

Systemic prednisone administration selectively alters granulocyte subsets in nasal polyps from aspirin-exacerbated respiratory disease and chronic rhinosinusitis patients

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Background: Nasal polyps (NPs) are hallmark inflammatory lesions of sinusitis. Despite the spectrum of NP conditions, cellular differences between NPs from patients with chronic rhinosinusitis with NPs (CRSwNP) and aspirin-exacerbated respiratory disease (AERD) are poorly understood. NPs are associated with abundant eosinophils; the contributions of neutrophil and basophil granulocytes are less defined. We therefore sought to assess granulocyte subpopulations, and differential effects following prednisone pretreatment, within NPs of CRSwNP and AERD patients.

Methods: NPs, adjacent ethmoid sinus tissue, and peripheral blood mononuclear cells (PBMCs) were obtained from patients undergoing endoscopic sinus surgery. Samples from 5 cohorts: CRSwNP \pm prednisone ($n = 6$ each), AERD \pm prednisone ($n = 6$ each), and controls ($n = 9$), were analyzed by high-dimensional flow cytometry to gate granulocyte populations. Specimens were also assessed using immunohistochemistry (IHC) staining.

Results: Systemic prednisone administration was associated with a lower frequency of eosinophils ($p < 0.0001$, $n = 6$) in NPs in both CRSwNP and AERD patients, whereas a decrease in neutrophils ($p = 0.0070$, $n = 6$) in NPs was only observed in CRSwNP patients after prednisone treat-

ment. In contrast, steroids do not alter basophil proportions ($p = 0.48$, $n = 6$) within NPs from either group. No significant shift in granulocyte subsets after steroid treatment was identified in the adjacent ethmoid mucosa or PBMCs from the same patients. Immunohistochemistry (IHC) staining supported these findings.

Conclusion: Granulocyte subpopulations are focally affected within NPs by systemic steroid exposure, without notable granulocyte alterations in the surrounding regional tissues. These data provide direct insights into the cellular effects of routine prednisone exposure in CRS patients, and highlight a unique microenvironment present within NP lesions. © 2013 ARS-AAOA, LLC.

Key Words:

flow cytometry; chronic rhinosinusitis; nasal polyp; aspirin-induced asthma; regulatory T cell; glucocorticoid; prednisone; eosinophil; neutrophil; granulocyte

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Chronic rhinosinusitis (CRS) is an inflammatory disease of the paranasal sinuses that affects more than 24 million individuals in the United States annually.¹ CRS is a heterogeneous group of inflammatory upper airway diseases that can be subdivided into 2 major categories: those patients with CRS who do not develop nasal polyps (CRSsNP), and CRS accompanied by nasal polyp formation (CRSwNP).² An additional subset of patients with CRSwNP are those with aspirin-exacerbated respiratory

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disease (AERD), sometimes referred to as Samter's triad, aspirin-induced asthma, or aspirin intolerance. AERD, occurring in approximately 7% of all asthmatic patients, is characterized by an excessive production of systemic cysteinyl leukotrienes and clinically by asthma, aspirin sensitivity, and nasal polyposis.³ Moreover, AERD patients present with the most recalcitrant form of nasal polyposis among CRS patients, with a higher likelihood of NP recurrence and lower long-term rate of NP control after surgery compared with CRSwNP patients.⁴⁻⁶

NPs are circumscribed, noninfiltrative benign lesions arising from sinus mucosa that have been generally characterized by an abundance of T helper 2 (Th2) cytokines, and the accumulation of eosinophils and lymphocytes within the polyp substance.⁷⁻¹⁰ Isolated studies have been performed to determine immune effector cell subpopulations in the sinus mucosa from CRSwNP patients,^{11,12} but a comprehensive simultaneous analysis comparing the immunologic environment within NPs to relevant regional and systemic compartments in the same patient has not been performed. This type of subsite analysis between NP, adjacent unaffected ethmoid mucosa, and blood in afflicted patients may elicit true cellular and microenvironmental disparities within NP lesions, and provide further insight into the pathogenesis of subtypes of CRSwNP.

Current treatments for afflicted patients with CRSwNP are limited, and include the use of topical and oral steroids,¹³ and ultimately to repeated rounds of sinus surgery to mechanically reduce the obstructive burden secondary to NPs. Administration of systemic corticosteroids such as prednisone is known to transiently improve quality of life and hyposmia,¹⁴⁻¹⁶ and has been shown to reduce inflammation and sputum cytokines in severe asthmatic patients.¹⁷ Isolated studies have demonstrated inhibitory effects of glucocorticoids on epithelial cells in NPs as well as a reduction in eosinophils and basophils in skin blisters after prednisone administration.^{18,19} Distinguishing the immunologic microenvironment housed within NP lesions, and any direct cellular changes conferred upon steroid exposure, may further assist in understanding this disease process.

Initial studies on CRS have focused on granulocyte populations, in particular eosinophils and neutrophils, and recent studies suggest that these granulocytes serve a fundamental role in the inflammatory response in CRS.²⁰ Another hallmark of inflammation is neutrophil recruitment, and these cells have been shown to infiltrate nasal tissue in CRSwNP patients via interleukin 8 (IL-8) chemotaxis.²¹ As neutrophils are the most abundant leukocytes in the blood, migration and activation of neutrophils to tissues typically occur when the microenvironment is perturbed, and can be correlated with myeloperoxidase enzyme expression.²² However, a comprehensive analysis of granulocyte subsets in different nasal tissues is lacking. Therefore, in this study, using high-dimensional flow cytometry, we attempted to quantitatively assess granulocyte subsets (eosinophils, neutrophils, basophils) in NPs, the unaffected adjacent eth-

moid sinus mucosa, and circulating peripheral blood between CRSwNP and AERD patients versus appropriate controls.

Patients and methods

Subjects and specimens

A total of 33 patients were included in this 10-month study, which was approved by the approved by the Institutional Review Boards of both Stanford University School of Medicine (Protocol ID: 18981, Stanford, CA) and the University of Colorado School of Medicine (COMIRB 11-1442, Aurora, CO). Subjects were recruited, and received informed consent, from the clinics within the Departments of Otolaryngology-Head and Neck Surgery at Stanford University in Stanford, CA and the University of Colorado in Aurora, CO. Individual patient data was obtained from questionnaires completed by the attending physician as well as from the medical records. NP, ethmoid sinus tissue, and blood samples were collected intraoperatively from the same individual patients. NP patients (n = 24) were divided into 2 main cohorts: CRSwNP ± prednisone (n = 6 each) and AERD ± prednisone (n = 6 each). Controls (n = 9) were also divided into 2 groups: normal (n = 3) and CRSsNP ± prednisone (n = 3 each).^{23,24} Patients placed on preoperative steroids received oral prednisone at a dose of 30 mg daily for 1 week prior to surgery. The assignment of patients to receive oral steroids was at the discretion of the treating physician and not randomized. Those patients without any history oral steroid treatment in the past 3 months were included in the nonsteroid cohort. Normal controls (n = 3) were recruited from subjects undergoing endoscopic transsphenoidal pituitary surgery. These patients had nonsecreting benign pituitary adenomas by final pathology. Furthermore, each showed no history of CRS or asthma and presented with normal preoperative imaging of the sinuses and normal sinonasal examinations intraoperatively. NPs and ethmoid sinus mucosa were isolated in the CRSwNP and AERD groups; mucosa from the ethmoid sinus alone was isolated in the CRSsNP group, and tissue from the sphenoid rostrum and peripheral regions of this sinus was used in the control group. Ten milliliters (10 mL) of peripheral blood was collected intraoperatively into heparinized tubes from all patients.

Antibodies

Fluorescent conjugated monoclonal antibodies against CD3, CD20, CD16, CD41a, CD45, CD66b, CD123, Ep-CAM, HLA-DR, and isotype controls were purchased from BD Biosciences (San Diego, CA). The full list of antibodies used in flow cytometry is presented in Table 1.

High-dimension 11-color flow cytometry

Peripheral blood mononuclear cells (PBMCs) from all patients were isolated by Ficoll-Hypaque density gradient centrifugation. Cells were then washed twice with

TABLE 1. Antibodies used in flow cytometry

Target antigen	Clone	Conjugation
CD45	HI30	Pacific Orange
EpCAM	EBA-1	PE
CD66b	G10F5	FITC
CD16	3G8	V450
CD41a	HIP8	APC
CD20	2H7	APC-H7
CD3	SK7	APC-H7
CD123	7G3	PerCP-Cy5.5
HLA-DR	G46-6	PE-Cy7

phosphate buffered saline (PBS) and used immediately. All nasal tissues from NPs, ethmoid sinus, and sphenoid sinus were washed twice with PBS containing 2 mM PBS/ethylenediamine tetraacetic acid (EDTA). Tissue samples were mechanically dissociated using microscissors and washed with PBS/EDTA. Single-cell suspensions were prepared by passing the tissue through a 45- μ m nylon mesh filter and collected in Roswell Park Memorial Institute (RPMI) culture medium containing 10% fetal bovine serum (FBS). Red blood cells were removed using red blood cell lysis buffer (BioLegend, San Diego, CA). Tissue samples retrieved naturally varied in size (range, 200–600 mg; average weight, 425 mg) with each patient and surgical field present. Therefore, all samples were uniformly treated with the same cell isolation protocol.

Single-cell suspensions obtained from fresh preparations were stained with a combination of anti-human fluorochrome-conjugated antibodies against CD3, CD20, CD16, CD41a, CD45, CD66b, CD123, EpCAM, HLA-DR, and appropriate isotype controls (BD Biosciences, San Diego, CA) (Table 1). The concentration of antibodies used for cell staining was according to manufacturer's instructions. A total of 1×10^6 cells per group were stained at room temperature for 25 minutes, washed in 1640 RPMI media (1600 revolutions per minute [rpm] or 515g, 5 minutes), and subsequently analyzed fresh without fixation. Propidium iodide 1 μ M was added to all samples prior to data collection to identify dead cells. High-dimension flow cytometry data were collected, with equal numbers of cells acquired (200,000 events) per sample, using an LSRII fluorescence-activated cell sorting (FACS) instrument (BD Biosciences). FLOWJO (TreeStar, San Carlos, CA) software was used for fluorescence compensation and analysis. Data are depicted as contour plots displaying fluorescence intensity (FI) axes plotted against cell numbers/FI interval with a total of 256 intervals per parameter. To eliminate possibilities of sample variation and pipetting error, samples from individual patients were stained simultaneously from a sin-

gle cocktail of antibodies with flow cytometry acquisition performed on the same day for all tissue subsites.

For each patient, both fresh peripheral blood and associated fresh nasal tissue samples were stained and acquired using FACS in parallel on the day of receipt (same day of surgery at Stanford University, or following overnight shipment on ice from the University of Colorado) to minimize the chances of artifactual results between samples. Approximately 70% of acquired events represented live cells from nasal tissue samples, while 99% of cells represented live cells from blood samples. Gating for a given patient's samples was determined based on the gating coordinate of blood for that patient, and inclusion gates were adjusted appropriately for that patient. Although gating thresholds varied slightly, samples generated similar analyses as gates were always normalized to blood for that patient.

Immunohistochemistry

Fresh NPs and ethmoid sinus mucosa from CRS patients were formalin-fixed and paraffin-embedded (FFPE) using standard protocols. Four micrometer-thick (4- μ m-thick) sections from paraffin-embedded tissue blocks were deparaffinized in xylene and hydrated in a series of graded alcohols. For neutrophil detection, sections were blocked for 10 minutes and stained with ready-to-use myeloperoxidase antibody and DAKO Envision kit (DAKO Corporation, Carpinteria, CA). Sections were counterstained with hematoxylin and subsequently dehydrated in alcohol and xylene before mounting. For the detection of eosinophil granules, sections were stained for 5 minutes in working Weigert's iron hematoxylin–Biebrich scarlet solution. Slides were differentiated in 1% HCl in 70% alcohol/water. Sections were rinsed in water and then subsequently dipped in lithium carbonate solution. After dehydration in alcohol and xylene, sections were mounted. Immunohistochemistry (IHC) experiments were performed in duplicate.

Immunostained sections were examined on a Zeiss Axio Imager 2 microscope. Microscopy images were captured using AxioCAM digital microscope cameras and Axio-Vision image processing (Carl Zeiss Vision, Oberkochen, Germany). The images were acquired using a 20 \times objective, resulting in an overall magnification of $\times 200$.

Statistical analysis

All statistical procedures were performed with Prism 5 Software (GraphPad Inc, San Diego, CA). Values of *p* were determined by applying a 2-tailed Student *t* test for independent samples assuming equal variances on all experimental data sets. Comparisons between the 4 polyp patient groups were performed by using an analysis of covariance (ANCOVA) model. Values of *p* < 0.05 were considered significant.

TABLE 2. Clinical characteristics of patient cohorts

	Control patients			Polyp patients			
	Normal	CRSsNP (-prednisone)	CRSsNP (+prednisone)	CRSwNP (-prednisone)	CRSwNP (+prednisone)	AERD (-prednisone)	AERD (+prednisone)
Patients (n)	3	3	3	6	6	6	6
Sex (male:female)	2:1	2:1	2:1	3:3	3:3	2:4	3:3
Age (years)	32 ± 13	50.7 ± 24.3	46.7 ± 11.8	43 ± 17	47.3 ± 14.6	41.2 ± 5.3	47.7 ± 6.5
Alcohol	1/3	1/3	0/3	2/6	1/6	3/6	1/6
Smoking	0/3	1/3	2/3	1/6	0/6	1/6	1/6
Revision FESS patient	0/3	1/3	0/3	1/6	3/6	1/6	3/6
Asthma	0/3	1/3	2/3	3/6	3/6	6/6	6/6
Endoscopic score (Lund-Kennedy)	0.0	2.3 ± 0.6	4.3 ± 0.6	8.2 ± 2.1	11.3 ± 3.1	9.3 ± 3.2	11.8 ± 1.3
CT score (Lund-Mackay)	0.0	9.0 ± 2.0	13.7 ± 5.9	14.3 ± 4.6	17.8 ± 5.9	16.0 ± 2.3	18.8 ± 2.1

Values are means ± SDs, number of patients, or number of patients out of the total number of patients in the column.

AERD = aspirin-exacerbated respiratory disease; CRS = chronic rhinosinusitis; CRSsNP = CRS without nasal polyps; CRSwNP = CRS with nasal polyps; CT = computed tomography; FESS = functional endoscopic sinus surgery; SD = standard deviation.

Results

Patient characteristics

Patients from 5 cohorts were used for this study: CR-SwNP ± prednisone (n = 6 each), AERD ± prednisone (n = 6 each), and controls (n = 9). The control group consisted of non-NP patients that can be subdivided into 3 categories: normal (n = 3) and CRSsNP ± prednisone (n = 3 each). There were no notable differences in patient characteristics between groups. Additional patient information is summarized in Table 2.

Steroid treatment reduces the eosinophil (CD66b⁺CD16⁻) and neutrophil (CD66b⁺CD16⁺) numbers in NPs

For each consented patient, NPs, adjacent ethmoid sinus tissue, and blood were collected in the process of performing standard comprehensive bilateral endoscopic sinus surgery. Initially, we resolved different granulocyte subsets in CRS patients (Fig. 1A). Eosinophils (CD66b⁺CD16⁻) and neutrophils (CD66b⁺CD16⁺) were classified according to relative CD16 and CD66b expression.^{25,26} Remaining granulocytes were resolved for basophils using further subgating by CD41, CD123, and HLA-DR. Analysis of 12 CRSwNP patients and appropriate controls revealed differences in granulocyte subsets in NPs from those patients treated preoperatively with prednisone (Fig. 1B). Steroid exposure was associated with a reduction in the cell density plots of eosinophils and neutrophils only in the NPs from CRSwNP patients, whereas basophil proportions remained unchanged. Interestingly, no decrement in the frequencies of granulocyte subsets in both the adjacent ethmoid sinus

mucosa and PBMCs was observed following preoperative steroid exposure.

Steroid treatment differentially alters granulocyte subsets in NPs in AERD and CRSwNP patients

Further analysis of AERD patients revealed a reduction of eosinophil numbers without apparent changes in neutrophil frequencies after steroid treatment. An associated reduction in eosinophil proportions is seen in both CR-SwNP and AERD NPs following exposure to steroids, but the loss of neutrophils within NPs found in CRSwNP patients is not seen in AERD patients under the same conditions (Fig. 2A). When depicted graphically, eosinophil ($p < 0.0001$) and neutrophil ($p = 0.0070$) frequencies in NPs from CRSwNP patients were reduced following preoperative prednisone treatment compared to non-steroid-treated controls (Fig. 2B). Quantitative analysis in NPs from AERD patients revealed a similar trend in eosinophil reduction ($p < 0.0001$) compared to CRSwNP patients after steroid treatment (Fig. 2C). However, a distinguishing feature observed in AERD patients within the granulocyte compartment is that neutrophil proportions remain unchanged after preoperative prednisone exposure. Steroid use did not significantly alter the percentage of basophils ($p = 0.48$) within NP in either CRSwNP or AERD patients.

IHC analysis revealed selective reduction of neutrophil numbers in NPs from CRSwNP patients following prednisone administration

IHC staining for myeloperoxidase in NP sections shows a readily appreciated reduction in neutrophil numbers in patients receiving preoperative steroids compared to NPs derived from steroid-naive CRSwNP patients (Fig. 3A).

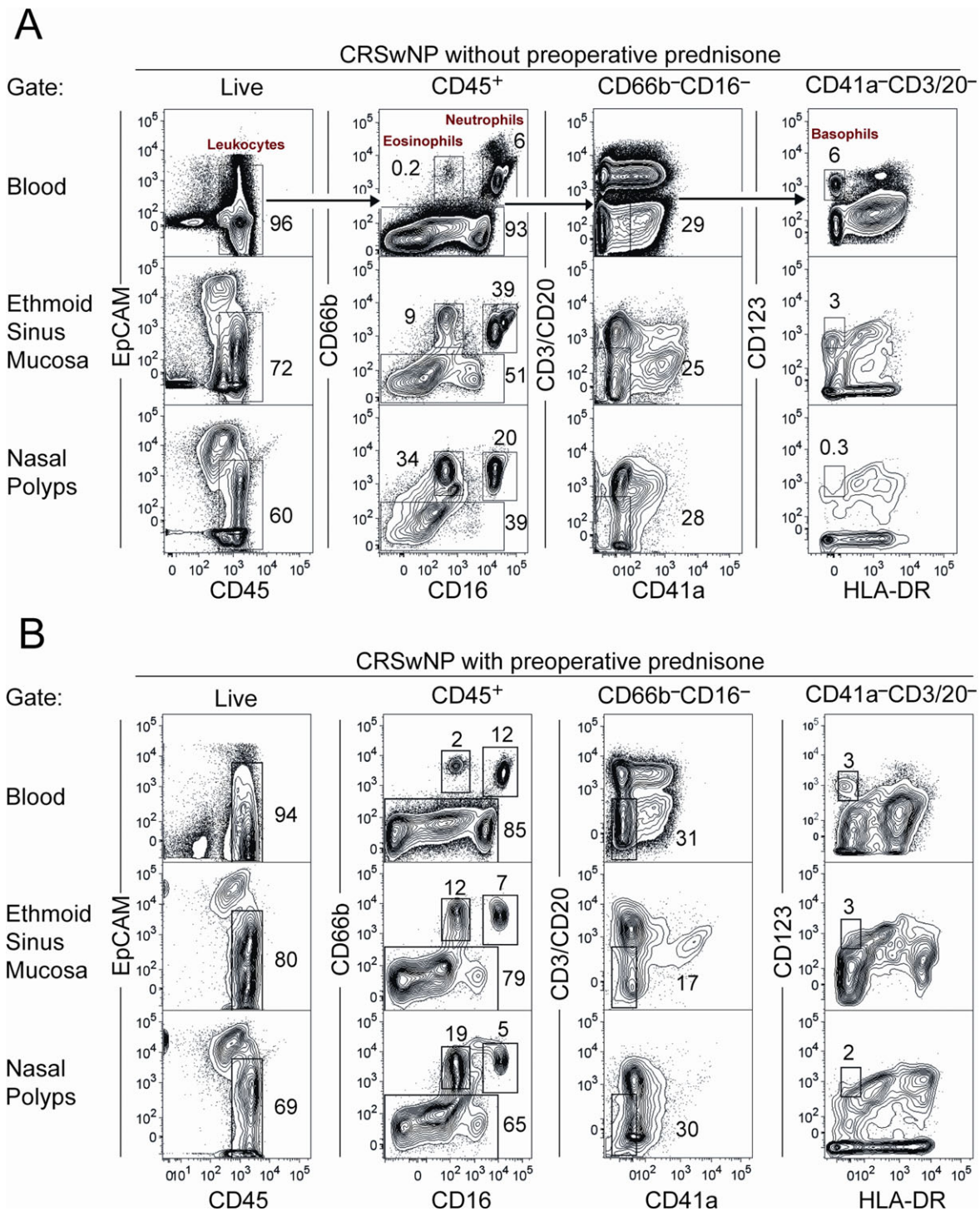


FIGURE 1. Phenotypic analyses of granulocytes from CRS patients reveal a selective decrease in eosinophils and neutrophils in NPs following prednisone administration. (A) PBMCs, adjacent ethmoid sinus mucosa, and NPs from CRS patients without preoperative prednisone were stained with fluorochrome conjugated antibodies to human EpCAM, CD45, CD66b, CD16, CD3, CD20, CD41a, CD123, and HLA-DR. Cells excluding propidium iodide were gated as live cells. Leukocytes (CD45⁺EpCAM⁻) were subgated from live cells. Eosinophils (CD66b⁺CD16⁻) and neutrophils (CD66b⁺CD16⁺) were then identified based on CD16 and CD66b expression. CD66b⁻CD16⁻ cells were subsequently gated using CD3, CD20, and CD41a to eliminate T cells, B cells, and platelets, respectively. Remaining cells were subsequently resolved for basophils (HLA-DR⁻CD123⁺). Arrows indicate progression of gating strategy. The same gating strategy shown in the top row panels (Blood) was applied to all FACS samples from individual patients. One characteristic representative from an analysis of 6 patients is shown here. Note that a significant number of eosinophils (CD66b⁺CD16⁻) and neutrophils (CD66b⁺CD16⁺) (second column from left) are present in NPs, while basophil numbers are low at this tissue subsite (far right column) compared to peripheral blood. (B) The CRS patient shown was treated preoperatively with prednisone 7 days prior to surgery. The same gating strategy shown in (A) was implemented. Note that steroid administration is associated with a decrease in the percentages of both eosinophils (CD66b⁺CD16⁻) and neutrophils (CD66b⁺CD16⁺) within NPs. One representative gating strategy from an analysis of 6 patients is shown here. Note the significant reduction in the number of eosinophils (CD66b⁺CD16⁻) and neutrophils (CD66b⁺CD16⁺) (second column from left) in NPs associated with prednisone treatment compared to NPs from CRSwNP-prednisone (see bottom panel in A). CRS = chronic rhinosinusitis; CRSwNP = CRS with nasal polyps; FACS = fluorescence-activated cell sorting; NP = nasal polyps; PBMC = peripheral blood mononuclear cell.

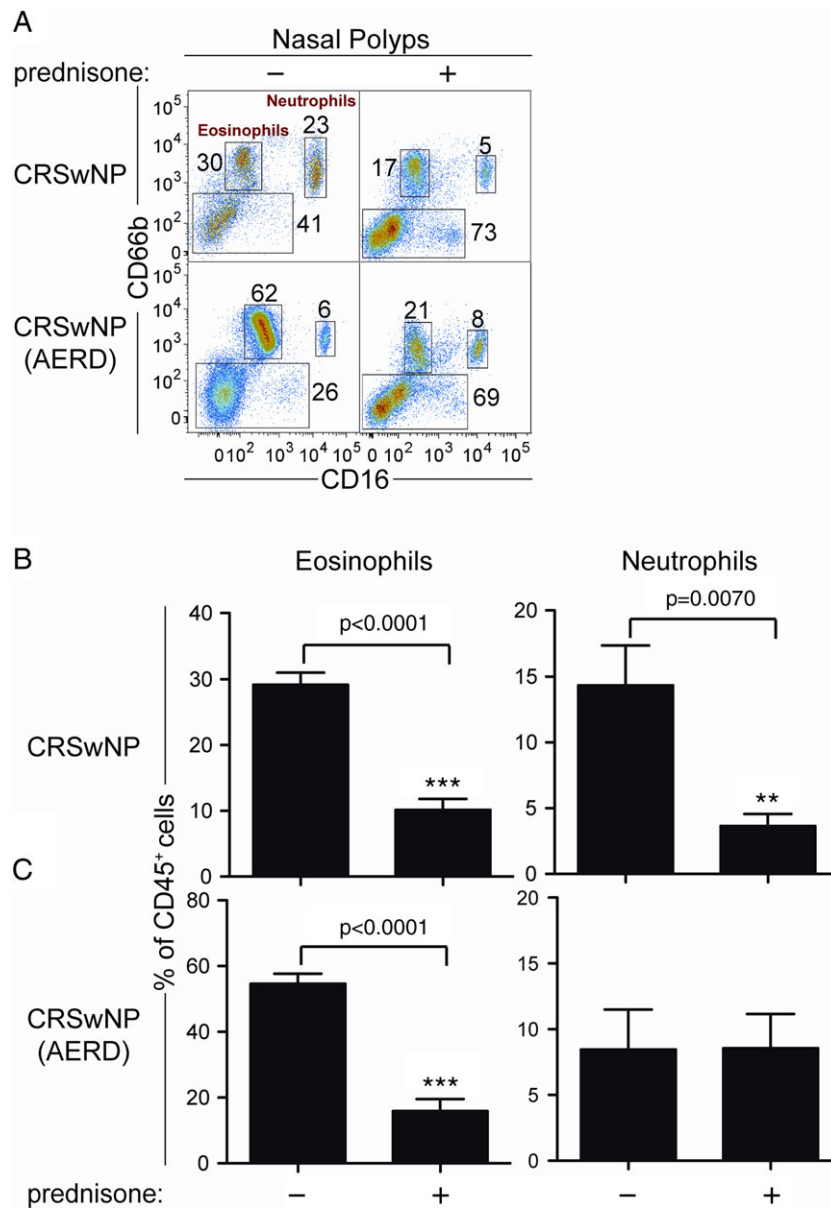


FIGURE 2. Prednisone administration causes selective alterations in granulocyte subsets in NPs from CRSwNP and AERD patients. (A) Single-cell suspensions prepared from NPs from both CRSwNP and AERD patients were gated using a known leukocyte marker (CD45) and analyzed for different granulocyte subsets. Eosinophils (CD66b⁺CD16⁻) and neutrophils (CD66b⁺CD16⁺) are represented in the same manner in each of the 4 panels. Prednisone administration was associated with a significant decrease in eosinophils in both CRSwNP and AERD patients. Though preoperative steroids resulted in a decrease in neutrophil percentages, no significant difference was observed in AERD patients. One representative FACS panel from each polyp patient cohort (n = 6) is presented here. (B) Quantitative analysis of eosinophils (CD66b⁺CD16⁻) and neutrophils (CD66b⁺CD16⁺) using flow cytometry in CRSwNP patients, where n = 6. Data shown are mean and standard deviations of percentages of each subset analyzed by flow cytometry. Significance of difference for each plot is denoted by asterisks: **p ≤ 0.01 and ***p ≤ 0.001. (C) Quantitative analysis of eosinophils (CD66b⁺CD16⁻) and neutrophils (CD66b⁺CD16⁺) using flow cytometry in AERD patients, where n = 6. Data shown are mean and standard deviations of percentages of each subset analyzed by flow cytometry. Significance of difference for each plot is denoted by asterisks: **p ≤ 0.01 and ***p ≤ 0.001. AERD = aspirin-exacerbated respiratory disease; CRSwNP = chronic rhinosinusitis with nasal polyps; FACS = fluorescence-activated cell sorting; NP = nasal polyps.

Myeloperoxidase-positive cells were observed in the subepithelial areas of NPs in focal clusters from steroid-naive CRSwNP patients (Fig. 3A); myeloperoxidase-positive cells are largely absent after prednisone use over 7 days. In agreement with findings from FACS analysis, prednisone administration was associated with no appreciable alteration in neutrophil numbers in NP sections from AERD patients. Changes in the phenotype and density of mucin-producing

goblet cells lining the apical (luminal) surface of the NP epithelium can also be appreciated.

Reduced numbers of eosinophils are observed in NPs from CRSwNP and AERD patients following prednisone treatment

Luna histochemical staining reveals a notable reduction in eosinophil numbers in both CRSwNP and AERD patients

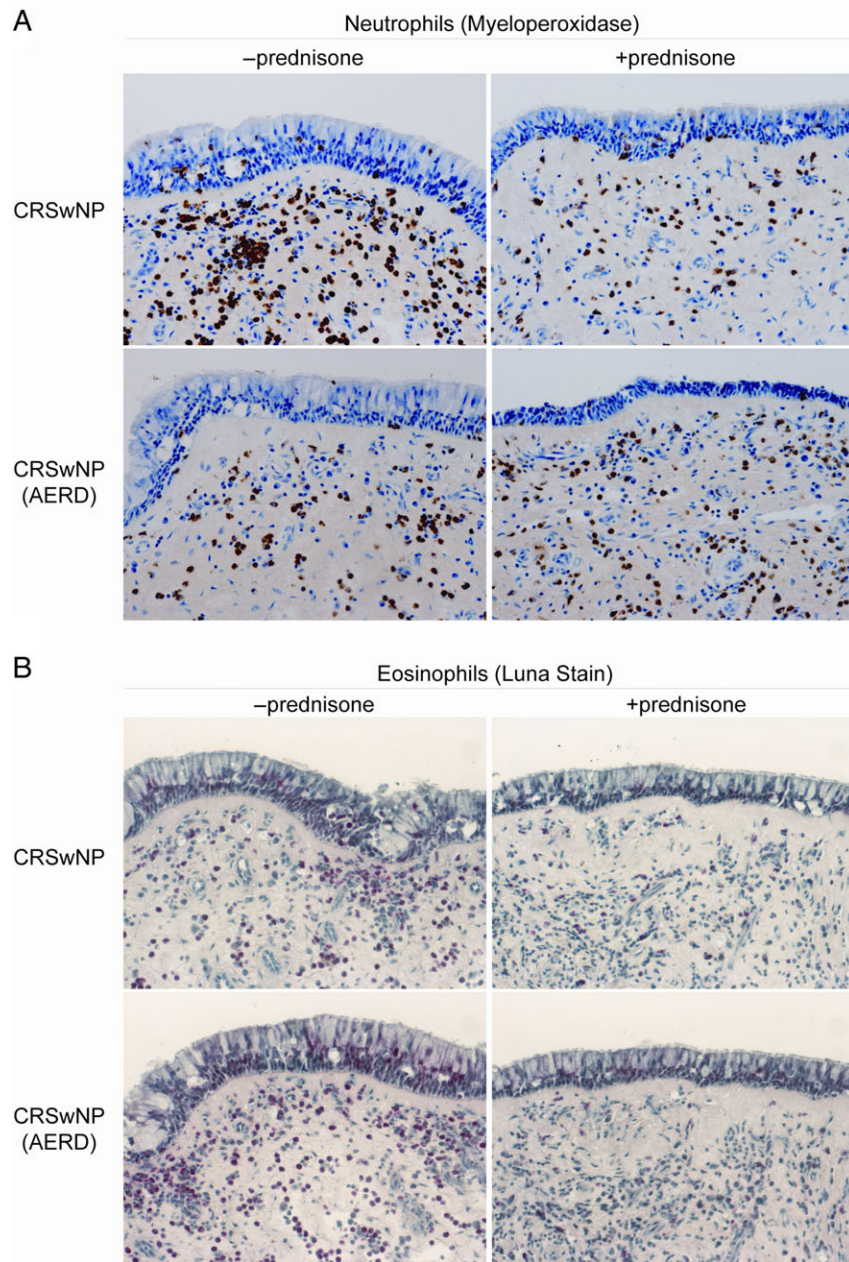


FIGURE 3. Immunohistochemical stains revealing neutrophil and eosinophil expression in NPs. (A) Formalin-fixed and paraffin-embedded polyp sections were stained for myeloperoxidase using antibody and counterstained with hematoxylin. Neutrophils were stained in brown and nuclei in blue (magnification = $\times 200$). This immunostaining revealed that NPs from CRSwNP patients after prednisone treatment (upper right panel) was nearly devoid of neutrophils compared to control (upper left panel). Bottom panels show myeloperoxidase staining in NPs from AERD patients. No significant decrease in neutrophils was seen following prednisone treatment, in agreement with flow cytometric findings. Note that the administration of oral prednisone over 1 week reduces lamina propria thickness and goblet cells numbers in these NP sections. (B) Histochemical Luna staining reveals a decrease in eosinophil expression in both CRSwNP and AERD groups following prednisone administration. Eosinophil granules are stained in red and nuclei in black (magnification = $\times 200$). Panels on the left are NP sections from patients without preoperative prednisone, revealing a localized infiltrate of eosinophils present in NPs. Following prednisone administration, the thickness of the lamina propria of the NP is reduced and eosinophil expression decreases in both CRSwNP and AERD patients. Sections were formalin-fixed and paraffin-embedded and counterstained with Weigert's iron hematoxylin. AERD = aspirin-exacerbated respiratory disease; CRSwNP = chronic rhinosinusitis with nasal polyps; NP = nasal polyps.

receiving preoperative steroids compared to NPs derived from steroid-naïve patients (Fig. 3B). The punctate eosinophilic clusters that were present directly below the epithelium in NPs prior to steroid use, were convincingly absent after 1 week of steroid use. This decrease in eosinophils across both CRS groups suggests that inflam-

mation is reduced after prednisone treatment and that both asthma and aspirin sensitivity in AERD do not impact directly eosinophil properties within NPs after steroid administration. Incidental note is again made that prednisone treatment reduces the profile of mucin-producing goblet cells that line the epithelial lumen (Fig. 3B).

TABLE 3. Granulocyte subsets in nasal tissues from steroid-treated and untreated cohorts

Cell type	Ag(s)	Control patients			Polyp patients			
		Normal	CRSsNP (-prednisone)	CRSsNP (+prednisone)	CRSwNP (-prednisone)	CRSwNP (+prednisone)	AERD (-prednisone)	AERD (+prednisone)
Blood (% of CD45⁺ cells)								
Eosinophils	CD16 ⁻ CD66b ⁺	2.6 ± 2.2	1.2 ± 1.1	1.1 ± 0.6	1.4 ± 0.9	0.8 ± 0.7	1.3 ± 2.0	0.9 ± 0.7
Neutrophils	CD16 ⁺ CD66b ⁺	36.5 ± 3.2	9.5 ± 5.9	8.5 ± 5.4	31.8 ± 10.5	23.7 ± 11.1	12.8 ± 13.9	26.2 ± 10.0
Basophils	CD123 ⁺ HLA-DR ⁻	0.3 ± 0.4	0.8 ± 0.6	0.6 ± 0.4	0.9 ± 0.3	0.4 ± 0.1	1.1 ± 0.4	0.6 ± 0.2
Ethmoid sinus (% of CD45⁺ cells)								
Eosinophils	CD16 ⁻ CD66b ⁺	6.8 ± 1.8	6.7 ± 3.1	6.1 ± 2.6	7.1 ± 2.9	7.7 ± 4.7	21.9 ± 4.6	14.6 ± 6.7
Neutrophils	CD16 ⁺ CD66b ⁺	16.6 ± 4.7	21.3 ± 9.6	17.3 ± 4.3	13.5 ± 5.1	7.6 ± 3.2	11.7 ± 6.9	10.8 ± 8.3
Basophils	CD123 ⁺ HLA-DR ⁻	0.8 ± 0.6	0.2 ± 0.3	0.4 ± 0.3	0.5 ± 0.4	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0.1
Nasal polyps (% of CD45⁺ cells)								
Eosinophils	CD16 ⁻ CD66b ⁺	—	—	—	29 ± 4.5	10.1 ± 4.1	51.7 ± 12.0	15.8 ± 9.1
Neutrophils	CD16 ⁺ CD66b ⁺	—	—	—	14.3 ± 3.0	3.6 ± 2.2	8.5 ± 7.3	8.6 ± 6.4
Basophils	CD123 ⁺ HLA-DR ⁻	—	—	—	0.4 ± 0.2	0.2 ± 0.1	0.3 ± 0.4	0.2 ± 0.1

Values are means ± SDs.

AERD = aspirin-exacerbated respiratory disease; Ag = antigen; CRS = chronic rhinosinusitis; CRSsNP = CRS without nasal polyps; CRSwNP = CRS with nasal polyps.

Changes in granulocyte subsets are not globally affected by steroid use and are restricted to NPs

For each enrolled patient, we performed a comprehensive analysis of granulocyte subsets in NPs, ethmoid sinus mucosa, and blood (Table 3). No notable differences in granulocytes were observed in either blood or ethmoid sinus mucosa from CRSwNP patients. In NPs, however, eosinophil and neutrophil numbers were decreased after prednisone administration (Fig. 4A). These comprehensive analyses highlight the fact that prednisone results in specific focal cellular changes within the polyp microenvironment. Trends toward higher baseline proportions of eosinophils were seen within NPs from AERD patients compared to CRSwNP patients, and also in internal sinus tissue controls and blood (Fig. 4B). Among AERD patients, there was a trend toward increased eosinophil numbers independent of steroid treatment in both ethmoid sinus tissue and NPs compared to blood from the same patients. No notable differences in AERD neutrophil levels could be observed at any tissue site regardless of steroid exposure. Analysis of granulocyte subsets in peripheral blood and ethmoid sinus tissue from CRSsNP controls yielded no apparent differences within these patient cohorts (Supporting Fig. S1).

Discussion

In this work, we examine the resident granulocyte infiltrate within NPs, ethmoid sinus mucosa, and circulating PBMCs from the same individual patients to assess for cel-

lular shifts in CRS patients following prednisone administration. We show here that eosinophils and neutrophils are selectively altered in the NPs of prednisone-treated CRSwNP and AERD patients compared to control patients and those with CRSsNP. Steroid treatment is associated with decrements in eosinophil levels in both CRSwNP and AERD patients, while neutrophil numbers appear to be selectively decreased only in the CRSwNP + prednisone cohort. No significant trends in either eosinophil or neutrophil frequencies were observed in ethmoid sinus mucosa adjacent to the NP within the surgical field in the same patient, or even between polyp patients or controls for these subsites outside of NPs. Furthermore, basophil numbers remained unchanged in steroid-treated tissues from CRSwNP and AERD patients when compared to control patients with steroid exposure. Taken together, these results demonstrate the selective cellular effects of oral corticosteroid administration on granulocyte subpopulations within NPs from CRSwNP and AERD patients.

We set out to investigate 2 overall aims with this study: to understand the direct cellular effects of steroid administration in CRSwNP patients, and to provide additional insight into the cellular differences that may be present in NPs of AERD patients. Due to the relative dearth of patients with AERD who present in a given experimental time frame, we formed a collaborative effort between Stanford University and University of Colorado to obtain sufficient numbers of patient samples. Because subjects were not randomized to treatment assignment, 1 potential limitation of our bi-institutional study is that there are inherent patient differences regarding steroid responsiveness. It is possible that

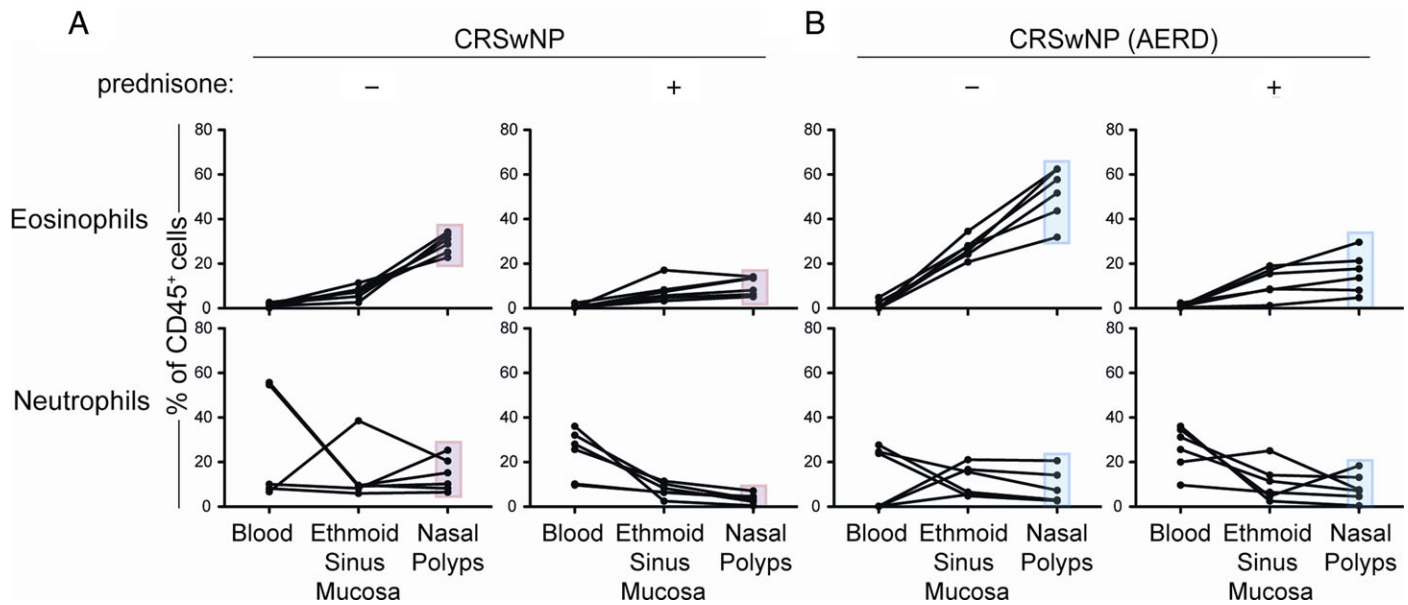


FIGURE 4. Eosinophils and neutrophils in regional nasal cavity tissues and systemic circulation following steroid exposure. (A) A thorough analysis of eosinophil and neutrophil percentages was performed in blood, ethmoid sinus mucosa, and NPs in both CRSwNP patients. Within each graph, each line represents tissue values for an individual patient, allowing tracing of granulocyte subset presence in different tissues in a single patient. Shaded red boxes over graphs denote changes in eosinophil and neutrophil percentages within NPs from CRSwNP patients. Note that preoperative prednisone administration causes a sizeable shift in the percentages of both eosinophils and neutrophils among total CD45⁺ cells in NPs in both CRSwNP patients. (B) Prednisone administration produces a selective decrease in eosinophil levels in NPs, but not in neutrophil percentages. Line tracings for individual patients are as described in (A). Shaded blue boxes group cellular alterations in NPs within the AERD patient population. Note that preoperative prednisone administration also causes a decrease in the percentage of eosinophils among CD45⁺ cells in NPs in AERD patients. In the bottom row, however, neutrophil expression remains unchanged in NPs from AERD patients. AERD = aspirin-exacerbated respiratory disease; CRS = chronic rhinosinusitis; CRSwNP = CRS with nasal polyps; NP = nasal polyps.

physicians may have inadvertently prescribed prednisone to patients who elicited a robust response to steroid treatment in the past. Despite this limitation, an unforeseen benefit of this study is that geographical variance did not affect baseline granulocyte percentages in our patient samples, but rather diversified our patient cohorts because approximately equal proportions of tissue samples were obtained from each institution (Table 1). Although previous studies have analyzed individual immune effector populations using IHC,^{11,27} this method limits the breadth and scope of the available analysis. We reasoned that FACS provided a more informative means to study multiple immune cell subsets within 1 sample. Using FACS, the capacity to perform ex vivo experiments in real-time with minimal sample processing time is possible. Here, we were able to study 3 individual tissue subsites using 9 discriminating antibodies (Fig. 1 and Table 1) simultaneously.

Granulocytes are a category of white blood cells that play a critical role in directing the Th1/Th2 immune response, thereby implicating them in the pathophysiology of CRS. There are 3 major types of granulocytes: neutrophils, eosinophils, and basophils. In healthy individuals, granulocytes account for approximately 65% of all blood leukocytes, with neutrophils comprising ~60%, eosinophils ~2%, and basophils <1%. Proportions of resident granulocytes within nasal mucosal tissues and the peripheral blood circulation may be altered in diseased states. Eosinophils are bone marrow-derived granulocytes present in limited numbers in peripheral blood and tissues.

During certain settings of inflammation, these cells can infiltrate affected tissues, where they become activated and degranulate, leading to the recruitment of other inflammatory cells. Neutrophils are another cellular component in the inflammatory cascade, and are the most abundant circulating leukocytes in the blood. Migration and activation of neutrophils into local tissues are considered uncommon unless the local microenvironment is perturbed. Basophils, the least common of the granulocytes, release histamine at tissue sites during allergic reactions. In this study, we attempted to understand the cellular dynamics of these granulocytes in control and polyp patient cohorts undergoing steroid treatment. To accomplish this objective, each of these granulocyte subpopulations was analyzed in NPs, adjacent ethmoid mucosa, and peripheral blood. Neutrophil (CD66b⁺CD16⁺) and eosinophil (CD66b⁺CD16⁻) populations were resolved based on established CD16 and CD66b coordinates in peripheral blood.²⁶ Although there remains a debate as to whether CD66b is a specific marker for eosinophils in nasal tissue samples, previous studies have shown that in airway sputum, CD66b⁻ cells are identifiable as macrophages,²⁵ ruling out the possibility that CD66b⁺ cells would include tissue macrophages. Resident eosinophil and neutrophil levels were found to be proportionally increased within ethmoid sinus tissues compared to blood in CRS patients in the absence of steroid administration (Fig. 1A). Eosinophils were further increased in NPs in CRSwNP patients, but there was a 2-fold decrease in neutrophil frequencies in the same tissue (Fig. 1A). This

result is not entirely unexpected, as eosinophils have been well characterized in NPs from CRSwNP patients.^{7,10}

Previous reports have shown that the sinus mucosa from CRSwNP patients contains elevated interleukins (IL-5, IL-22, IL-33) and an elevated ratio of eosinophilic cationic protein (ECP) to myeloperoxidase (MPO), suggesting an increased prevalence of eosinophils within nasal tissue compared to neutrophils in this disease.^{2,28,29} In other studies, no difference in MPO expression, a marker for neutrophil activation, was identified between inferior turbinate and maxillary sinus tissues.³⁰ Although these latter data suggest that neutrophil levels remain balanced between different nasal tissue subsites, the NP itself is distinctly an abnormal, mucosal inflammatory lesion and is potentially immunologically divergent in its properties. Thus, a comparison of neutrophil levels between sinus tissue and NPs from the same patient was warranted. Our analysis suggests that there is a decrease in neutrophil levels between ethmoid sinus mucosa and NPs from CRSwNP patients. Granulocytes have been well studied in nasal tissue from CRSwNP patients, but how these levels may shift after steroid administration and in the setting of AERD was unknown prior to initiating this work.

Preoperative prednisone administration in CRSwNP patients was associated with notable differences in both eosinophils and neutrophils frequencies in NPs (Fig. 2A). Prednisone is a glucocorticoid that is used to treat inflammatory diseases such as asthma, allergic disorders, and CRS, and is a particularly potent inhibitor of eosinophil-mediated inflammation.^{19,31} In peripheral blood, corticosteroids have been shown to have proapoptotic effects on eosinophils.³² However, glucocorticoids have the opposite effects on neutrophils, causing decreased margination in bone marrow.³³ Furthermore, a study in asthmatic patients confirmed that blood neutrophils were not inhibited after oral corticosteroid treatment.³⁴ The direct effect of glucocorticoids on granulocyte subsets in NPs, however, remains unknown. As a result of the relative inhibitory nature of steroid activity, we anticipated that there would be a decrease in all granulocyte levels following steroid exposure due to a suppression of the entire immune system axis. Our findings, however, demonstrate that steroids are associated with a decrease in eosinophils in NPs from both AERD and CRSwNP patients, but are associated only with a reduction in neutrophil frequencies in CRSwNP patients (Fig. 2B and C). While no significant differences in granulocyte subsets were observed in the adjacent ethmoid tissue or peripheral blood from any patient cohort after prednisone treatment, trends were noted between patient cohorts (Table 3). These results suggest that the immunologic microenvironment within NP lesions is entirely distinct from the surrounding mucosa and should be studied further. His-

tological staining of NPs further confirmed flow cytometric analyses (Fig. 3A and B).

Finally, AERD, a subset of CRSwNP, typically presents with a more aggressive form of polyposis. Patients with AERD are often treated with oral prednisone to reduce inflammation and with functional endoscopic sinus surgeries to reduce polyp burden. Despite these efforts, the recovery of a normal sense of smell after medical treatment is less likely in AERD patients compared with patients with allergic rhinitis.^{14,15} Recurrence of nasal polyposis is common in AERD patients and is nearly 3 times higher than in aspirin-tolerant asthmatic patients.¹⁶ We posited that a thorough analysis of the granulocyte subsets in NPs from AERD patients might provide a better understanding of this phenomenon. AERD is characterized by an excessive production of cysteinyl leukotrienes (cysLTs).⁶ CysLT metabolites have a variety of downstream effects, including the constriction of smooth muscle³⁵ and the accumulation of eosinophils in respiratory mucosa,³⁶ which may contribute to the chronic inflammation present in both the nasal and pulmonary respiratory tissues in AERD patients. CysLTs derive from the metabolism of arachidonic acid by effector cells of the immune system. Neutrophils are unique in that LTA₄ is preferentially converted by LTA₄ hydrolyase into LTB₄. In monocytes, mast cells, basophils, and eosinophils, LTA₄ can be conjugated by glutathione to form LTC₄, the parent cysLT.³⁷ As a working hypothesis, it is conceivable that the elevated levels of cysLTs in AERD patients, or the specific inability of neutrophils to form the parent cysLT metabolite, may be related to their resistance to prednisone in AERD patients. Further molecular and cell-signaling studies are warranted to determine the basis of these findings in AERD patients.

Conclusion

Systemic steroid exposure is associated with focal changes in both eosinophil and neutrophil populations exclusively in NPs, without notable granulocyte alterations found in the surrounding regional tissues. These data provide direct insights into the cellular effects of prednisone exposure in patients with CRSwNP and AERD.

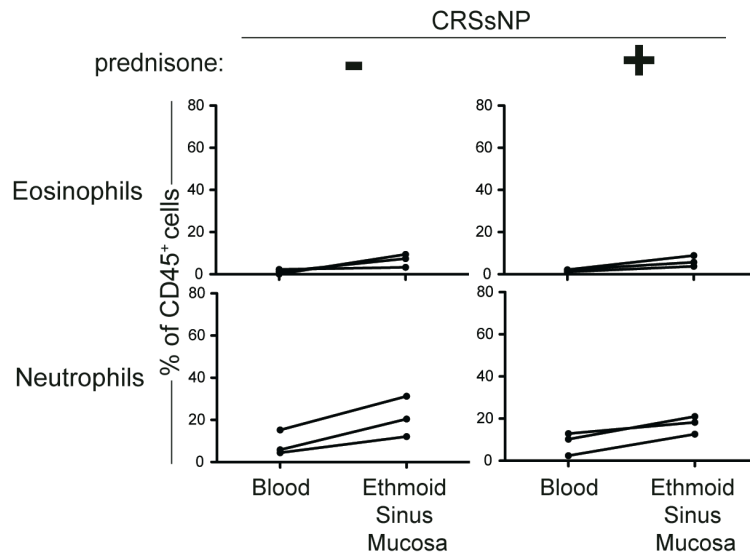
Acknowledgments

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Figure S1



SUPPORTING FIGURE S1. Eosinophils and neutrophils are not altered in CRSsNP patients following steroid exposure. A thorough analysis of eosinophil and neutrophil percentages was performed in blood and ethmoid sinus mucosa from CRSsNP patients to determine differences associated with prednisone exposure. Within each graph, the presence of granulocyte subsets in different tissues in a single patient can be traced. No notable differences in granulocyte proportions between peripheral blood and ethmoid sinus tissue were found.