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Decreased nasal polyp eosinophils but increased mast cells in an AERD patient treated with reslizumab.

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Chronic sinusitis; nasal polyps; CRSwNP; AERD; Aspirin Exacerbated Respiratory Disease; reslizumab; biologics; eosinophil; mast cell

Chronic rhinosinusitis with nasal polyps (CRSwNP) affects 3-6% of the population and is associated with increased morbidity and health care costs^{1,2}. A subset of CRSwNP patients have Aspirin Exacerbated Respiratory Disease (AERD), which is associated with asthma and an intolerance to medications that inhibit the cyclooxygenase-1 enzyme³. In the US, CRSwNP and AERD are predominantly characterized by type 2 inflammation⁴. Eosinophils, a hallmark of type 2 inflammation, have been associated with more severe CRSwNP⁵ and are enriched in AERD⁶. The mechanisms by which eosinophils contribute to chronic inflammation in CRSwNP and AERD are unclear, but ongoing trials suggest biological therapies targeting eosinophils are safe and effective in CRSwNP⁷. However, not all patients who receive these treatments clinically improve. Understanding why biologic treatments fail in certain CRSwNP patients may provide insight into disease heterogeneity and the mechanisms driving pathogenesis.

In this study, we identified a 47 year-old woman with AERD with significant sinonasal disease despite having undergone three prior sinus surgeries. She noted severe persistent sinonasal symptoms, chronic sinonasal inflammation on sinus CT scan, and only moderate and temporary improvement following systemic steroid courses. Her daily medical regimen included intranasal steroid spray, large volume saline rinses, combined inhaled corticosteroid

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Conflicts of Interest

AS, RC, and KW report no conflicts of interest. WS served on an advisory board for GlaxoSmithKline. AP has served as an advisor to Sanofi Regeneron, AstraZeneca, and OptiNose.

This article has an online data supplement.

with long-acting beta-agonist, and 15mg of prednisone. Given the severity of her asthma, she was not a candidate for aspirin desensitization.

The patient underwent her fourth endoscopic sinus surgery in 2012. Within the year, her sinonasal disease and asthma again worsened which required frequent bursts of systemic steroids. As her disease continued to worsen, the decision was made to add a biologic and reslizumab 3mg/kg IV every 4 weeks was started in January 2017. On reslizumab, the patient reported improvement in her asthma with a decrease in exacerbations. Her peripheral eosinophils became undetectable. However, her sinonasal symptoms continued to worsen and her sinus CT scan showed persistent sinus disease. The patient underwent her fifth sinus surgery in August 2017 (8 months after starting reslizumab).

Given this unique circumstance, we examined reslizumab's effects on inflammatory cells by comparing NP tissue surgically removed during reslizumab therapy (in 2017) with NP tissue obtained from her prior sinus surgery before reslizumab (in 2012). To serve as standards of comparison, we performed the same analyses in NP of separate AERD and CRSwNP patients and in uncinate tissue (UT) of control patients without sinonasal disease; none of whom received biologics. This study was approved by the Northwestern University IRB with additional information provided in the Online Supplement.

Gene expression levels of the eosinophil granule protein *CLC* (Figure 1A) were elevated in our patient prior to reslizumab and were equivalent to levels in NP from AERD patients not on biologics. During reslizumab, gene expression of *CLC* were substantially reduced by 274-fold (Figure 1A). A similar trend was observed for eosinophil cationic protein (ECP), an eosinophil granule protein (Figure 1B). ECP levels prior to reslizumab (1,515ng/mg protein) were similar to untreated NP (2,507±706ng/mg protein). During treatment, ECP decreased (17ng/mg protein) to levels more closely resembling control UT (50±15ng/mg protein) (Figure 1B). Reslizumab reduced the average number of ECP+ eosinophils detected in NP by immunohistochemistry from 122 to 12 cells (Figure 1C).

When using flow cytometry to characterize NP inflammatory cells during reslizumab, we surprisingly identified a population of CD45⁺Siglec-8⁺FcERI⁻CCR3⁺ cells. Although expression of these surface markers suggested this population could be eosinophils, these cells did not express CD69, a marker found to be upregulated on nearly all NP eosinophils (eFigure 1, unpublished observations). These cells may represent a distinct non-eosinophil cell subset. Alternatively, this population may represent “ghost” or “inactive” eosinophils, *i.e.* ones that do not express stereotypical eosinophil granule proteins but still retain traditional eosinophil surface markers. It is also possible that CD69 was uniquely dysregulated on eosinophils but other activation markers (*e.g.* CD32, CD62L) were not affected. The true identity, function, and relevance of this population warrant further investigations.

Gevaert and colleagues reported that a single injection of reslizumab was associated with improved NP scores in half of their study population⁸. Patients who responded to treatment had higher levels of IL-5 in nasal fluid than non-responders. While we were unable to measure protein levels of IL-5 in nasal lavage from our patient, levels of IL-5 gene

expression in her NP prior to reslizumab was within the range of what was observed in CRSwNP and AERD NP not on biologics (Figure 1D). IL-5 gene expression was reduced 2.6-fold following reslizumab, suggesting reslizumab did not significantly affect NP IL-5 transcript levels.

Interestingly, we detected by flow cytometry a large population of mast cells (CD45⁺Siglec-8⁺FcERI⁺) in reslizumab-treated NP (eFigure 1). This was confirmed by RT-PCR and immunohistochemistry showing increased gene expression of tryptase (*TPSB2*, Figure 1E) and elevated numbers of tryptase⁺ cells (Figure 1F) in NP during reslizumab compared to before treatment. This is similar to a study of dexamipexole in CRSwNP where eosinophils in the blood and NP were reduced but clinical symptoms and NP size did not significantly improve and NP mast cells were elevated⁹.

While conclusions from this study are limited by sample size, reslizumab appears to have been biologically effective in depleting peripheral eosinophils. However, the drug was clinically ineffective as the patient continued to have persistent sinonasal disease. The underlying mechanisms contributing to her worsening symptoms are unknown, but it could be that eosinophils were not the primary mediator of her disease. Instead, mast cells, which increased following treatment, may play a more prominent role. Mast cells have been implicated in other type 2 diseases including severe asthma¹⁰. Alternatively, the CD45⁺Siglec-8⁺FcERI⁺CD69⁻ cell population detected in reslizumab-treated NP may also be contributing. These cells were either refractory to, or were somehow altered by, reslizumab compared to more “conventional” eosinophils. Interestingly, after the patient's fifth sinus surgery, she switched to dupilumab. By global physician assessment, her upper and lower respiratory disease improved with less exacerbations and corticosteroid requirements.

In summary, this study provides interesting observations into potential mechanisms by which CRSwNP patients may fail biologic therapies targeting eosinophils. More comprehensive work is needed to validate these findings and ultimately identify mechanisms leading to biologic treatment non-responsiveness in CRSwNP.

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Supplementary data

Methods

Study Population

Subjects who met the criteria for CRS as previously defined¹ were recruited from Northwestern Medicine. All patients with AERD had physician-diagnosed asthma, CRSwNP, and documentation of at least 1 respiratory reaction to a COX-1 inhibitor. AERD patients were excluded if they were taking daily aspirin after undergoing a desensitization. Control subjects had no history of sinusitis and were undergoing surgery for other indications that required access to the sinuses. The Institutional Review Board of Northwestern University Feinberg School of Medicine approved this study.

Quantitative Real-time PCR

RNA was isolated from samples and cDNA was prepared as previously described². The following Taqman primer and probe sets from Applied Biosystems (Carlsbad, CA) were used: *CLC* (Hs00171342_m1), *TPSB2* (Hs02576518_gH), *IL-5* (Hs01548712_g1) and *GUSB* control. RT-PCR was run on a StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA). All values were normalized to β -glucuronidase and expressed as 2^{-C_t} .

Immunochemistry

Paraffin-embedded formalin-fixed nasal tissue was prepared and stained for immunohistochemistry as previously described² using the following antibodies: a 1:800 dilution of mouse monoclonal (EG2) anti-ECP (Diagnostics Development, Uppsala, Sweden), a 1:2,000 dilution of mouse monoclonal (AA1) anti-tryptase Ab-2 (Thermo Scientific, Waltham, MA), a 1:500 dilution of biotinylated horse-anti-mouse secondary antibody (Vector Labs, Burlingame, CA). Images were obtained at 10× and 20× objective magnification using a using an Olympus IX71 inverted microscope (Olympus, Waltham, MA). ECP⁺ stained cells were counted using thresholded region of interest analysis with the Nikon NIS Elements software (Melville, NY). Tryptase stained slides were blinded and the number of tryptase⁺ cells in 10 random fields per slide were counted by two independent observers and averaged together.

Cell isolation and flow cytometric analysis

Cells were isolated from nasal polyps for evaluation by flow cytometry as previously described³. Cells were first treated with Aqua LIVE/DEAD fixable dead cell staining reagent (Thermo Fisher, Waltham, MA). Then, the following antibodies were used: 1:20 dilution of V450 anti-CD45 (HI30, BD Horizon, San Jose, CA), 1:10 dilution of Alexa647 anti-Siglec-8 (gift from Dr. Bruce Bochner), 1:20 dilution of PE anti-Fc ϵ RI α (AER37 (CRA-1), Biolegend, San Diego, CA), 1:20 dilution of FITC anti-CCR3 (5E8, Biolegend, San Diego, CA), 1:20 dilution of PECy7 anti-CD69 (FN50, Biolegend, San Diego, CA). A LSRII flow cytometer (BD Biosciences, San Jose, CA) was used and data was compensated and analyzed with FlowJo software v9.9.6 (TreeStar, Ashland, OR). Proper single-stained control beads (BD Biosciences) and fluorescence minus one negative controls were used. A

total of 100,000 events was collected with the subsequent gating strategy: 1) exclusion of debris; 2) exclusion of doublets; 3) exclusion of dead cells; 3) inclusion of CD45⁺ immune cells; and 4) inclusion of Siglec-8⁺ FcεRI⁻ cells (eFigure 3). Eosinophils were defined as CD45⁺ Siglec-8⁺ FcεRI⁻ cells⁴. Expression of CCR3 and CD69, a marker of eosinophil activation⁵, was subsequently evaluated among CD45⁺ Siglec-8⁺ FcεRI⁻ cells.

Eosinophil Cationic Protein Measurement

Eosinophil cationic protein (ECP) was measured using the MESACUP ECP TEST per the manufacturer's instructions (MBL International, Woburn, MA). Tissue ECP concentrations were corrected for total protein concentration.

Statistics

Statistical differences in demographics were determined by Chi-squared or Mann-Whitney analyses. Differences among control, CRSwNP, and/or AERD nasal tissues were first analyzed by one-way ANOVA Kruskal-Wallis test and, if significant, then by Dunn's test for multiple comparisons. All calculations were completed using Graphpad Prism v7.0 software (La Jolla, CA). A p-value < 0.05 was considered significant.

Figure 1. Reslizumab was associated with an increase in mast cells and detection of a possible eosinophil subtype in nasal polyps as measured by flow cytometry.

Nasal polyp tissue was excised from our study patient during reslizumab treatment and, for comparison, a patient with CRSwNP not taking a biologic and prepared for flow cytometry. Eosinophils were identified as Live/Dead⁻ CD45⁺ Siglec-8⁺ FcεRI⁻ CCR3⁺ cells and mast cells were identified as Live/Dead⁻ CD45⁺ Siglec-8⁺ FcεRI⁺ cells.

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Figure 1. Reslizumab therapy is associated with a reduction in eosinophils but an increase in mast cells in nasal polyps.

Total RNA was extracted from UT isolated from controls, nasal polyps from patients with CRSwNP or AERD, and, in our study patient, nasal polyps before and after the initiation of reslizumab. mRNA expression of eosinophil-associated genes *CLC* (A) and *IL-5* (D) as well as a mast cell-associated gene encoding tryptase, *TPSB2* (E), were measured by real-time PCR. Protein levels of eosinophil cationic protein (ECP) were measured by ELISA (B) and immunohistochemistry was utilized to identify and enumerate ECP⁺ eosinophils (C) and

tryptase⁺ mast cells (F) in control UT and nasal polyps. Dot plots illustrate individual data points with solid lines representing the mean $\pm$ SEM.

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