.7\%$ of cells were OSM⁺. **B**, Within the OSM⁺ gate, $80.4\% \pm 5\%$ of cells were neutrophils (CD16⁺Siglec-8⁻), and $14.1\% \pm 5\%$ of cells were either eosinophils, mast cells, or basophils (CD16⁺Siglec-8⁺). **C**, Quantification of the percentage of OSM⁺ cells within the CD45⁺ gate and percentage of neutrophils (CD16⁺Siglec-8⁻) and CD16⁺Siglec-8⁺ cells within the OSM⁺ gate (n = 9). **D**, In matched blood and NPs $0.60\% \pm 0.36\%$ of CD45⁺ cells in the blood were OSM⁺, whereas $6.3\% \pm 2.9\%$ of CD45⁺ cells in NPs were OSM⁺ (n = 5; *P < .05, Mann-Whitney U test), suggesting that OSM was locally induced (n = 5).

active than GM-CSF alone (Fig 4, A). These data were analyzed by using both means and medians, and the statistics were the same; the data represented in Fig 4, A, show the mean values. Analysis of *OSM* mRNA after culture showed that both GM-CSF and FSTL1-treated neutrophils expressed *OSM* transcripts, suggesting that neutrophils are capable of *de novo* production of *OSM*, although *OSM* levels were not increased compared with control values (see Fig E4, A, in this article's Online Repository at www.jacionline.org). We next wanted to determine whether other type 2 mediators were sufficient to induce neutrophil-derived OSM. Neutrophils were either left unstimulated or stimulated with GM-CSF (25 ng/mL), IL-25 (10-100 ng/mL), IL-33 (10-100 ng/mL), thymic stromal lymphopoietin (10-100 ng/mL), or leukotriene C₄ (10^{-6} to 10^{-7} mol/L; Fig E4, B). Only GM-CSF was sufficient to induce OSM in neutrophils. We also wanted to determine whether neutrophil ligation with E-selectin and ICAM would induce neutrophil-derived OSM. We coated plates with E-selectin, ICAM, and BSA as a control and then cultured neutrophils for 20 hours in the coated wells. We did not observe any induction of OSM with E-selectin or ICAM ligation (see Fig E4, C).

OSM has been shown to be important for the early stages of epithelial repair, specifically by inducing basal cell proliferation and migration.³⁸ We next hypothesized that injured epithelium may upregulate GM-CSF to induce OSM production from infiltrating neutrophils, which would then promote the early stages of epithelial repair. To determine whether GM-CSF could potentially be produced from injured epithelium, we injured NHBE cells that were grown at ALI and when they were fully differentiated they were treated with HKSA, as previously described.³⁹ Exposure for 48 hours to HKSA at a concentration of 5×10^8 particles/mL induced GM-CSF secretion into cell-culture supernatants (Fig 4, B). Although HKSA can activate epithelium through various pathways, these data support the concept that injured epithelium is capable of producing GM-CSF, which has the potential to induce OSM expression in neutrophils that migrate to the site of injury. Because GM-CSF was sufficient to induce neutrophil-derived OSM secretion, we next hypothesized that GM-CSF levels would be increased in NPs compared with control UT. To do this, we measured GM-CSF and FSTL1 protein expression in NPs and UT from patients with CRS and control subjects. GM-CSF levels were

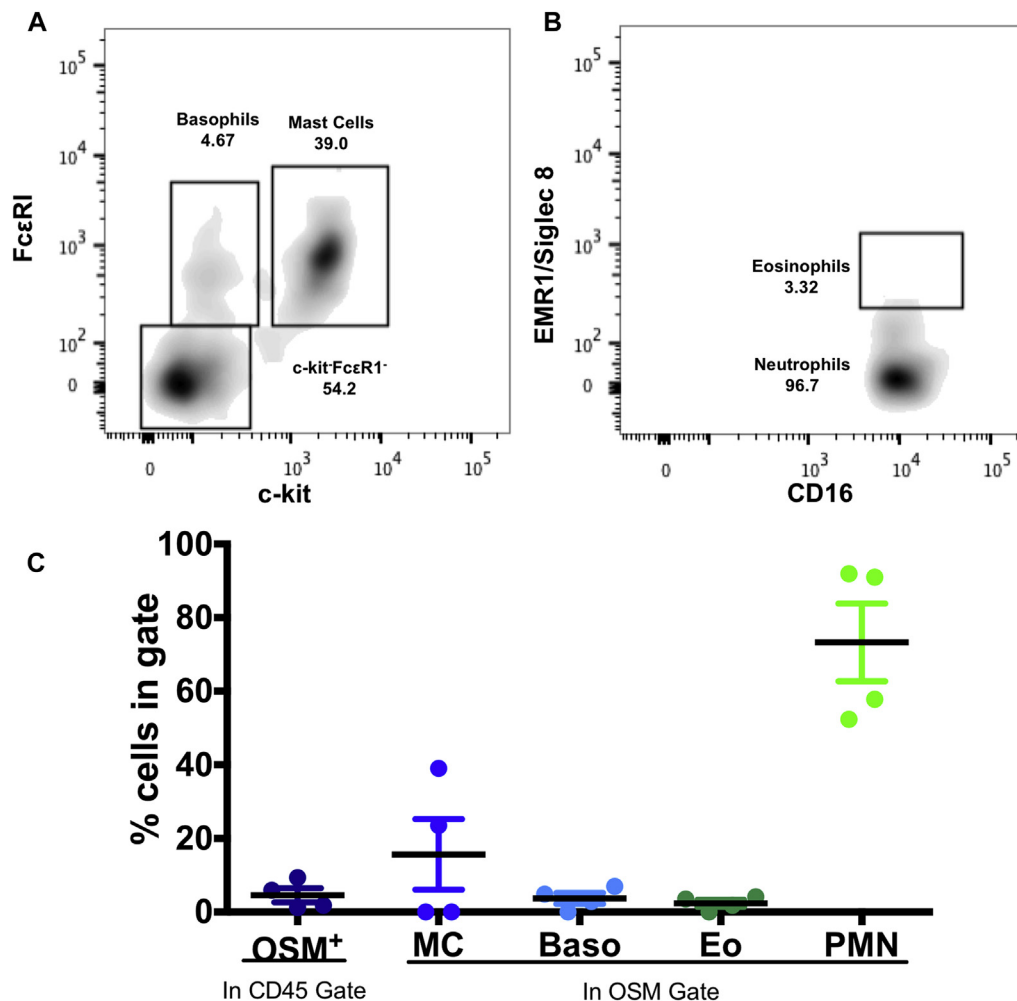


FIG 3. Flow cytometric analysis showed that mast cells were a minor source of OSM-producing cells in NPs. **A**, Representative flow cytometric plot of the OSM⁺ gate. **B**, Representative flow cytometric plots of the c-kit⁺FcεRI⁻ gate. **C**, Quantification of the relative representation of different cell types among OSM⁺ cells. Mast cells (MC) were 15.7% ± 9.6%, basophils (Baso) were 3.7% ± 1.5%, eosinophils (Eo) were 2.3% ± 0.9%, and neutrophils (PMN) were 73.3% ± 10.6% within the OSM⁺ gate (n = 4). OSM⁺ cells were 4.6% ± 1.9% of the CD45⁺ cells.

increased in NPs compared with that in control UT, whereas FSTL1 expression was not increased, suggesting that GM-CSF could be responsible for the induction of neutrophil OSM expression in NPs (Fig 4, C and D).

To determine the cell types responsible for GM-CSF production in NPs, we stained NP sections (n = 4) for OSM, neutrophil elastase, and GM-CSF (Fig 4, E, and see Fig E4, D and E). Surprisingly, the neutrophils that expressed OSM also expressed GM-CSF. GM-CSF has been shown to protect neutrophils against apoptosis,⁴⁰ suggesting that neutrophils might have an autocrine pathway through which GM-CSF expression prolongs their own survival and promotes OSM expression. We did not observe GM-CSF expression in the epithelium in NPs.

OSM-producing neutrophils might have a nonclassical phenotype

We next tested whether OSM-producing neutrophils were phenotypically classical inflammatory N1 neutrophils or tissue repair-associated N2 neutrophils. We used flow cytometry and

stained cells isolated from NPs for CD45, OSM, CD16, IL-5 receptor (IL-5R), myeloperoxidase (MPO), and ARG1 (see Fig E5, A, in this article's Online Repository at www.jacionline.org). No flow cytometric antibodies were available for elastase, and therefore we used anti-MPO instead. Of cells within the CD45⁺ gate, 3.7% ± 1% were OSM⁺, and of the OSM⁺ cells, 56.0% ± 8% were CD16⁺IL-5R⁻ neutrophils. In the neutrophil gate we found 2 populations: MPO^{hi}ARG1⁺ (72.5% ± 12%) and MPO^{lo}ARG1⁻ (22.1% ± 8%) cells (Fig 5, A and B). The expression of ARG1 suggests that the majority of neutrophils taken from NP tissue *in vivo* were not classical N1 neutrophils. As discussed above, GM-CSF and FSTL1 are 2 potential inducers of OSM *in vivo*.

To determine whether GM-CSF induced neutrophils to assume a nonclassical phenotype, we compared neutrophils treated *in vitro* with GM-CSF to neutrophils that were polarized either toward an N1 phenotype using LPS and IFN-γ or toward an N2 phenotype using IL-4 and IL-13. As expected, we saw increased levels of OSM protein released into the culture supernatants in response to GM-CSF, but we did not see any increased OSM

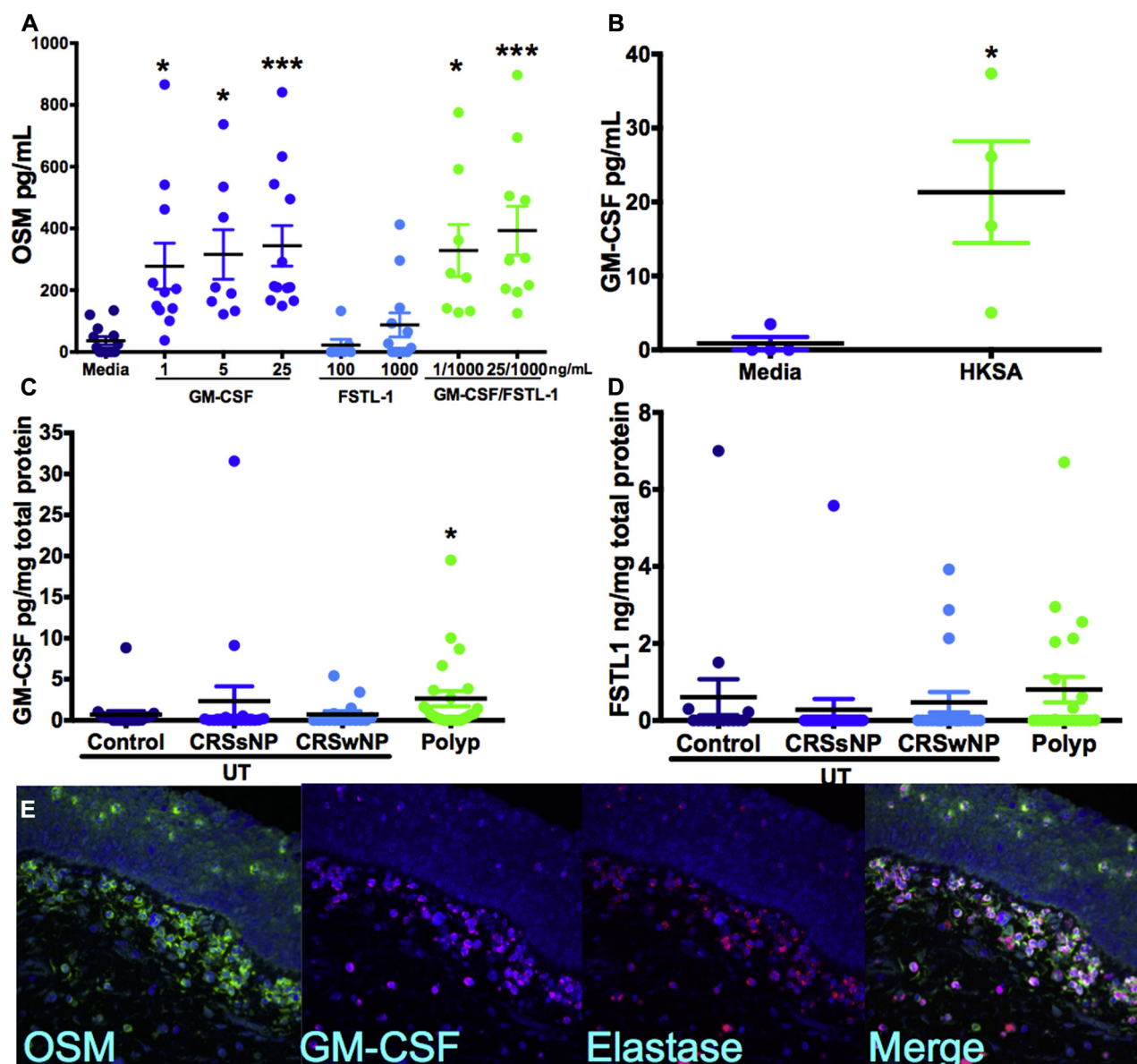


FIG 4. GM-CSF levels were increased in NPs and induced neutrophil-derived OSM. **A**, Blood neutrophils were stimulated with indicated concentrations of GM-CSF, FSTL1, or both, and levels of OSM protein in the culture supernatant were measured ($n = 7-13$). * $P < .05$ and *** $P < .001$, Kruskal-Wallis test. **B**, Fully differentiated NHBE cells were either unstimulated or stimulated with HKSA, and levels of GM-CSF protein in cell-culture supernatants were measured. HKSA-treated NHBE cells released more GM-CSF (21.3 ± 6.9 pg/mL) than control NHBE cells (0.87 ± 0.87 ; $n = 4$; $P < .05$, Mann-Whitney U test). **C**, GM-CSF protein levels (2.6 ± 0.93 pg/mg total protein) were increased in NPs compared with control UT (0.68 ± 0.42 pg/mg total protein; $n = 16-24$; $P < .05$, Mann-Whitney U test). **D**, FSTL1 protein levels (0.60 ± 0.47 ng/mL) were increased in NPs compared with control UT (0.80 ± 0.33 ; $n = 15-23$; $P = .31$, Mann-Whitney U test). **E**, OSM (Alexa Fluor 488), GM-CSF (Alexa Fluor 568), and neutrophil elastase (Alexa Fluor 647) colocalized in NPs ($n = 4$).

levels in the supernatants in either the N1- or N2-polarizing conditions (Fig 5, C).

To determine whether neutrophil phenotypes influence OSM expression, we polarized neutrophils to N1 or N2 conditions or left them unstimulated for 24 hours. After polarization, the neutrophils were either left unstimulated or stimulated with GM-CSF. Unactivated, N1, and N2 neutrophils had no difference in OSM expression, suggesting that all neutrophils are capable of OSM expression (data not shown).

To further define the phenotype of our GM-CSF- and FSTL1-treated neutrophils, we analyzed mRNA expression of the type 2 markers *MRC1* and *ARG1*. Neither GM-CSF, FSTL1 nor their combination caused any induction of *ARG1* (see Fig E5, B). However, GM-CSF and FSTL1 were both sufficient to induce increased mRNA for another N2 marker, *MRC1*, suggesting that GM-CSF and FSTL1 can induce neutrophils to assume elements of an N2-like phenotypic state (Fig 5, D).

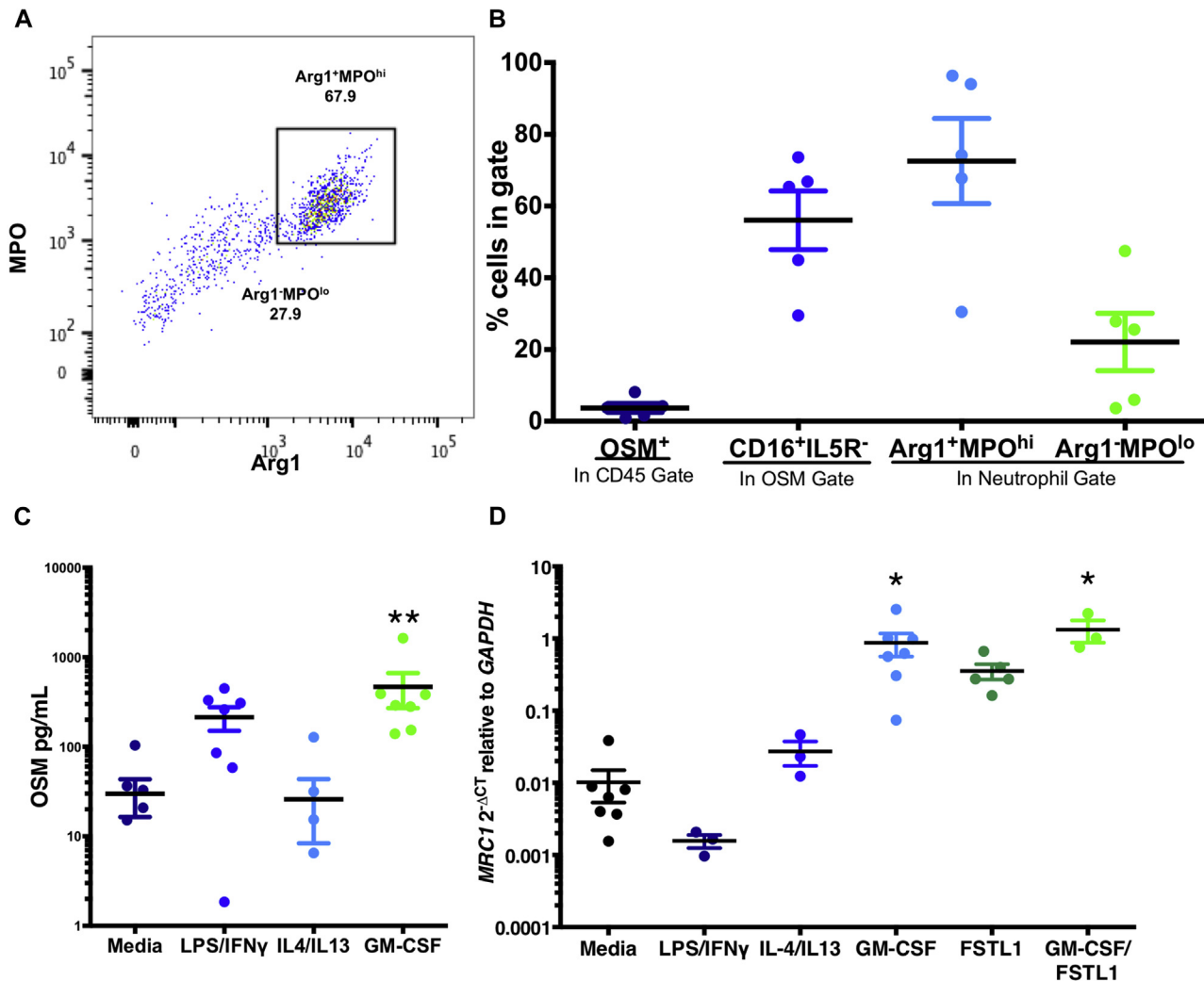


FIG 5. OSM-producing neutrophils did not express a classical N1 phenotype. **A**, OSM⁺ neutrophils contained a distinct population of Arg1⁺MPO^{hi} cells (representative data). **B**, 3.7% $\pm$ 1.3% of cells in the CD45 gate were OSM⁺; within the OSM⁺ gate, 56.0% $\pm$ 8.2% of the cells were CD16⁺IL-5R⁻ neutrophils. Within the neutrophil gate, 72.56% $\pm$ 11.9% of the cells were Arg1⁺MPO^{hi}, and 22.1% $\pm$ 8.0% were Arg1⁻MPO^{lo} (n = 5). **C**, Blood neutrophils were either left untreated or treated under N1-polarizing conditions (LPS/IFN- γ), N2-polarizing conditions (IL-4/IL-13), or GM-CSF. GM-CSF-treated neutrophils secreted increased levels of OSM into the cell-culture supernatants, whereas N1- and N2-polarizing conditions did not induce OSM (n = 4-7; **P < .01, Kruskal-Wallis test). **D**, Levels of *MRC1* mRNA were increased in neutrophils stimulated with GM-CSF either alone or together with FSTL1 (n = 3-7; *P < .05, Kruskal-Wallis test).

Neutrophil expression of OSM in asthmatic patients

To determine whether neutrophils were also a source of OSM in asthmatic patients, we stained bronchial biopsy specimens from control subjects (n = 4), as well as those from patients with moderate (n = 5) and severe (n = 6) asthma, for OSM and neutrophil elastase (Fig 6, A). We counted neutrophils and OSM⁺ cells in each specimen, and the tabulated data are represented in Table E3 in this article's Online Repository at www.jacionline.org. We did not observe any OSM⁺ cells in the control biopsy specimens, but we detected OSM⁺ cells in 60% of biopsy specimens from patients with moderate asthma and 100% of biopsy specimens from patients with severe asthma (see Fig E6, A, in this article's Online Repository at www.jacionline.org). Of the neutrophils counted in each specimen, 0% $\pm$ 0% were OSM⁺ in control biopsy specimens, 14.0% $\pm$ 9.8% were OSM⁺ in

biopsy specimens from patients with moderate asthma, and 30.1% $\pm$ 10.2% were OSM⁺ in biopsy specimens from patients with severe asthma (see Fig E6, B). Additionally, of the OSM⁺ cells counted, 0% $\pm$ 0% were neutrophils in control biopsy specimens, 35.0% $\pm$ 21.8% were neutrophils in biopsy specimens from patients with moderate asthma, and 52.1% $\pm$ 15.9% were neutrophils in biopsy specimens from patients with severe asthma (Fig 6, B). Neutrophil counts were normalized per millimeter of basement membrane within the biopsy section, and no differences in the total neutrophil counts per millimeter of basement membrane were observed between control subjects and asthmatic patients, suggesting an increased state of activation of neutrophils rather than increased neutrophil numbers (see Fig E6, C).

To further test whether OSM is produced in the airways of patients with asthma, we evaluated a set of induced sputum

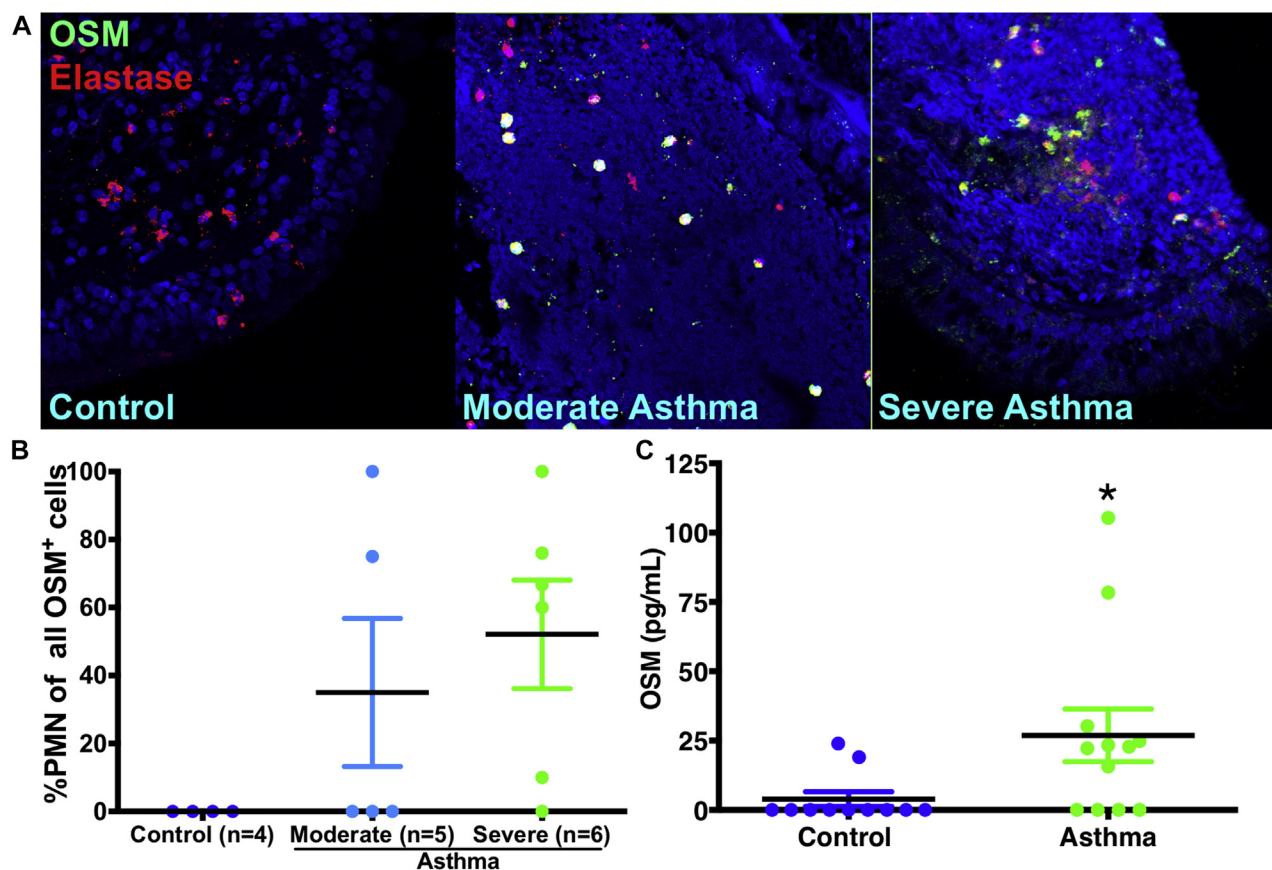


FIG 6. OSM levels were increased in asthmatic patients. **A**, Bronchial biopsy specimens from control subjects, patients with moderate asthma, and patients with severe asthma were stained for OSM in green, and neutrophil elastase was stained in red (n = 4-6). **B**, Counts were obtained of neutrophils and OSM⁺ cells; none of the control biopsy specimens had any OSM⁺ cells, and of the OSM⁺ cells, 35% ± 21.8% were neutrophils in patients with moderate asthma, and 52.1 ± 15.9% were neutrophils in patients with severe asthma. **C**, OSM levels in sputum of asthmatic patients (26.9 ± 9.5 pg) were increased compared with those in control subjects (3.9 ± 2.6 pg; n = 11-12; *P < .05, Mann-Whitney U test).

samples from control subjects and asthmatic patients. We observed increased OSM protein levels in induced sputum from asthmatic patients compared with that from control subjects (Fig 6, C). Finally, we previously reported that segmental allergen challenge in allergic asthmatic patients leads to increased levels of OSM protein in BAL fluids.⁸ We reevaluated the previous data set to test the role of neutrophils in the response and found that OSM levels correlated with total neutrophil counts, total lymphocyte counts, and total cell counts in BAL fluid (see Fig E6, D, E, and H) but not with total eosinophil counts or total macrophage counts (see Fig E6, F and G). Additionally, we previously reported that a concentration of 50 pg/mL GM-CSF was measured in the BAL fluid in a separate cohort of allergen-challenged allergic asthmatic patients, suggesting that GM-CSF can also induce neutrophil-derived OSM production in asthmatic patients.²⁰ Together, these data suggest that OSM is produced by neutrophils in both sinus and lung tissue undergoing allergic inflammation and might play a role in epithelial barrier dysfunction.

DISCUSSION

We previously showed that OSM levels were increased in NP tissue from patients with CRS and that physiologic levels of OSM

are sufficient to induce profound loss of barrier function in differentiated bronchial and nasal epithelial cells cultured at ALI.⁸ We hypothesized that increased OSM levels in patients with CRS can mediate epithelial barrier dysfunction *in vivo*. NPs have been shown to express robust type 2 inflammation,⁴¹⁻⁴³ and we originally speculated that the OSM producers in NPs were likely to be eosinophils, M2 macrophages, or mast cells. Instead, in the present article we found that neutrophils are a major population of cells expressing OSM in tissue from patients with CRS (Figs 1-3). We also identified a minor population of OSM⁺ cells that were Siglec-8⁺, and these cells were found to be mast cells (Figs 2 and 3).

Neutrophils are classically thought to be a first line of defense in response to tissue injury,⁴⁴ and neutrophil-derived OSM has been shown to be important in the early stages of epithelial repair when basal cells proliferate and cover a wound.⁴⁵ Neutrophils are known to be increased in patients with CRSsNP, patients with CRSwNP, and patients with certain forms of asthma, and production of OSM by neutrophils could both participate in ongoing epithelial repair and promote the epithelial barrier dysfunction that is observed in these diseases. Although we did not investigate whether neutrophils were responsible for OSM production in patients with CRSsNP, we suspect that neutrophil-derived OSM might

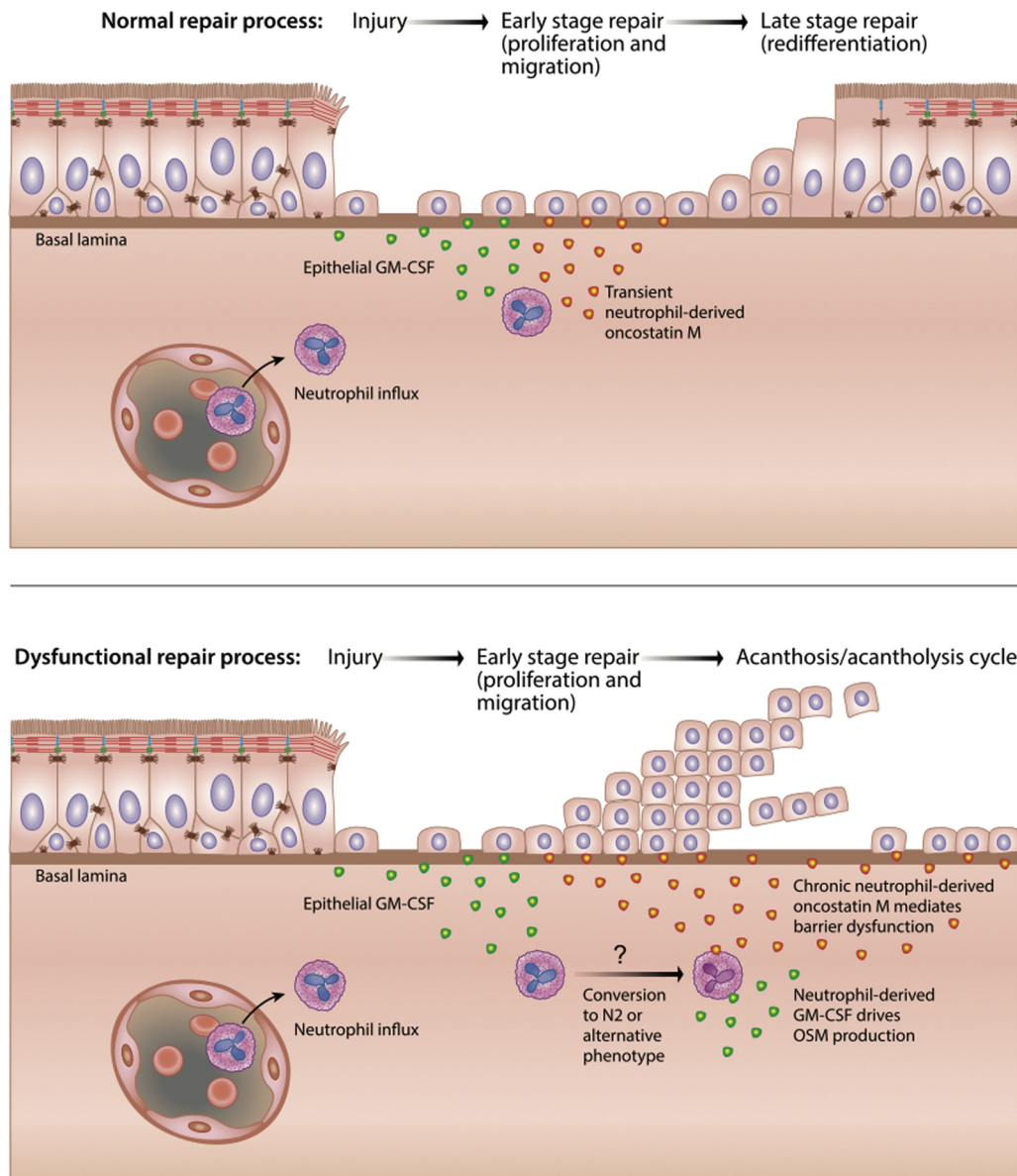


FIG 7. Proposed mechanism of OSM-mediated barrier dysfunction in patients with mucosal disease. Under normal circumstances, when epithelium is injured, neutrophils are recruited to the site of injury and, potentially, transiently make OSM to promote the early stages of repair. Because of transient OSM expression, once epithelial cells become contact inhibited, they are able to enter the later stages of repair and redifferentiate back into functional epithelium. However, under pathogenic conditions, we hypothesize that neutrophils are recruited to the injury site, and once at the site of injury, neutrophils are converted into an alternative phenotype, potentially N2, which makes both OSM and GM-CSF. GM-CSF alone is sufficient to induce production of OSM in neutrophils and will also contribute to long-term survival of OSM-producing neutrophils that might prevent late-stage repair, causing a long-term state of barrier dysfunction.

also play a role in barrier dysfunction in patients with this nonpolypoid form of CRS. It is possible that under normal circumstances, neutrophil-derived OSM production is transient, allowing basal cells to reinitiate contact inhibition, stop proliferating, and then differentiate back into mature epithelium. However, chronic expression of OSM by neutrophils in patients with mucosal disease might prolong or even perpetuate the state of the epithelial repair process (Fig 7). OSM has been shown to induce epithelial-mesenchymal transition,⁴⁶ which antagonizes epithelial differentiation, and expression of

epithelial-mesenchymal transition biomarkers is well established in both patients with CRS and those with asthma.⁴⁷

Neutrophils are not classically associated with type 2 immune responses and are generally considered a type 1 immune response effector cell. However, neutrophilic subsets of CRS and asthma have been described. Although NPs in Western countries tend to be highly eosinophilic, a significant proportion of NPs from patients in Eastern Asian countries are neutrophilic.⁴⁸⁻⁵⁰ In unpublished studies not shown, we found that NPs, which are highly eosinophilic in our Chicago cohort, nonetheless have

significant levels of neutrophils and neutrophil elastase. Increased neutrophilia within NPs has been linked to decreased responsiveness to corticosteroid treatment.⁵¹ Severe asthma is a subtype of asthma that is often characterized by neutrophilia.⁵²⁻⁵³ It is thought that neutrophilia in asthmatic patients associates with more severe disease, again because neutrophils are not particularly responsive to corticosteroid treatment.⁵⁴ However, little is known about the role of neutrophils in eosinophilic mucosal disease outside of these known neutrophilic subtypes of CRS and asthma. Our data suggest that neutrophils might be capable of playing a pathogenic role in patients with eosinophilic mucosal disease through induction of epithelial barrier dysfunction mediated by the concurrent production of OSM and GM-CSF.

We present circumstantial evidence that the neutrophils responsible for OSM production are of the N2 phenotype. Recent studies have shown that neutrophils can be polarized into either classical N1 neutrophils, which are thought to have a more inflammatory phenotype, and N2 neutrophils, which promote tissue repair and tumorigenesis and are thought to have an anti-inflammatory or regulatory phenotype.²³⁻²⁶ We showed that the majority of OSM⁺ neutrophils from NPs expressed the type 2 marker *ARG1* (Fig 5), indicating a phenotype resembling N2 neutrophils more than N1 neutrophils. We also showed that both GM-CSF and FSTL1 induced *in vitro*-stimulated neutrophils to express increased *MRC1* compared with unstimulated neutrophils and N1-polarized neutrophils, also indicating that OSM-producing neutrophils could be skewed toward the N2 phenotype (Fig 5). Because both GM-CSF and FSTL1 have been shown to be important inducers of OSM,^{11,16,18} we tested their ability to induce OSM *in vitro* in cultured neutrophils; of these 2 stimuli, only GM-CSF induced OSM production. However, because we did not see any induction of *ARG1* in response to GM-CSF, another factor might be necessary for full induction of the N2 phenotype. These studies implicate GM-CSF as a potential inducer of OSM in neutrophils *in vivo*. Because circulating neutrophils do not express OSM in patients, this induction likely takes place within NP or lung tissue, where these inducers can be found.

Interestingly, immunofluorescent staining showed that GM-CSF localized to OSM-producing neutrophils in NPs, suggesting that OSM-producing neutrophils can induce their own OSM through autocrine production of GM-CSF (Fig 4, E). There is precedent for such a concept because Anwar et al⁵⁵ showed that eosinophils produce GM-CSF to prolong their own survival. In addition, Durand et al⁴⁰ have shown that autoantibody ligation of FcγRIIIb (CD16) receptors on the surfaces of neutrophils protects against apoptosis through induction of autocrine GM-CSF. We have previously shown increased autoantibody levels in NPs, suggesting that these increased autoantibody levels can trigger GM-CSF expression in the infiltrating neutrophils through a similar mechanism.⁵⁶ Whether production of OSM by neutrophils through an autocrine GM-CSF-dependent pathway occurs in patients with CRSwNP and asthma is worthy of further investigation.

Importantly, we have also shown that OSM levels were increased in induced sputum from asthmatic patients compared with those in control subjects and that OSM⁺ cells were present in bronchial biopsy specimens from 100% of patients with severe asthma, 60% of patients with moderate asthma, and none of the healthy control subjects. These results suggest that expression of OSM and its association with barrier disruption applies to

CRSwNP, asthma, and, as shown in our previous study, eosinophilic esophagitis. In the present study 52% of the OSM⁺ cells were neutrophils in patients with severe asthma, and 35% of the neutrophils were OSM⁺ in patients with moderate asthma, suggesting that neutrophil-derived OSM plays a role in asthma pathogenesis. OSM has previously been implicated in the induction of fibrosis in a mouse model of asthma, although it was derived from macrophages.^{16,57}

Given that GM-CSF might promote prolonged survival in neutrophils, as well as induce OSM, therapeutic targeting of GM-CSF could potentially be beneficial in the treatment of CRS and possibly other mucosal diseases, such as asthma and eosinophilic esophagitis. Inhibition of GM-CSF could block neutrophil-derived OSM production, allowing epithelium to enter the late phase of repair and differentiation, potentially resolving chronic inflammation resulting from barrier dysfunction. Two strategies have been used to inhibit GM-CSF in human trials: mAbs against GM-CSF and an mAb against GM-CSF receptor α. Clinical trials have been conducted with mAbs against GM-CSF (MOR103, KB003, and namilumab) in patients with rheumatoid arthritis, plaque psoriasis, and severe asthma. A phase II trial was conducted by using KB003 in asthmatic patients whose symptoms were poorly controlled with long-acting bronchodilators and either inhaled or oral corticosteroids. They did not observe improved FEV₁ in the entire patient cohort. However, when they separated patients based on markers of poorly controlled asthma, peripheral blood eosinophilia, low baseline FEV₁, and high bronchodilator reversibility, they did detect an improvement in FEV₁ in response to treatment with KB003.⁵⁸

In summary, we have shown that neutrophils are a major source of OSM-producing cells in NPs using both immunofluorescence and flow cytometric analyses. We have also shown that GM-CSF levels are increased in NPs and that physiologic levels of GM-CSF are sufficient to induce OSM production in cultured neutrophils. Interestingly, GM-CSF is expressed by many of the same neutrophils that produce OSM in NPs, suggesting the presence of an autocrine activation. OSM-producing neutrophils phenotypically resemble N2 neutrophils based on limited biomarker evaluation, but further characterization of these cells will be required. OSM⁺ cells are also present in biopsy specimens from patients with severe asthma, and approximately half of the OSM-producing cells are neutrophils, suggesting that neutrophil-derived OSM might also play a role in asthma. Taken together, these studies further implicate OSM in barrier dysfunction in patients with CRSwNP, as well as in those with asthma, and suggest that the OSM found in these diseases is produced by neutrophils. Thus therapeutic intervention targeting either GM-CSF or OSM might be beneficial in the treatment of CRS and asthma.

We thank Dr Bruce Bochner for the very kind gift of anti-Siglec-8 antibody and Ms Jacqi Schaffer for the outstanding medical illustrations.

Clinical implications: Neutrophils are a major source of OSM-producing cells in patients with CRS and those with severe asthma. GM-CSF levels were increased in patients with CRS at levels sufficient to induce neutrophil-derived OSM production. Therapeutic targeting of OSM, neutrophils, or GM-CSF might be beneficial in patients with mucosal disease through restoration of epithelial barrier function.

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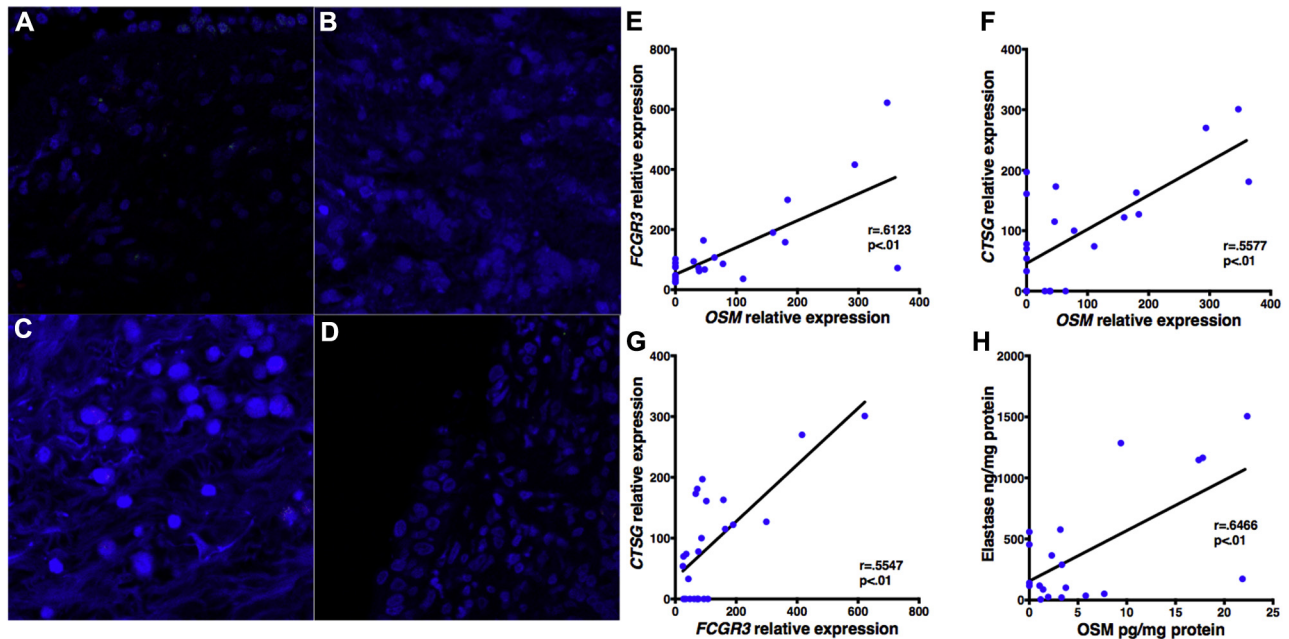


FIG E1. **A-D**, Control images from OSM staining with CD68 (Fig E1, **A**), ECP (Fig E1, **B**), tryptase (Fig E1, **C**), and elastase (Fig E1, **D**). **E** and **F**, OSM expression levels correlated with expression of the granulocyte marker FCGR3 (Fig E1, **E**) and with the neutrophil-specific protease cathepsin G (*CTSG*; Fig E1, **F**) in the microarray data set. **G**, FCGR3 levels correlated with CTSG levels in the microarray. **H**, OSM protein levels correlated with elastase protein levels in lysates from both NPs and UT from patients with CRS and control subjects.

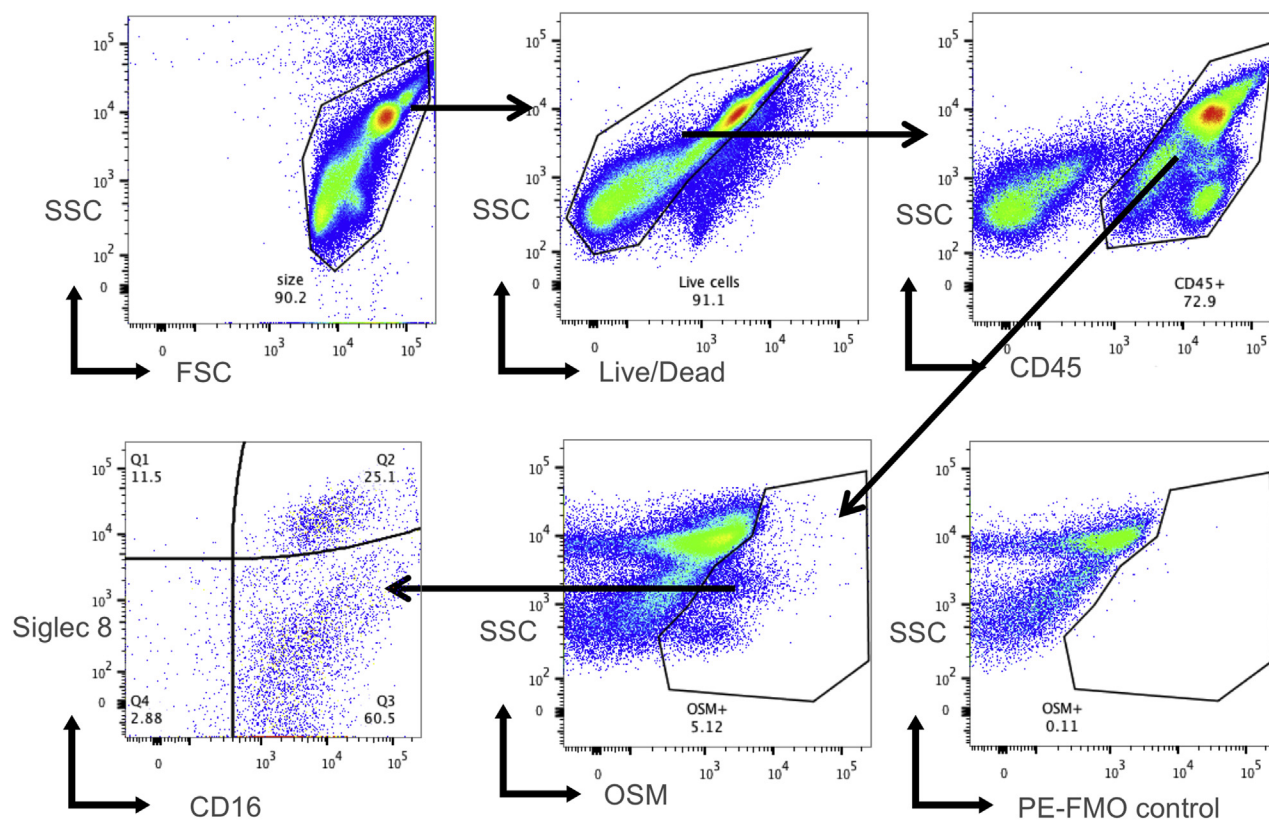


FIG E2. Gating strategy for identification of OSM⁺ cells. Cells were first gated on forward scatter (FSC) and side scatter (SSC), and doublets (data not shown) and dead cells were gated out. Cells were then gated on the CD45⁺ population and then subsequently gated on the OSM⁺ population. The OSM⁺ population was then plotted based on CD16 and Siglec-8 expression, and the CD16⁺Siglec-8⁻ and CD16⁺Siglec-8⁺ populations were quantified.

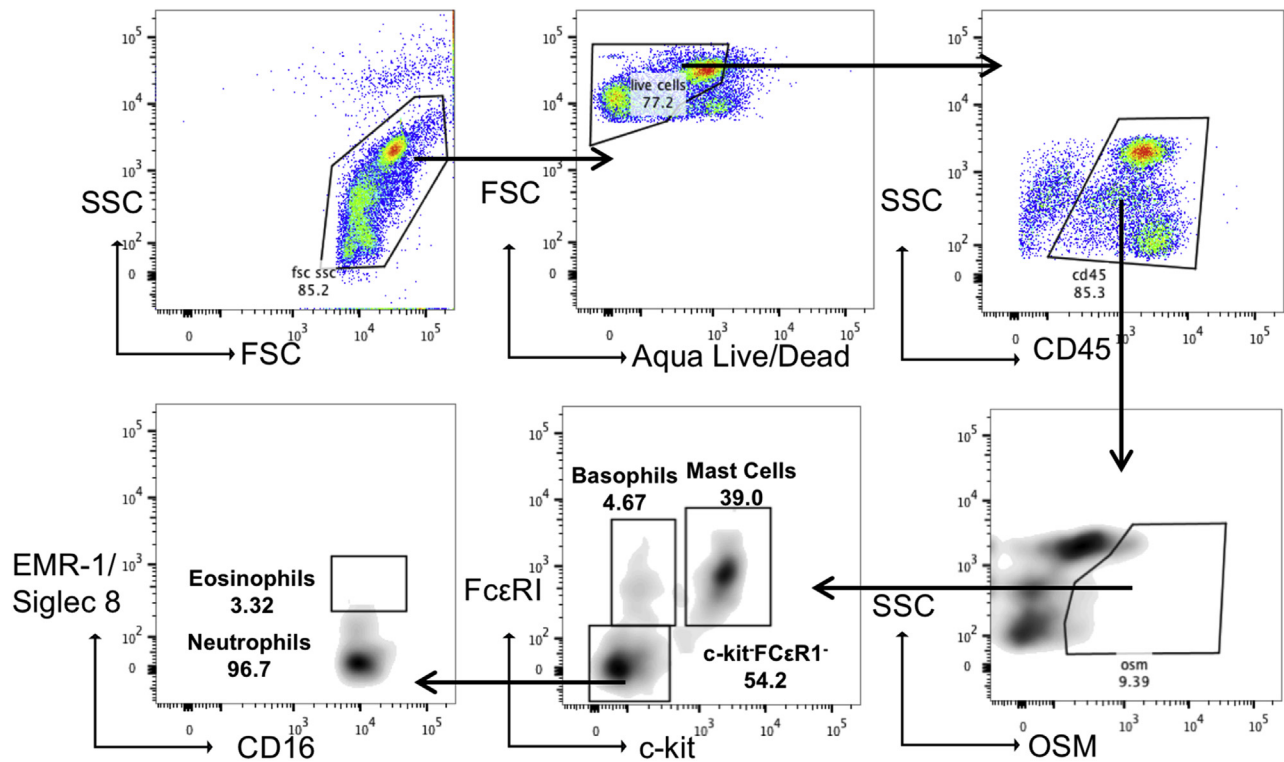


FIG E3. Contribution of mast cells, eosinophils, and basophils to the OSM⁺ cell population. Cells were first gated on forward scatter (FSC) and side scatter (SSC), and doublets (not shown) and dead cells were gated out. Cells were then gated on the CD45⁺ population and then gated on the OSM⁺ population. The OSM⁺ population was then plotted on c-kit and FcεRI expression, and the mast cell, c-kit⁺FcεRI⁺, and basophil c-kit⁺FcεRI⁻ populations were quantified. The c-kit⁺FcεRI⁻ population was then plotted on CD16 and either EMR1 or Siglec-8 to discriminate neutrophils (CD16⁺EMR1/Siglec-8⁻) from eosinophils (CD16⁺EMR1/Siglec-8⁺).

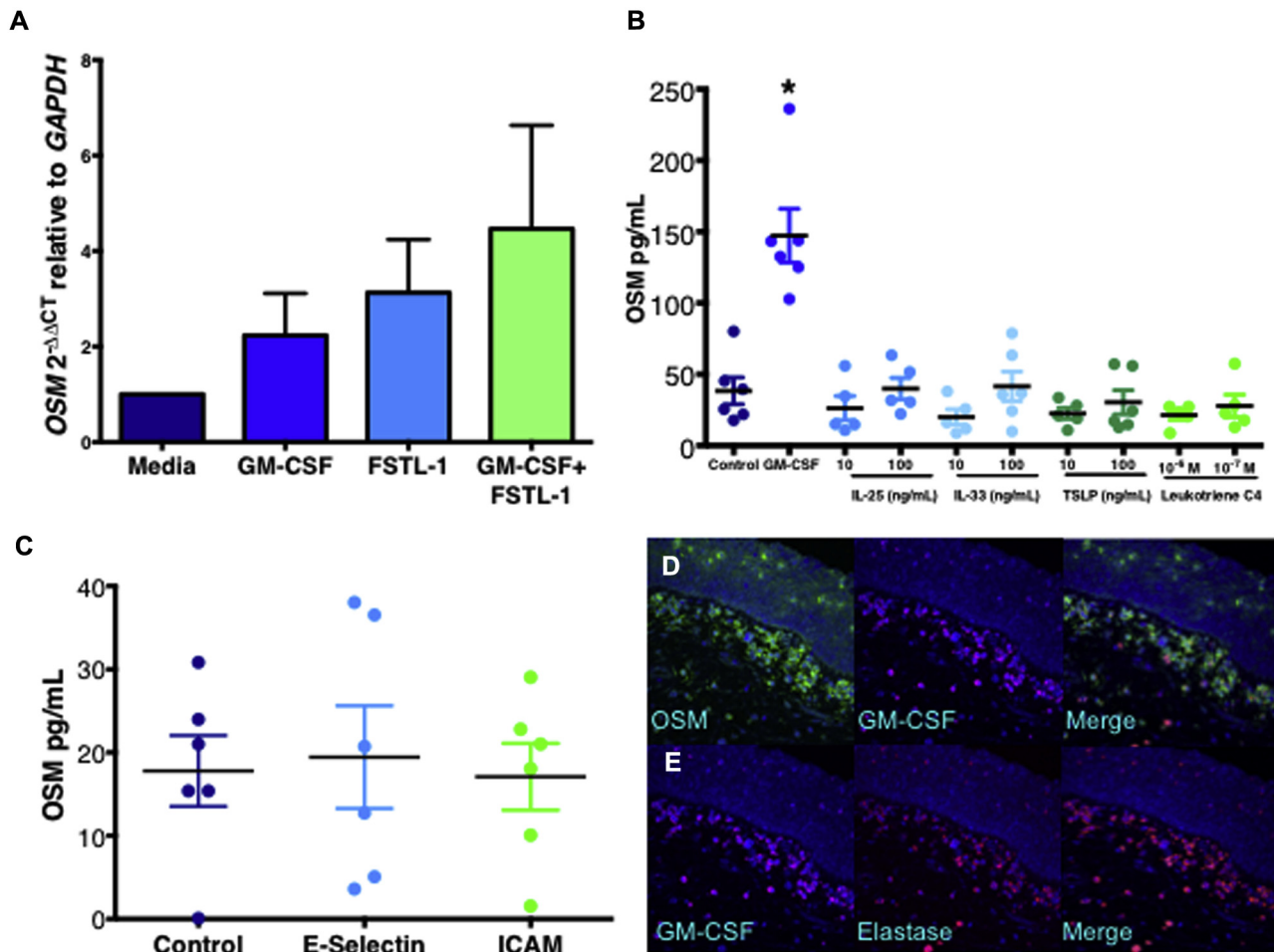


FIG E4. **A**, Blood neutrophils were isolated and either left unstimulated or stimulated with GM-CSF, FSTL1, or both. There was a trend toward both GM-CSF and FSTL1 to increase *OSM* mRNA expression compared with that seen in unstimulated control cells. **B**, Neutrophils were either left unstimulated or stimulated with 25 ng/mL GM-CSF or the indicated concentrations of IL-25, IL-33, thymic stromal lymphopoietin (TSLP), or leukotriene C₄ for 20 hours ($n = 5-6$). Only GM-CSF was sufficient to induce OSM. * $P < .05$. **C**, E-selectin and ICAM ligation did not induce neutrophil-derived OSM ($n = 5-6$). **D**, OSM (Alexa Fluor 488) and GM-CSF (Alexa Fluor 568) colocalized in NPs ($n = 4$). **E**, GM-CSF (Alex Fluor 568) and neutrophil elastase (Alexa Fluor 647) colocalized in NPs ($n = 4$).

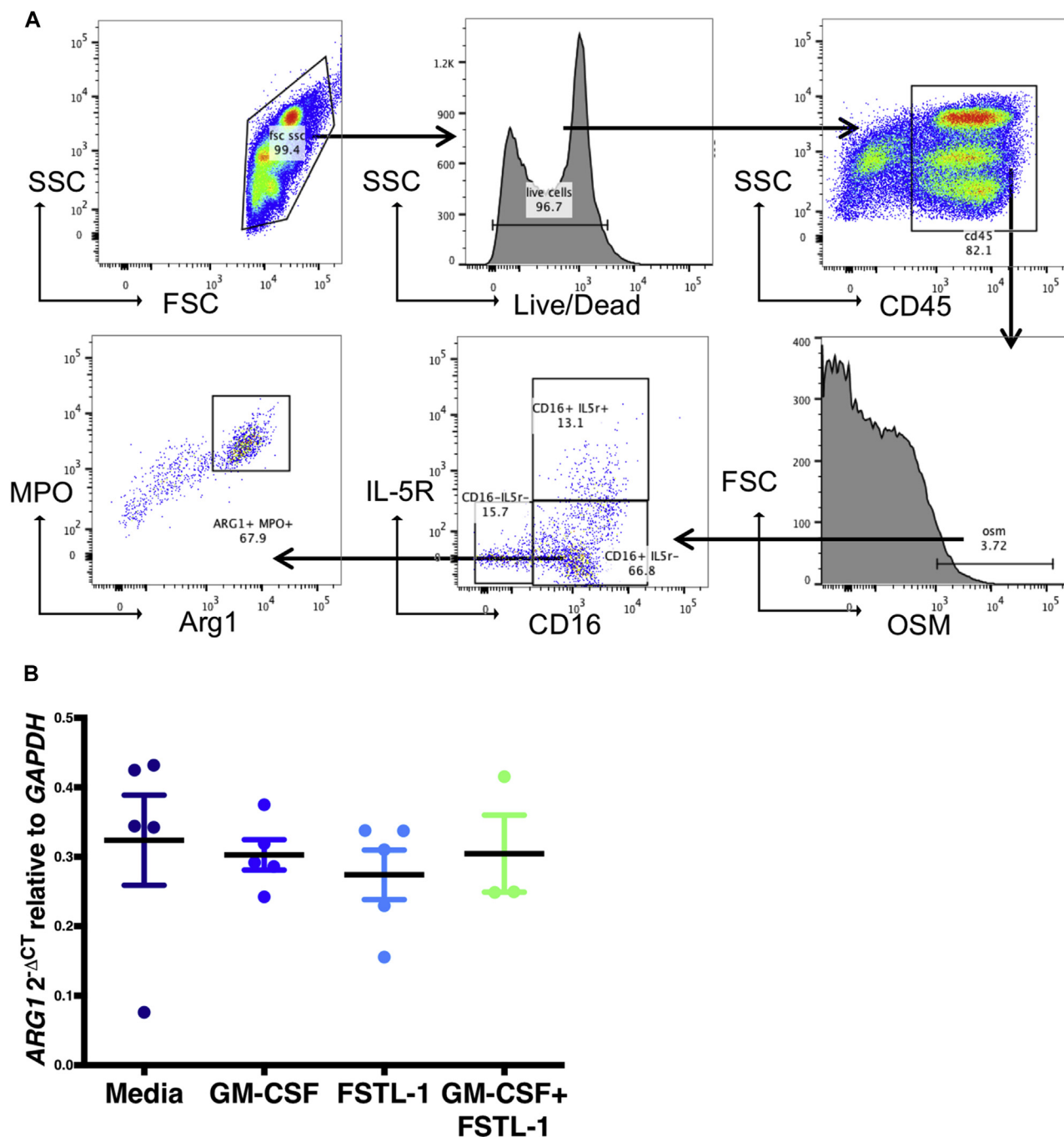


FIG E5. Identification of putative N1 and N2 cells among OSM⁺ neutrophils. **A**, Cells were first gated on forward scatter (FSC) and side scatter (SSC), and doublets and dead cells were gated out. Cells were then gated on the CD45⁺ population and then gated on the OSM⁺ population. The OSM⁺ population was then plotted on CD16 and IL-5R to discriminate neutrophils (CD16⁺IL-5R⁻) and eosinophils (CD16⁺IL-5R⁺). Cells were then gated on the neutrophil population and plotted on MPO and Arg1, and the Arg1⁺MPO^{hi} and Arg1⁺MPO^{lo} populations were quantified. **B**, Levels of ARG1 mRNA were not increased in neutrophils stimulated with GM-CSF either alone or together with FSTL1 compared with control values.

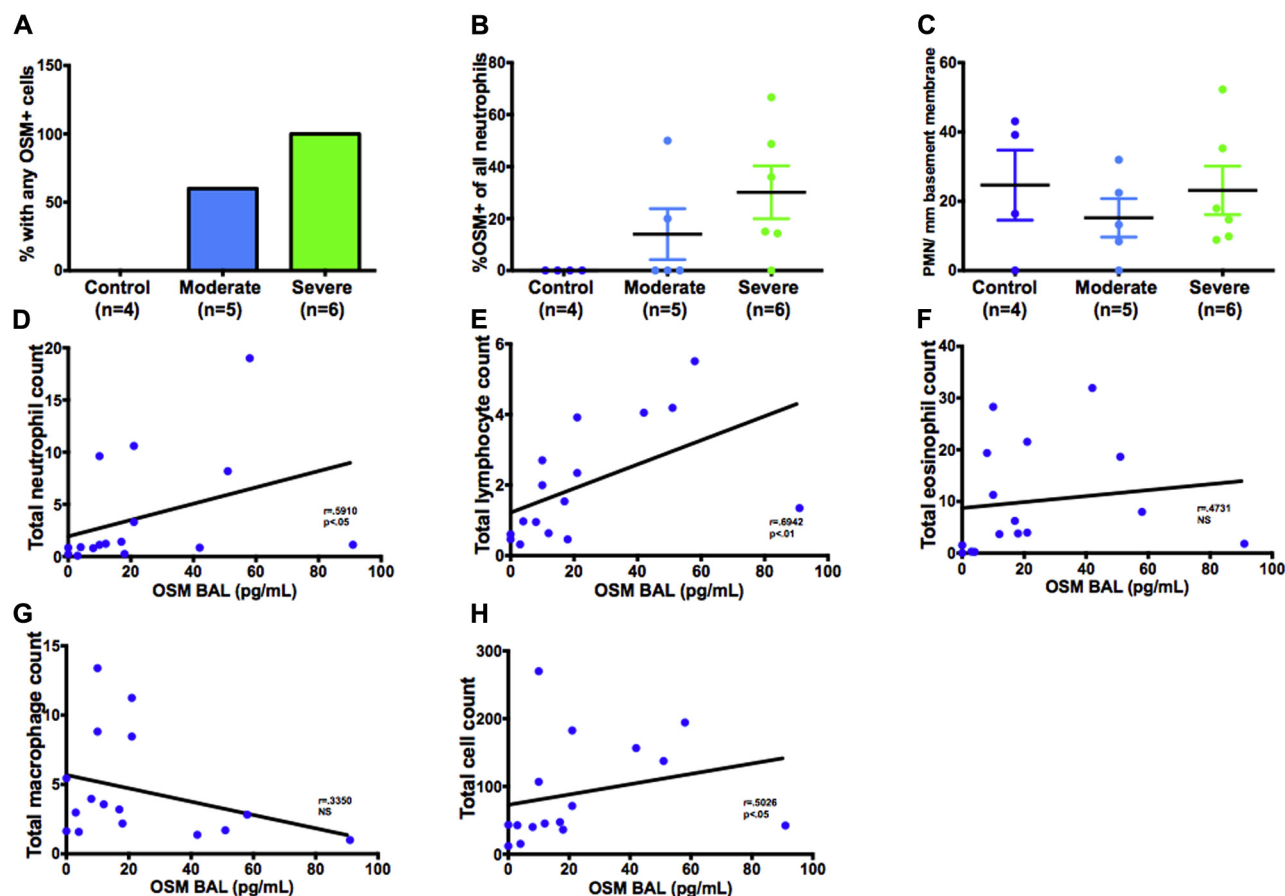


FIG E6. **A**, Bronchial biopsy sections from healthy control subjects and asthmatic patients were stained for OSM and neutrophil elastase: none of the control biopsy specimens had any OSM⁺ cells, 60% of biopsy specimens from patients with moderate asthma had OSM⁺ cells. **B**, Of the neutrophils counted, 0% ± 0% were OSM⁺ in control subjects, 14.0% ± 9.8% were OSM⁺ in patients with moderate asthma, and 30.1% ± 10.1% were OSM⁺ in patients with severe asthma. **C**, No differences were seen in numbers of neutrophils per millimeter of basement membrane. **D-H**, OSM levels in BAL fluid from segmental allergen challenge of allergic asthmatic patients correlated with total neutrophil counts (Fig E6, *D*), total lymphocyte counts (Fig E6, *E*), and total cell counts (Fig E6, *H*) but not with total eosinophil counts (Fig E6, *F*) or total macrophage counts (Fig E6, *G*).

TABLE E1. Patients' demographics

		Control subjects			Patients with CRSsNP			Patients with CRSwNP			Patients with CRSwNP, polyps
Total no. of subjects		n = 33 (15 F/18 M)			n = 24 (14 F/10 M)			n = 89 (36 F/53 M)			
Age (y), median (range)		45.5 (22-73)			48 (19-75)			48 (18-69)			
	χ^2 Test	Yes	No	Unknown	Yes	No	Unknown	Yes	No	Unknown	
Atopy	$P < .0001$	5	15	13	10	10	4	53	29	7	
Asthma	$P < .0001$	1	32	0	4	20	0	43	45	1	
Perioperative systemic steroids	$P = .0142$	1	30	2	1	23	0	18	68	2	
Antibiotics	$P = .2659$	3	18	1	0	24	0	10	78	1	
Methodology used											
Flow cytometry											n=16 (8 F/8 M)
Age (y), median (range)											44 (18-69)
Immunofluorescence											n = 21 (8 F/13 M)
Age (y), median (range)											45 (25-66)
Neutrophil culture		n = 13 (3 F/10 M)			n = 6 (3 F/3 M)			n = 8 (2 F/6 M)			
Age (y), median (range)		37 (22-63)			53 (25-75)			36 (28-65)			
Protein lysates		n = 20 (12 F/8 M)			n = 18 (11 F/7 M)			n = 20 (7 F/13 M)			n = 24 (11 F/13 M)
Age (y), median (range)		58 (25-73)			46 (19-67)			48 (28-69)			54 (25-69)

F, Female; M, male.

TABLE E2. Flow cytometric antibodies

	Antibody	Clone	Dilution	Manufacturer
Figs 2 and 3	Anti-human OSM-PE	17022	1:10	R&D Systems, Minneapolis, Minn
	Anti-human CD45-V450	HI30	1:40	BD Biosciences, San Jose, Calif
	Anti-human CD16-APC-H7	3G8	1:40	BD Biosciences
	Anti-human Siglec 8-Alexa Fluor 647		1:10	Gift from Dr Bruce Bochner
	Anti-human EMR1-Alexa Fluor 647	A10	1:20	AbD Serotec, Raleigh, NC
	Anti-human FcεR1-Pe-Cy7	MHE-18	1:100	BioLegend, San Diego, Calif
Fig 5	Anti-human c-kit-Alexa Fluor 488	104D2	1:50	BioLegend
	Anti-human OSM-PE	17022	1:10	R&D Systems
	Anti-human CD45-V450	HI30	1:40	BD Biosciences
	Anti-human CD16-APC-H7	3G8	1:40	BD Biosciences
	Anti-human IL-5R-APC	26815	1:20	R&D Systems
	Anti-human MPO-FITC	MPO455-8E6	1:20	eBioscience, San Diego, Calif
	Anti-human Arg1-Alexa Fluor 700	658922	1:20	R&D Systems

APC, Allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

TABLE E3. Bronchial biopsy cell counts

Patient groups	Patient	OSM, yes/no	Total PMN count	OSM ⁺ PMN count	OSM ⁺ count	OSM ⁺ non-PMN count	% OSM ⁺ PMNs	% PMNs that are OSM ⁺	PMN/mm basement membrane
Patients with severe asthma	abb 0120908	Y	20	3	5	2	60	15	35.27
	abb 0151108	Y	7	1	10	9	10	14.29	14.69
	abb 0308207	Y	6	0	2	2	0	0	9.85
	abb 0235206	Y	6	4	6	2	66.67	66.67	8.86
	abb 0120709	Y	39	19	25	6	76	48.72	52.25
	abb 0305807	Y	25	9	9	0	100	36	17.93
Patients with moderate asthma	abb 0167606	N	0	0	0	0	0	0	0
	abb 0144408	Y	20	10	10	0	100	50	31.97
	abb 0240008	Y	7	0	2	2	0	0	8.41
	abb 0200412	N	8	0	0	0	0	0	13.24
	abb 0164406	Y	15	3	4	1	75	20	22.44
Control subjects	abb 349911	N	23	0	0	0	0	0	43.08
	abb 0150705	N	11	0	0	0	0	0	16.41
	abb 0160706	N	22	0	0	0	0	0	39.15
	abb 0165706	N	0	0	0	0	0	0	0