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

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Abstract

Background—We have previously shown that Oncostatin M (OSM) is elevated in nasal polyps of chronic rhinosinusitis (CRS) patients, as well as in bronchoalveolar lavage (BAL) fluids after segmental allergen challenge in allergic asthmatics. We also showed *in vitro* that physiological levels of OSM impair barrier function in differentiated airway epithelium.

Objective—We sought to determine which hematopoietic or resident cell type(s) were the source of the OSM expressed in 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 NP and matched blood samples for flow cytometric analysis. Neutrophils were isolated from whole blood, cultured with the known OSM inducers GM-CSF and FSTL1, and levels of OSM were measured in the supernatants. Bronchial biopsy sections from controls, moderate asthmatics and severe asthmatics were stained for OSM and neutrophil elastase.

Results—OSM staining was observed in NP, showed co-localization with neutrophil elastase (n=10), and did not co-localize with markers for eosinophils, macrophages, T cells or B cells (n=3–5). Flow cytometric analysis of NP (n=9) showed that 5.1±2% of $CD45^+$ cells were OSM^+, and of the OSM^+ cells, 56±7% were $CD16^+Siglec8^-$, indicating neutrophil lineage. Only.6±.4% of

CD45$^+$ events from matched blood samples (n=5) were OSM$^+$, suggesting that elevated OSM in CRS was locally stimulated and produced. A majority of OSM$^+$ neutrophils expressed Arginase 1 (72.5±12%), suggesting a N2 phenotype. GM-CSF was elevated in nasal polyp tissue compared to control, and was sufficient to induce OSM production (p<.001) in peripheral blood neutrophils *in vitro*. OSM$^+$ neutrophils were also observed at elevated levels in biopsies from patients with severe asthma. Additionally, OSM protein was elevated in induced sputum from asthmatic patients compared to controls (p<.05).

Conclusions—Neutrophils are a major source of OSM producing cells in CRS and severe asthma.

Keywords

Oncostatin M; Epithelial barrier; Neutrophils; Chronic rhinosinusitis; Atopic asthma; Granulocyte-Monocyte Colony Stimulating Factor

Introduction

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 (EoE)[1, 2, 3, 4, 5, 6, 7]. Our laboratory has previously shown that the cytokine Oncostatin M (OSM) was elevated in nasal polyps of chronic rhinosinusitis (CRS) patients, in esophageal biopsies from eosinophilic esophagitis (EoE) patients, and in BAL fluids after allergen challenge in allergic asthmatic patients[8]. Physiological levels of OSM were sufficient to induce barrier dysfunction in *ex vivo* cultured airway epithelium as measured by decreased epithelial resistance, increased epithelial permeability, and loss of tight junction structure. Additionally, levels of OSM in both nasal tissue from CRS patients and bronchoalveolar lavage fluid (BAL) from allergen-challenged allergic asthma patients correlated with markers of epithelial leak, suggesting that OSM may mediate barrier dysfunction *in vivo* in 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 mucosal disease in order to understand the mechanism of barrier disruption in 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, 10, 11, 12]. Although osteopontin, prostaglandin E2 (PGE2), follistatin-like 1 (FSTL1), complement factor 5a, and thrombin have all been shown to induce OSM expression in various cell types[13, 14, 15, 16, 17], only GM-CSF was sufficient to induce OSM in neutrophils[11, 18]. Elbjeirami et al. showed that OSM was induced during neutrophil transendothelial migration in response to endothelial production of GM-CSF[18]. Queen et al. showed that co-culture 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[11]. Additionally, Miller et al, have shown that follistatin like-1 (FSTL1) was necessary for OSM induction in a mouse model of chronic asthma[16]. Both GM-CSF and FSTL1 have been shown to be

elevated in airways disease[19, 20, 21, 22], and we hypothesized that one or both of these cytokines may induce neutrophil derived OSM in 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 specifically have been shown to promote tumor growth and metastasis, as well as play an important role in repair processes and the resolution of inflammation[24, 25, 26]. N1 and N2 neutrophil polarization has been best studied in mice, and N2 neutrophils have been shown to express the macrophage mannose receptor (MMR), arginase 1 (ARG1), chitinase-like 3 (Ym1), IL-10 and TGFβ, which are also characteristic markers of M2 macrophages. Both M2 macrophages and N2 neutrophils have been associated with tissue repair mechanisms[25]. Compared to N2, N1 neutrophils have been shown to express more proinflammatory cytokines and chemokines such as TNF, IL-1β, CCL3, CCL5, IL-6 and IL-12[25]. Although progress is being made, N2 neutrophils in humans are much less extensively studied, and the markers used to define N2 neutrophils in mice may 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 CRS. In this manuscript we show that neutrophils are a major source of OSM producing cells in nasal polyps and neutrophils also express OSM in severe asthma. We also show that the OSM inducer, granulocyte-macrophage colony-stimulating factor (GM-CSF), was elevated in nasal polyps 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 the Northwestern Medicine and the Northwestern Sinus Center at Northwestern Medicine. Nasal polyps 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. 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 include: pituitary adenoma (5), chordoma (4), meningioma (2), esthesioneuroblastoma (1), clival metastatic carcinoma (1), inverted Schneiderian papilloma (1) and osteoma (1). Asthma and atopic status were determined by the surgeon treating the patient. Segmental allergen challenge and bronchoalveolar lavage (BAL) were performed in a patient cohort of allergic asthmatics, as previously described[29, 30]. Bronchial biopsies were obtained from asthmatics during bronchoscopy at the Meakins-Christie Laboratories at McGill University Montreal, Quebec 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

Nasal polyp specimens were processed for histology as previously described[34]. Sections were blocked with PBS containing 5% goat serum (Vector Laboratories, Burlingame, Calif) and .5% Triton X-100 (Sigma Aldrich, St Louis, MO) for 1 hour at room temperature (RT), then incubated with either rabbit anti-OSM (N-1, 1:400, Santa Cruz Biotechnology, Dallas, TX), mouse anti-neutrophil elastase (NP57, 1:100, Dako North America, Carpinteria, CA), mouse anti-CD68 (PG-M1, 1:50, Thermofisher), mouse anti-ECP (EG2, 1:1000, Diagnostics Development, Uppsala, Sweden), mouse anti-tryptase (AA1, 1:10000, Thermofisher), mouse anti-CD3 (SP7, 1:1, Thermofisher) mouse anti-CD20 (L26, 1:250, Thermofisher) or rat anti-GM-CSF (BVD2-21C11, 1:100, Abcam, Cambridge, MA) 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(Thermofisher). Slides were mounted with slowfade gold antifade reagent with DAPI counter stain (Thermofisher). To control for background staining, additional slides were stained with the secondary antibody only. Imaging was performed using a Nikon A1R confocal microscope using the 20× objective. Images were processed using ImageJ software.

Flow Cytometric Analysis

Live cells were isolated from nasal polyp tissues obtained during endoscopic sinus surgery. Nasal polyps were incubated overnight on an oscillator in 1mg/mL collagenase I (Worthington Biochemical Corporation, Lakewood, NJ) and 30ug/mL DNase (Worthington Biochemical Corporation) in RPMI at 4 degrees in a gentleMACS tube. Cells were then dissociated using a gentleMACS cell dissociator (Miltenyi Biotec, San Diego, CA) and then passed through a 70 μm cell strainer (BD Biosciences, San Jose, CA). 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 using the LIVE/DEAD Aqua dead cell staining kit (Thermofisher, Waltham, MA), to facilitate gating only on live cells. Cells were then blocked using 10μL of Fc blocking reagent for 15min (Miltenyi Biotec, San Diego, CA) and then cells were stained at RT for 15min with one of 3 panels of antibodies listed in supplemental table E2. Following cell surface staining, cells were then fixed and permeablized using the BD cytofix/cytoperm kit for intracellular staining following manufacturer's protocols (BD Biosciences, San Jose, CA), and cells were stained intracellularly for 30min at 4°C. Cells were analyzed on an LSRII flow cytometer with FACSDiva software. All analysis and compensation were done using FlowJo software (Treestar, Ashland, OR). All flow cytometric studies were prepared using the appropriate single stained control beads (BD Biosciences, San Jose, CA and eBioscience, San Diego, CA) and fluorescence minus one (FMO) controls.

Neutrophil isolation and cell culture

Neutrophils were isolated from peripheral blood using an EasySep direct human neutrophil isolation kit (Stemcell, Vancouver, BC). Cells were then counted and cultured at 10^7

cells/mL in RPMI supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. Neutrophil cultures were treated with GM-CSF (1–25 ng/mL, R&D Systems, Minneapolis, MN), FSTL1 (100–1000 ng/mL, R&D Systems), Interferon-γ (IFNγ) (10 ng/mL, R&D Systems), lipopolysaccharide (LPS) (1 μg/mL, Enzo Life Sciences, Farmingdale, NY), Interleukin 4 (IL-4) (20 ng/mL, R&D Systems), Interleukin 13 (IL-13) (20 ng/mL, R&D Systems), Interleukin 25 (IL-25) (10–100ng/mL, R&D Systems), Interleukin 33 (IL-33) (10–100 ng/mL, R&D Systems), thymic stromal lymphopoietin (TSLP) (10–100 ng/mL, R&D Systems) or Leukotriene C_4 (LTC_4) (10^{-6} –10^{-7} Cayman Chemical, Ann Arbor, MI) 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, ICAM (50ng/well, Peprotech, Rocky Hill, NJ) or bovine serum albumin (BSA) (50ng/well, Sigma Aldrich) in pH 8 tris buffered saline (TBS) overnight at 4 degrees. Neutrophils were seeded into the wells and the culture supernatants were harvested at 20 hours.

NHBE cell culture and injury

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

Protein quantification

Oncostatin M protein was detected in neutrophil cell culture supernatants using a DuoSet ELISA kit (R&D Systems, Minneapolis, MN). FSTL1 and GM-CSF protein were detected in neutrophil cell culture supernatants and FSTL1 was detected in sinus tissue extracts using DuoSet ELISA kits (R&D Systems). GM-CSF protein was detected in sinus tissue lysates 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, MA) were detected using 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 0.5μg of total RNA with SuperScript II reverse transcriptase and random primers (Invitrogen, Carlsbad, Calif). Quantitative real-time RT-PCR was performed using an Applied Biosystems 7500 Sequence Detection System (Applied Biosystems, Foster City, Calif) in 20μL reactions (10μL of 2x TaqMan Master mix [Applied Biosystems], 400nmol/L of each primer and 200nmol/L of TaqMan probe plus 10μg of cDNA). Primer and probe sets for *OSM* (Hs00968300_g1) *ARG1* and *MRC1* (Hs00267207_m1) were purchased from Applied Biosystems. Primers (Forward:GAAGGTGAAGGTCGGAGTC, Reverse: GAAGATGGTGATGGGATTTC) and probe (6-VIC-CAAGCTTCCCGTTCTCAGCC-MGB) for *GAPDH* were synthesized by IDT technologies (IDT Technologies, Coralville, IA) 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 gene expression omnibus: GSE36830.

Statistical Analysis

All data were analyzed using GraphPad Prism 6 software (GraphPad Software, La Jolla, Calif). All data are reported as mean ± SEM. Differences between groups were analyzed using a non-parametric Kruskal-Wallis ANOVA or Mann-Whitney U test. Correlations were assessed using the Spearman correlation. Significance: *p<0.05, **p<0.01, ***p<0.001, ****p<.0001.

Results

Neutrophils are a major source of OSM in Nasal Polyps

To determine which cell type was making OSM in CRS, we first obtained sections from nasal polyps 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 co-localization of OSM with markers for macrophages (Figure 1A–B) or eosinophils (Figure 1C–D), while we observed some co-localization with a minor population of mast cells (Figure 1E–F). We also did not observe co-localization of OSM with T cells or B cells (data not shown). Elastase positive cells made up the majority of the OSM positive cells, suggesting that neutrophils are an important source of OSM (Figure 1G–H). Control images with no primary antibody for CD68, ECP, tryptase, and elastase staining strategies are shown in Figure E1A–D. Further analysis of our previously published microarray database (GEO: GSE36830) showed that *OSM* mRNA expression correlated with the granulocyte marker CD16 *(FCGR3)* (r = .61, p<.01; Figure E1E), and the neutrophil specific protease, cathepsin G *(CTSG)* (r = .56, p<.01; Figure E1F) in nasal polyps and uncinate tissue from control, CRSsNP and CRSwNP patients, suggesting a potential relationship between *OSM* expression and neutrophils[36]. While *FCGR3* is not neutrophil specific, *FCGR3* and *CTSG* correlated with each other in the database (r =.56, p<.01; Figure E1G), suggesting that neutrophils are responsible for much of the *FCGR3* expression in sinonasal tissue. We also observed a positive correlation between elastase and OSM using lysates from both nasal polyps, and uncinates from control, CRSsNP, and CRSwNP patients (r = .65, p<.01; Figure E1H).

Flow cytometric analysis of OSM producing cells in nasal polyps

To further validate the observation that neutrophils are a major source of OSM producers in nasal polyps, we utilized flow cytometry. Live cells were isolated from nasal polyps, and stained with CD45, OSM, CD16 and Siglec-8. Within the CD45 gate, 5.1±2% of the cells were OSM^+, and the OSM^+ cells were determined using the appropriate FMO control. Of the OSM^+ cells, 29.8±8% were excluded during analysis of the data because the wavelengths of the fluorophores used to stain for CD16-APCH7 siglec 8-Alexa 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 two main populations; the major population was

CD16^{+}Siglec-8^{-} (80.4±5%), which were neutrophils, and the minor population was CD16^{+}Siglec-8^{+} (14.1±5%), which were either mast cells, basophils, or eosinophils. We did observe a small CD16^{-}Siglec-8^{-} population (5.5±3%) that was present in less than half of the polyps we analyzed (Figure 2A–C). Additionally, neutrophils were isolated from peripheral blood and nasal polyp tissue of five matched CRSwNP patients, and OSM expression in blood neutrophils was only minimally observed in one patient (.6±.4%), indicating that *in vivo* OSM induction was likely a locally induced event (Figure 2D). The full gating strategy for this antibody panel, including the OSM FMO control is available in Figure E2.

OSM was expressed in a minor population of mast cells

To further identify the cells within the minor CD16^{+}Siglec8^{+} population of OSM producers, we stained live cells isolated from nasal polyps with CD45, OSM, CD16, FcεRI, c-kit, and either EMR-1 or Siglec8. In the CD45^{+} gate, 4.6±2% events were OSM^{+}. Within the OSM^{+}, 15.6±10% were FcεRI^{+}c-kit^{+}, indicating mast cells, 3.7±1% were FcεRI^{+}c-kit^{-}, indicating basophils, and of the FcεRI^{-}c-kit^{-} cells, only 2.3±1% were emr1^{+} or siglec8^{+}, which would indicate eosinophils, leaving the remaining 73.3±11% of the cells neutrophils (Figure 3). The full gating scheme for this antibody panel is available in Figure E3, and the sample chosen as the representative example was selected to best show the gating strategy for basophils and mast cells.

GM-CSF was elevated in nasal polyps, and induces neutrophil derived OSM production *in vitro*

Since GM-CSF and FSTL-1 have been shown to be important inducers of OSM, our next aim was to determine whether GM-CSF and/or FSTL1 may be playing 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 and/or FSTL1[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 1000ng/mL did not induce OSM in purified neutrophils; the combination of 1 or 25 ng/mL GM-CSF with 1000ng/mL FSTL1 was not more active than GM-CSF alone (Figure 4A). This data was analyzed using both mean and median and the statistics were the same; the data represented in Figure 4A shows the mean values. Analysis of *OSM* mRNA following 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 levels of *OSM* were not elevated compared to control. (Figure E4A). 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 (25ng/mL), IL-25 (10–100 ng/mL), IL-33 (10–100 ng/mL), TSLP (10–100 ng/mL) or leukotriene C_4 (10^{-6}–10^{-7} M) Figure E4B). 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 (Figure E4C).

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 normal human bronchial epithelial (NHBE) cells that were grown at air-liquid interface, and when they were fully differentiated they were treated with heat killed *Staphlococcus aureus* (HKSA) as described[39]. Exposure for 48 hr to HKSA at a concentration of 5×10^8 particles/mL induced GM-CSF secretion into cell culture supernatants (Figure 4B). Although HKSA can activate epithelium by 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. Since GM-CSF was sufficient to induce neutrophil-derived OSM secretion, we next hypothesized that GM-CSF would be elevated in nasal polyps compared to control UT. To do this, we measured GM-CSF and FSTL1 protein expression in nasal polyps and UT from CRS patients and controls. GM-CSF was elevated in nasal polyps compared to control UT while FSTL1 was not elevated, suggesting that GM-CSF could be responsible for induction of neutrophil OSM expression in nasal polyps (Figure 4C, D).

To determine the cell types responsible for GM-CSF production in nasal polyps, we stained nasal polyp sections (n=4) for OSM, neutrophil elastase, and GM-CSF (Figure 4E and Figure E4D–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 may have an autocrine pathway by which GM-CSF expression prolongs their own survival and promotes expression of OSM. We did not observe GM-CSF expression in the epithelium in nasal polyps.

OSM producing neutrophils may have a non-classical phenotype

We next tested whether OSM-producing neutrophils were phenotypically classical, inflammatory N1 neutrophils or tissue repair-associated N2 neutrophils. We utilized flow cytometry, and stained cells isolated from nasal polyps for CD45, OSM, CD16, IL-5R, myeloperoxidase (MPO) and Arg1 (Figure E5A). No flow cytometry antibodies were available for elastase, so 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 $CD16^+IL\text{-}5R^-$ neutrophils. In the neutrophil gate, we found two populations; $MPO^{hi}Arg1^+$ (72.5±12%) and $MPO^{lo}Arg1^-$ (22.1±8%) (Figure 5A–B). The expression of Arg1 suggests that the majority of neutrophils taken from polyp tissue *in vivo* were not classical N1 neutrophils. As discussed above, GM-CSF and FSTL1 are two potential inducers of OSM *in vivo*. To determine whether GM-CSF induced neutrophils to assume a non-classical phenotype, we compared neutrophils treated *in vitro* with GM-CSF to neutrophils that were polarized either toward an N1 phenotype using LPS and IFNγ 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 in the supernatants in either the N1 or N2 polarizing conditions (Figure 5C). To determine whether neutrophil phenotypes influence OSM expression, we polarized neutrophils to N1 or N2

conditions, or left them unstimulated for 24 hours. Following polarization, the neutrophils were either left unstimulated or stimulated with GM-CSF. Unactivated, N1 and N2 neutrophils had no difference in expression of OSM, 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 mannose receptor (MMR) and arginase-1 (Arg1). Neither GM-CSF or FSTL1 or their combination caused any induction of Arg1 (Figure E5B). However, GM-CSF and FSTL1 were both sufficient to induce elevated mRNA for another N2 marker, *MRC1*, suggesting that GM-CSF and FSTL1 may induce neutrophils to assume elements of an N2-like phenotypic state (Figure 5D).

Neutrophil expression of OSM in asthmatic patients

To determine whether neutrophils were also a source of OSM in asthma, we stained bronchial biopsies from control patients (n=4) as well as those with moderate (n=5) and severe (n=6) asthma for OSM and neutrophil elastase (Figure 6A). We counted neutrophils and OSM^+ cells in each specimen, and the tabulated data are represented in Table E3. We did not observe any OSM^+ cells in the control biopsies, but we detected OSM^+ cells in 60% of biopsies from moderate asthmatics and 100% of biopsies from severe asthmatics (Figure E6A). Of the neutrophils counted in each specimen, 0±0% were OSM positive in control biopsies, 14.0±9.8% were positive in moderate asthmatic biopsies, and 30.1±10.2% were OSM positive in severe asthmatic biopsies (Figure E6B). Additionally, of the OSM^+ cells counted, 0±0% were neutrophils in control biopsies, 35.0±21.8% were neutrophils in moderate asthmatic biopsies, and 52.1±15.9% were neutrophils in severe asthmatic biopsies (Figure 6B). Neutrophil counts were normalized per mm of basement membrane within the biopsy section, and no differences in the total neutrophil counts/mm basement membrane were observed between controls and asthmatics, suggesting an increased state of activation of neutrophils rather than increased neutrophil numbers (Figure E6C).

To further test whether OSM is produced in the airways of patients with asthma, we evaluated a set of induced sputum samples from controls and asthmatic subjects. We observed elevated levels of OSM in induced sputum from asthmatic patients compared to controls (Figure 6C). 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 the BAL (Figure E6D–E, H), but not with total eosinophil counts or total macrophage counts (Figure E6F–G). Additionally, we previously reported that a concentration of 50pg/mL of GM-CSF was measured in the BAL in a separate cohort of allergen challenged allergic asthmatics, suggesting that GM-CSF may 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 may play a role in epithelial barrier dysfunction.

Discussion

We have previously showed that OSM was elevated in nasal polyp tissue from CRS patients, and that physiological levels of OSM are sufficient to induce profound loss of barrier function in differentiated bronchial and nasal epithelial cells cultured at air-liquid interface[8]. We hypothesized that elevated OSM in CRS may mediate epithelial barrier dysfunction *in vivo*. Nasal polyps have been shown to express robust type 2 inflammation[41, 42, 43], and we originally speculated that the OSM producers in nasal polyps were likely to be eosinophils, M2 macrophages, or mast cells. In the present manuscript, we found rather that neutrophils are a major population of cells expressing OSM in CRS tissues (Figure 1–3). We also identified a minor population of OSM^+ cells that were siglec 8^+, and these cells were found to be mast cells (Figure 2–3). 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 CRSsNP, CRSwNP and in certain forms of asthma, and production of OSM by neutrophils could participate in both ongoing epithelial repair as well as promote the epithelial barrier dysfunction that is observed in these diseases. While we did not investigate whether neutrophils were responsible for OSM production in CRSsNP, we suspect that neutrophil-derived OSM may also play a role in barrier dysfunction in this non-polypoid 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 mucosal disease may prolong or even perpetuate the state of the epithelial repair process (Figure 7). OSM has been shown to induce epithelial-mesenchymal transition (EMT)[46], which antagonizes epithelial differentiation, and expression of EMT biomarkers is well established in both CRS and asthma[47].

Neutrophils are not classically associated with type 2 immune responses, and are generally considered to be a type 1 immune response effector cell. However, neutrophilic subsets of CRS and asthma have been described. While nasal polyps in Western countries tend to be highly eosinophilic, a significant proportion of nasal polyps from patients in Eastern Asian countries are neutrophilic[214, 215, 216]. In unpublished studies not shown, we found that nasal polyps, that are highly eosinophilic in our Chicago cohort, nonetheless have significant levels of neutrophils and neutrophil elastase. Increased neutrophilia within nasal polyps has been linked to decreased responsiveness to corticosteroid treatment[217]. Severe asthma is a subtype of asthma that is often characterized by neutrophilia[218, 219]. It is thought that neutrophilia in asthma associates with more severe disease, again, because neutrophils are not particularly responsive to corticosteroid treatment[220]. 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 may be capable of playing a pathogenic role in eosinophilic mucosal disease through the 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, 24, 25, 26]. We showed that the majority of OSM$^+$ neutrophils from nasal polyps expressed the type 2 marker Arg1 (Figure 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 elevated MMR compared to unstimulated neutrophils and N1 polarized neutrophils, also indicating that OSM producing neutrophils could be skewed toward the N2 phenotype (Figure 5). Since 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 two stimuli, only GM-CSF induced OSM production. However, since we did not see any induction of Arg1 in response to GM-CSF, another factor may be necessary for full induction of the N2 phenotype. These studies implicate GM-CSF as a potential inducer of OSM in neutrophils *in vivo*. Since circulating neutrophils do not express OSM in patients, this induction likely takes place within nasal polyp or lung tissue where these inducers can be found.

Interestingly, immunofluorescent staining showed that GM-CSF localized to OSM-producing neutrophils in nasal polyps, suggesting that OSM producing neutrophils may induce their own OSM through autocrine production of GM-CSF (Figure 4E). There is precedent for such a concept, as Wardlaw and colleagues showed that eosinophils produce GM-CSF to prolong their own survival[48]. In addition, Durand et al, have shown that autoantibody ligation of FcγRIIIb (CD16) receptors on the surface of neutrophils protects against apoptosis through the induction of autocrine GM-CSF[40]. We have previously shown elevated levels of autoantibodies in nasal polyps, suggesting that elevated autoantibodies in nasal polyps may trigger GM-CSF expression in the infiltrating neutrophils by a similar mechanism[49]. Whether production of OSM by neutrophils via an autocrine GM-CSF dependent pathway occurs in CRSwNP and asthma is worthy of further investigation.

Importantly, we have also shown that OSM was elevated in induced sputum from asthmatics compared to controls and that OSM$^+$ cells were present in bronchial biopsies from 100% of severe asthma patients, 60% of moderate asthma patients and none of the healthy controls. These results suggest that the 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 severe asthmatics and 35% of the neutrophils were OSM$^+$ in moderate asthmatics, suggesting that neutrophil derived OSM plays a role in the pathogenesis of asthma. OSM has previously been implicated in the induction of fibrosis in a mouse model of asthma, though it was derived from macrophages[16, 50].

Given that GM-CSF may 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 like 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, monoclonal antibodies against GM-CSF, and a monoclonal antibody against GM-CSFRα.

Clinical trials have been conducted with monoclonal antibodies against GM-CSF (MOR103, KB003, and namilumab) in rheumatoid arthritis, plaque psoriasis, and severe asthma. A phase II trial was conducted using KB003 in asthmatic patients that were poorly controlled with long-acting bronchodilators and either inhaled or oral corticosteroids. They did not observe improved forced expiratory volume in 1 second (FEV_1) in the entire patient cohort. However, when they separated patients based on markers of poorly controlled asthma, peripheral blood eosinophilia, low baseline FEV_1, and high bronchodilator reversibility, they did detect an improvement in FEV_1 in response to treatment with KB003[51].

In summary, we have shown that neutrophils are a major source of OSM producing cells in nasal polyps using both immunofluorescence and flow cytometric analyses. We have also shown that GM-CSF is elevated in nasal polyps, and that physiological 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 nasal polyps, 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 biopsies from severe asthmatics, and approximately half of the OSM producing cells are neutrophils, suggesting that neutrophil-derived OSM may also play a role in asthma. Taken together, these studies further implicate OSM in barrier dysfunction in CRSwNP as well as in asthma, and suggest that the OSM found in these diseases is produced by neutrophils. Therapeutic intervention targeting either GM-CSF or OSM may thus be beneficial in the treatment of CRS and asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ARG1	Arginase 1
BAL	Bronchoalveolar lavage
CRS	Chronic rhinosinusitis
CRSsNP	CRS without nasal polyps
CRSwNP	CRS with nasal polyps

ECP	Eosinophil cationic protein
FEV_1	Forced Expiratory Volume in 1 Second
FSTL1	Follistatin-like 1
GM-CSF	Granulocyte-Macrophage colony stimulating factor
HKSA	Heat killed *staphylococcus aureus*
NHBE	Normal human bronchial epithelial cells
MMR	Macrophage mannose receptor
PMN	Polymorphonuclear leukocyte/neutrophil
OSM	Oncostatin M
UT	Uncinate tissue

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Clinical implications

Neutrophils are a major source of OSM producing cells in CRS, and severe asthma. GM-CSF was elevated in CRS, at levels sufficient to induce neutrophil derived OSM production. Therapeutic targeting of OSM, neutrophils or GM-CSF may be beneficial in mucosal disease through the restoration of epithelial barrier function.

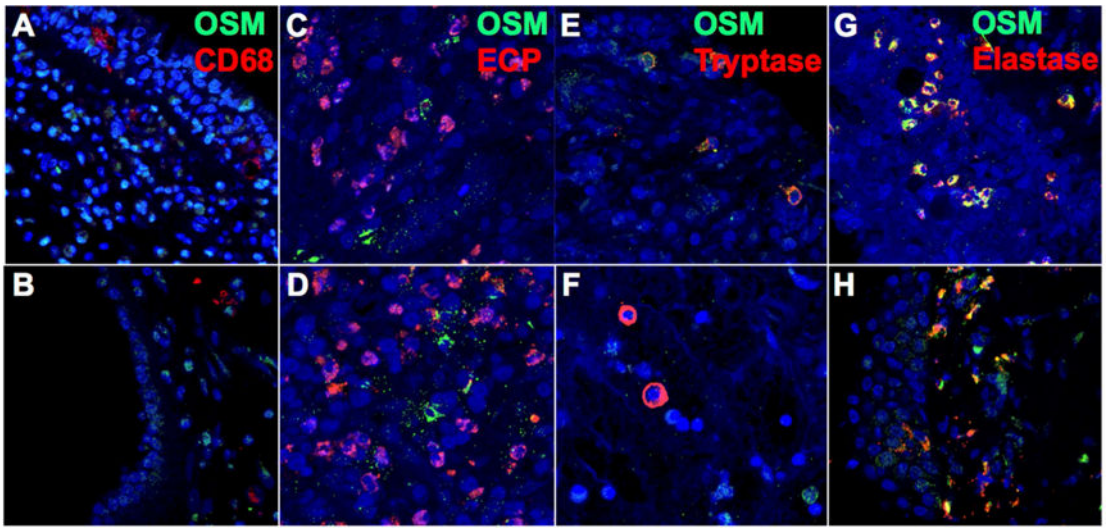

Figure 1. Oncostatin M was expressed in neutrophils

Nasal polyp sections were stained for OSM in green and various cell type specific markers as indicated in red; two representative examples from separate patients are shown for each staining strategy. OSM did not co-localize with macrophages (A, B) (n=3) or eosinophils (C,D) (n=5) and had minimal co-localization with mast cells (E,F) (n=6). OSM did co-localize with neutrophils (G,H) (n=10).

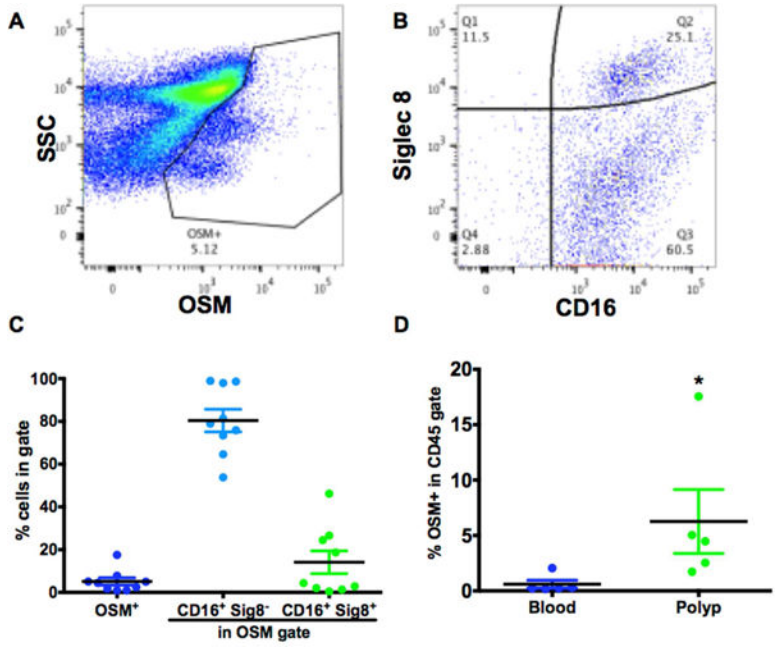

Figure 2. Flow cytometric analysis showed that neutrophils are a major source of OSM producing cells in nasal polyps

(A) Within the CD45 gate, 5.1± 1.7% of cells were OSM^+. (B) Within the OSM^+ gate, 80.4±5% of cells were neutrophils ($CD16^+Sig8^-$) and 14.1±5% of cells were either eosinophils, mast cells or basophils ($CD16^+Sig8^+$). (C) Quantification of the percentage of OSM^+ cells within the $CD45^+$ gate, and the percentage of neutrophils ($CD16^+Sig8^-$), and $CD16^+Sig8^+$ cells within the OSM^+ gate, n=9. (D) In matched blood and nasal polyps, .60± . 36% of $CD45^+$ cells in the blood were OSM^+ while 6.3± 2.9% of $CD45^+$ cells in nasal polyps were OSM^+ (n=5, $p< .05$, Mann-Whitney U test), suggesting that OSM was locally induced, n=5.

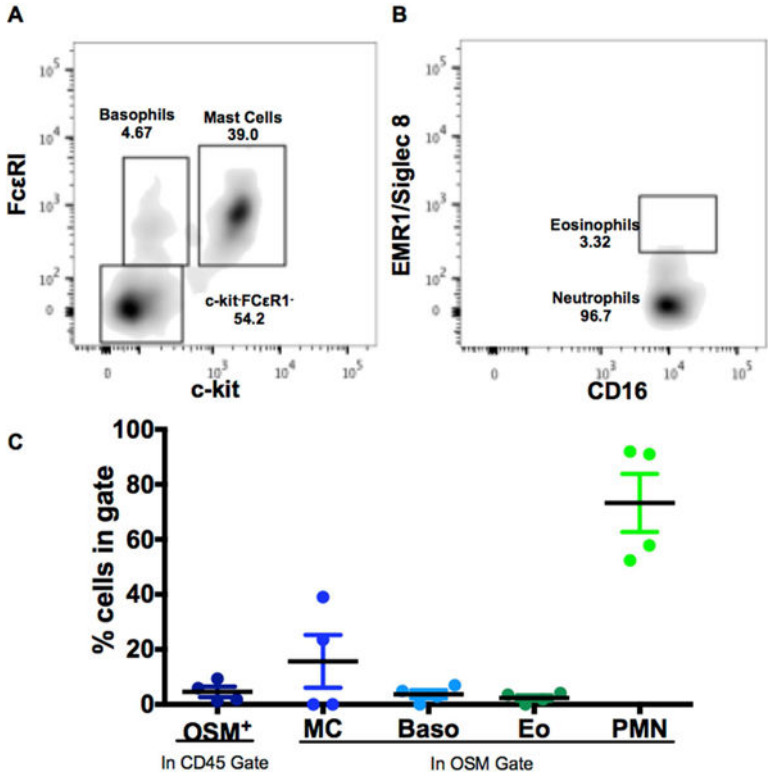

Figure 3. Flow cytometric analysis showed that mast cells were a minor source of OSM producing cells in nasal polyps

(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 the OSM^+ cells. Mast cells (MC) were 15.7±9.6%, basophils (Baso) were 3.7±1.5%, eosinophils (Eo) were 2.3±.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.

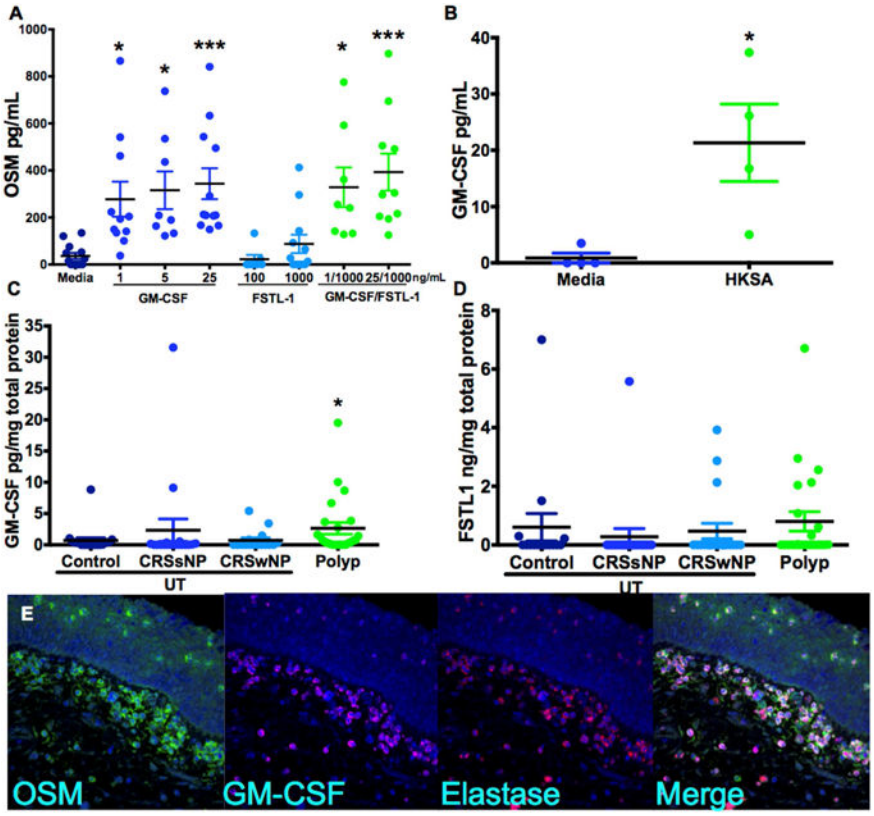

Figure 4. GM-CSF was elevated in nasal polyps and induced neutrophil derived OSM

(A) Blood neutrophils were stimulated with the indicated concentrations of GM-CSF, FSTL1 or both and levels of OSM protein in the culture supernatant were measured; (n=7–13, * p< .05, * p< .001, Kruskal-Wallis test). (B) Fully differentiated NHBE cells were either unstimulated, or stimulated with HKSA, and levels of GM-CSF protein in the cell culture supernatants were measured. HKSA treated NHBE released more GM-CSF, 21.3± 6.9pg/mL, than control NHBE, .87±.87 (n=4, p< .05, Mann-Whitney U test). (C) GM-CSF protein, 2.6±.93pg/mg total protein, was elevated in nasal polyps compared to control UT, .68±.42pg/mg total protein, (n=16–24, p< 05, Mann-Whitney U test). (D) FSTL1 protein, .60±.47ng/mL, was elevated in nasal polyps compared to control UT, .80±.33, (n=15–23, p=.31, Mann-Whitney U test). (E) OSM (alexa 488), GM-CSF (alexa 568) and neutrophil elastase (alexa 647) colocalized in nasal polyps (n=4).

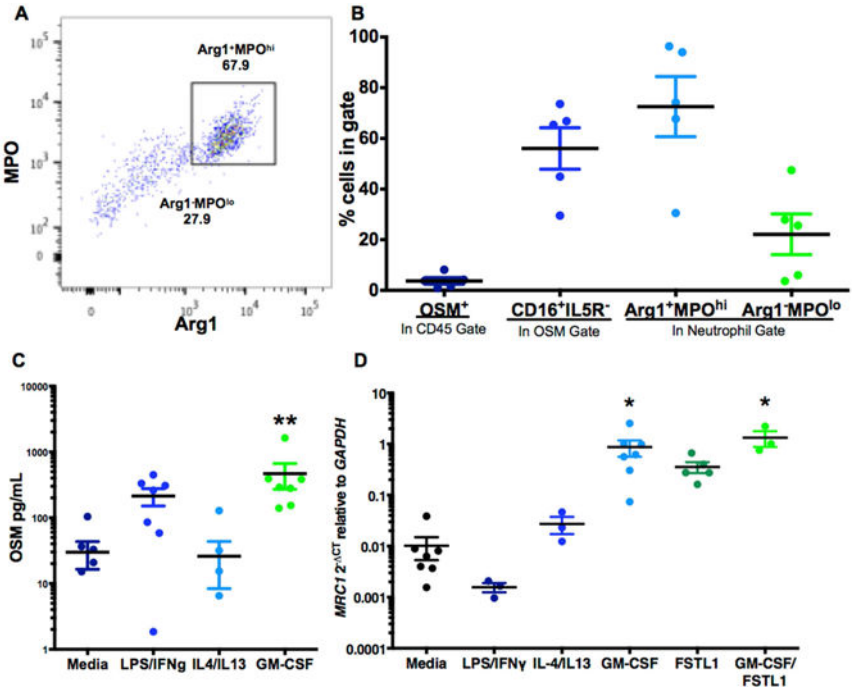

Figure 5. OSM producing neutrophils did not express a classical N1 phenotype

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

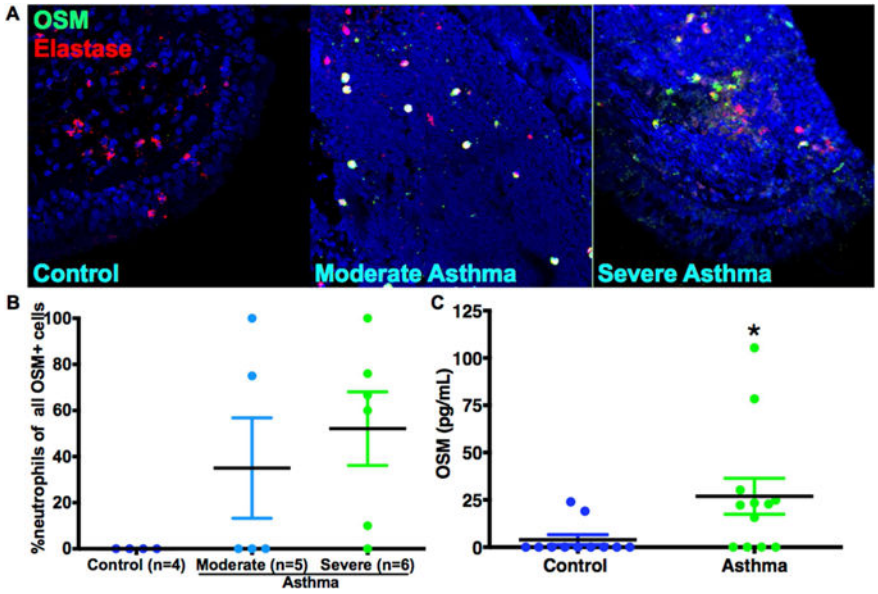

Figure 6. OSM was elevated in asthmatic patients

(A) Bronchial biopsies from control patients, moderate asthmatics and severe asthmatics were stained for OSM in green and neutrophil elastase in red, n=4–6. (B) Counts were obtained of neutrophils and OSM^+ cells; none of the control biopsies had any OSM^+ cells, and of the OSM^+ cells 35± 21.8% were neutrophils in moderate asthmatics, and 52.1± 15.9% were neutrophils in severe asthmatics. (C) Levels of OSM in the sputum of asthmatic patients, 26.9± 9.5pg were elevated compared to control patients 3.9± 2.6pg, (n=11–12, $p < .05$, Mann-Whitney U test).

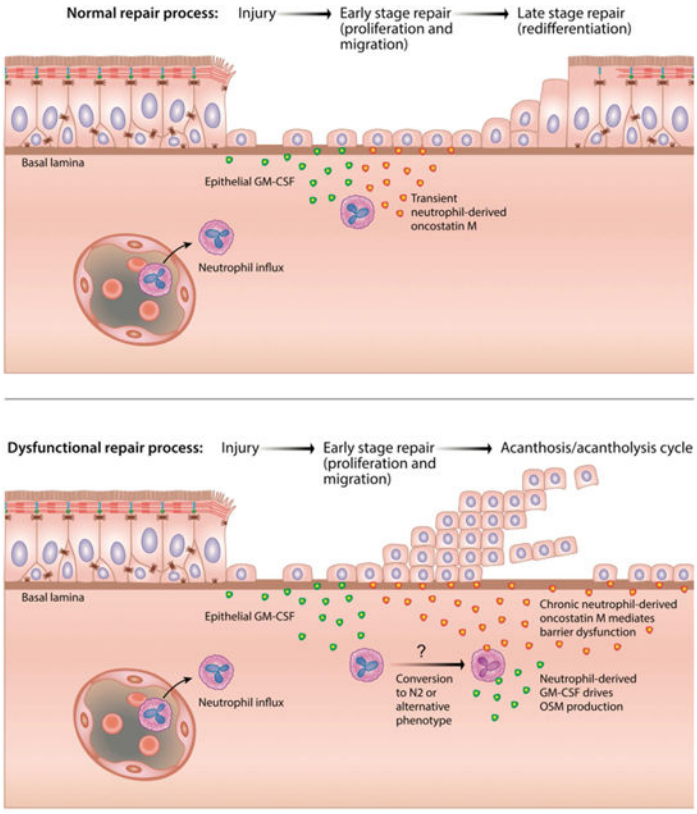

Figure 7. Proposed mechanism of OSM mediated barrier dysfunction in mucosal disease

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. Due to transient OSM expression, once the 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 the neutrophils are converted into an alternative phenotype, potentially N2, that makes both OSM and GM-CSF. The 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 may prevent late stage repair, causing a long-term state of barrier dysfunction.