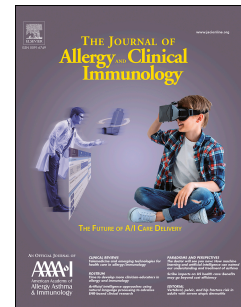


Journal Pre-proof

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PII: S0091-6749(21)02681-6

DOI: <https://doi.org/10.1016/j.jaci.2021.11.023>

Reference: YMAI 15401

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 16 September 2021

Revised Date: 28 October 2021

Accepted Date: 12 November 2021

Please cite this article as: Poposki JA, Klingler AI, Stevens WW, Suh LA, Tan BK, Peters AT, Abdala-Valencia H, Grammer LC, Welch KC, Smith SS, Conley DB, Kern RC, Schleimer RP, Kato A, Elevation of activated neutrophils in chronic rhinosinusitis with nasal polyps, *Journal of Allergy and Clinical Immunology* (2022), doi: <https://doi.org/10.1016/j.jaci.2021.11.023>.

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Elevation of activated neutrophils in chronic rhinosinusitis with nasal polyps

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Word Count: 4117 (Introduction to Discussion)

Funding: This research was supported in part by NIH grants, R01 AI104733, R01 AI137174, U19 AI106683 and P01 AI145818 and by a grant from the Ernest S. Bazley Foundation.

Conflicts of interest: WWS served on advisory boards for GlaxoSmithKline, Genentech, and Bristol Myers Squibb. ATP has served on advisory boards for Sanofi-Genzyme/Regeneron, Optinose, AstraZeneca, Novartis, and GSK. ATP also has research support from Optinose and Sanofi-Regeneron. LCG reports personal fees from Astellas Pharmaceuticals. KCW reports

consultant fees from Baxter, OptiNose and Acclarent. RCK reports consulting fees from Lyra Therapeutics, Medtronic, GSK, Genentech and Sanofi/Regeneron. RPS reports personal fees from Intersect ENT, Merck, GlaxoSmithKline, Sanofi, AstraZeneca/Medimmune, Genentech, Actobio Therapeutics, Lyra Therapeutics, Astellas Pharma Inc., and Otsuka Inc. RPS also has royalty rights to Siglec-8 and Siglec-8 ligand related patents licensed by Johns Hopkins to Allakos Inc. AK reports a consultant fee from Astellas Pharma and a gift for his research from Lyra Therapeutics. JAP, AIK, LAS, SSS and DBC report no conflicts of interest.

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Abstract

Background: Chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP) is well characterized by type 2 (T2) inflammation characterized by eosinophilia in Western countries. However, the presence and the roles of neutrophils in T2 CRSwNP are poorly understood.

Objective: To clarify accumulation and inflammatory roles of neutrophils in Western CRSwNP.

Methods: Sinonasal tissues and nasal lavage fluids (NLFs) were obtained from control patients and patients with CRS, and neutrophil markers were determined by ELISA. The presence of neutrophils in tissue was determined by flow cytometry. The gene expression profiles in neutrophils were determined by RNA-Sequencing.

Results: A neutrophil marker elastase was selectively elevated in nasal polyp (NP) tissue whereas eosinophilic cationic protein (an eosinophil marker) was elevated in both uncinata and NP tissues of CRSwNP patients. NLF myeloperoxidase (another neutrophil marker) was also significantly elevated in CRSwNP compared to control patients. Neutrophil markers were more greatly elevated in CRSwNP patients with recurrent disease. Flow cytometric analysis confirmed that neutrophil numbers were significantly elevated in NPs compared to control tissues. RNA-Sequencing analysis found that 344 genes were >3-fold and significantly elevated in NP neutrophils compared to peripheral blood neutrophils. Gene ontology analysis suggested that elevated genes in NP neutrophils were significantly associated with activation. Results suggest that neutrophils are accumulated in T2 NP tissues and that accumulated neutrophils are highly activated and contribute to inflammation in NPs.

Conclusions: Neutrophils may play a heretofore unrecognized meaningful role in the pathogenesis of CRSwNP in western countries and may be considered to be a potentially important therapeutic target in T2 CRSwNP.

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60

61 Key messages

62 • Neutrophils are highly elevated in NPs and neutrophils, but not eosinophils, are key
63 immune cells that distinguish between NP tissue and other inflamed areas in CRSwNP in
64 the US.

65 • Accumulated neutrophils are highly activated in NPs and neutrophil numbers are further
66 elevated in tissues from patients undergoing revision surgery.

67 • Neutrophils may be an important therapeutic target in CRSwNP.

68

69 Capsule summary

70 Neutrophils are highly elevated and activated in NPs in the US and may be an important therapeutic
71 target in CRSwNP, even in type 2 inflammatory conditions.

72

Key words

Neutrophils; Nasal polyps; Chronic rhinosinusitis; Endotype; Type 2 inflammation.

Abbreviations

CASP; Caspase

CLC; Charcot-Leyden crystal galectin

CRS; Chronic rhinosinusitis

CRSsNP; CRS without nasal polyps

CRSwNP; CRS with nasal polyps

ECP; Eosinophil cationic protein

GO; Gene ontology

LT; Leukotriene

MPO; Myeloperoxidase

NE; Neutrophil elastase

NLF; Nasal lavage fluid

NP; Nasal polyp

PB; Peripheral blood

RNA-Seq; RNA-sequencing

NE; Neutrophil elastase

INTRODUCTION

Chronic rhinosinusitis (CRS) affects approximately 12.5% of Americans, is responsible for over 400,000 surgeries annually, produces significant morbidity and costs our health system an estimated \$22-32 billion annually to manage.¹⁻³ CRS is frequently divided into two main phenotypes: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). In Western countries including the US, CRSwNP is primarily characterized by type 2 (T2) inflammation with eosinophilia and the elevation of T2 cytokines including IL-5 and IL-13.³⁻⁸ Indeed, recent studies confirmed the current knowledge that 87% and 85% of CRSwNP patients had T2 inflammation in inflamed areas including nasal polyps (NPs) in Chicago and Benelux, respectively.^{7,8} In contrast, both T2 and non-T2 endotypes are found in Asian patients.^{7,9}

Although neutrophilic inflammation plays an important role in non-T2 NPs in Asia, the presence of neutrophils in NPs in western countries has not been well examined, except in the case of cystic fibrosis.^{4, 10} Recently, two groups presented potential evidence that neutrophilic inflammation may also play a role in the pathogenesis of Western T2 NPs.¹¹⁻¹³ Delemarre et al. examined eosinophils and neutrophils by immunohistochemistry and found that neutrophils are elevated in eosinophilic NPs and that the presence of eosinophil extracellular traps and deposition of Charcot-Leyden crystals (CLC, an eosinophil related crystal) are associated with neutrophil infiltration in Belgium.¹² Succar et al. examined eosinophils and neutrophils by histochemistry and found that patients with mixed granulocytic CRS had the highest SNOT-22 scores, indicative of worse disease-specific quality of life and higher symptom burden in the US.¹¹ However, the accumulation of neutrophils in T2 CRSwNP has only been evaluated by histological examination. Furthermore, it is still largely unclear whether accumulated neutrophils in NPs are activated or play an important role in inflammation and pathogenesis in T2 CRSwNP. In the present study, we

117 set out to better understand the presence, accumulation and activation status of neutrophils in
118 CRSwNP by ELISA and flow cytometry and assess the potential role of neutrophils in NPs by
119 bulk RNA-Sequencing.

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METHODS

Patients and tissue collection

Patients with CRS and control patients were recruited from the Otolaryngology clinic and the Northwestern Sinus Center of Northwestern Medicine. Uncinate tissue, inferior turbinate and nasal polyp (NP) tissue were obtained during routine endoscopic sinus surgery performed on patients with CRS. All CRS patients met the criteria for the International Consensus Statement on Allergy and Rhinology: Rhinosinusitis.¹ All CRS patients recruited had failed appropriate and maximal medical therapy prior to surgery. We included a mixed population regarding the timepoint of surgery: the cohort included patients with earlier stage disease as well as some patients that were receiving revision surgery after a recurrence. Patients with an established immunodeficiency, pregnancy, coagulation disorder or diagnosis of eosinophilic granulomatous polyangiitis (eGPA or Churg-Strauss syndrome) or cystic fibrosis were excluded from the study. Disease-free control tissues were obtained mainly from skull base tumor cases, and some from septoplasty cases, without evidence of ethmoid inflammation on scans. Characteristics of subjects in this study are shown in Supplementary Table E1-E4. All patients signed informed consent forms and the protocol governing procedures for this study was approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine.

Protein assays

Freshly obtained tissue specimens were weighed, and 1 ml of PBS supplemented with 0.05 % Tween 20 (Sigma-Aldrich, St. Louis, MO) and 1% protease inhibitor cocktail (PIC, PN; P8340, Sigma-Aldrich) was added for every 100 mg of tissue. Tissue extracts were prepared by

homogenization with a Bullet Blender Blue (Next Advance, Averill Park, NY) as described previously.¹⁴ Nasal lavage fluids (NLF) were obtained from patients before surgery. After suctioning the nasopharynx, 8 ml of PBS was sprayed through a syringe toward the middle meatus, and the resultant fluid was collected as described previously.¹⁴ Before analysis, tissue extracts and NLFs were centrifuged at 16,000*g* for 5 minutes and supernatants were used for each assay.

The protein concentrations of eosinophil cationic protein (ECP), neutrophil elastase (NE) and myeloperoxidase (MPO) were measured by commercial ELISA kits from MBL (Woburn, MA), Hycult Biotech, (Wayne, PA) and R&D systems (Minneapolis, MN), respectively. The minimal detection limits for ECP, NE and MPO are 125, 400 and 62.5 pg/ml, respectively. The concentration of these proteins in tissue extracts was normalized to the concentration of total protein as detected by BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL).

Flow cytometric analysis and cell sorting

Tissue samples were fragmented and incubated with 30 µg/ml DNase I and 1 mg/ml type I collagenase-containing media at 37°C for 45 minutes. Following this, tissues were minced using a gentleMACS dissociator (Miltenyi Biotec, Auburn, CA) and the cells were filtered through 70 µm nylon mesh (BD Biosciences, San Jose, CA). Contaminated red blood cells were then removed by EasySep™ RBC Depletion Reagent (STEMCELL Technologies, Vancouver, British Columbia, Canada) before counting and staining the cells. After isolation, cells were first treated with Aqua LIVE/DEAD fixable dead cell staining reagent (Invitrogen, Carlsbad, CA) at room temperature in the dark. Cells were then blocked by Fc Block reagent and incubated with FITC anti-CD62L (DRG-56), PerCP/Cy5.5 anti-CD66b (G10F5), PE/Cy7 anti-CD16 (3G8) and BV421 anti-CD45 (HI30) at 4°C in the dark. After washing, cells were resuspended in MACS buffer and stored at

4°C in the dark before analysis on a CytoFLEX flow cytometer (Beckman Coulter, Indianapolis, IN). All analysis was performed with FlowJo software, version 10.7 (TreeStar, Ashland, OR), and the experimental method was established and verified with the proper single-stained control beads (BD Biosciences) and fluorescence minus one (FMO) negative controls (not shown). In the case of neutrophil sorting, isolated cells were stained with Alexa Fluor 700 anti-CD45 (HI30), BUV395 anti-CD16 (3G8), PE anti-CCR3 (5E8), FITC anti-FcεRI (AER-37), BV421 anti-CD117 (104D2) and APC/H7 anti-HLA-DR (G46-6). We sorted neutrophils (Aqua⁻ CD45⁺ CD117⁻ FcεRI⁻ CCR3⁻ CD16^{high} HLA-DR^{low} SSC^{high}) with a BD FACS Aria SORP cell sorter (BD Biosciences) (Supplementary Fig. E1). The purity of peripheral blood and NP neutrophils was greater than 98.5% (not shown).

Bulk RNA-Sequencing

Total RNA from FACS sorted human neutrophils was extracted using an Arcturus PicoPure RNA isolation kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The quality of total RNA from neutrophils was assessed with a 2100 Bioanalyzer using a RNA 6000 Pico LabChip (Agilent Technologies, Santa Clara, CA). RNA for which the RNA integrity number (RIN) was greater than 7.0 was used for library preparation and RNA-Sequencing (RNA-Seq). RNA-Seq was performed by the NextSeq 500 system (Illumina, San Diego, CA) according to the manufacturer's instructions. FASTQ sequence data was trimmed and filtered by fastp (v0.21.0), was aligned by HISAT2 (v2.1.0) and then the expression levels were calculated by StringTie (v2.1.3) using Subio platform ver. 1.24 (Subio Inc., Kagoshima, Japan). The count data were log 2 transformed, normalized by global normalization, centered and then analyzed using Subio Platform. Gene ontology (GO) enrichment analysis was performed by

g:Profiler. GO associated with up-regulated genes in NP neutrophils was identified based on an adjusted p value <0.05 by g:SCS algorithm.¹⁵ We also used the TPM (transcripts per million) data for individual comparison of each gene.

Statistical analysis

All statistical calculations were performed using Graphpad Prism version 6.07 (GraphPad Software, La Jolla, CA), Subio Platform and g:Profiler. Differences between groups were analyzed using the 1-way ANOVA Kruskal-Wallis Dunn's multiple comparison test or the Mann-Whitney test. A p value of less than 0.05 was considered significant.

RESULTS

Elevation of neutrophil markers in CRSwNP

To examine evidence of the presence of neutrophils in CRSwNP in our cohort at Northwestern in Chicago, we first searched our record of repository samples (from 2007-2014) in which we assayed for both an eosinophil marker (eosinophil cationic protein [ECP]) and a neutrophil marker (neutrophil elastase [NE]). We confirmed the well-known elevation of ECP but also found that NE was significantly elevated in NPs compared to control and CRSsNP patients (Fig. 1A). Importantly, elevation of NE was only found in NP tissues whereas ECP was elevated in both uncinate and NP tissues of patients with CRSwNP (Fig. 1A). We previously found similar patterns of elevation of T2 markers in sinus tissue. Eosinophils and other T2 markers including ECP, CLC, IL-5 and IL-13 were elevated in ethmoid and NP tissues of CRSwNP compared to control ethmoid tissue.⁶ This suggests that elevation of neutrophils is a selective phenomenon, occurring only in NP tissue but not in the surrounding inflamed uncinate and ethmoid tissues. Although we initially hypothesized that eosinophils and neutrophils would be inversely correlated, based upon the notion that T1 and T2 inflammation would be inverse phenomena, surprisingly we found that levels of NE were not negatively correlated with ECP in NPs ($r = 0.151$, $p = 0.245$, Fig. 1B). Furthermore, elevation of NE, but not ECP, was found in NPs at significantly higher levels in CRSwNP patients who required revision surgery (Fig. 1C). We also examined the effect of asthmatic, atopic status, smoking status and steroid treatment on the levels of ECP and NE in NPs. We found that smokers only ($n=4$) presented significantly higher levels of NE ($p<0.01$) and relatively higher levels of ECP ($p=0.078$) compared to nonsmokers ($n=57$) (Supplementary Fig. E2).

Since we found evidence of accumulation of neutrophils in NPs in our repository data, we next assayed for another established neutrophil marker, myeloperoxidase (MPO), in nasal lavage fluids in a new cohort (in 2017-2019). We found that both ECP and MPO were significantly elevated in nasal lavage fluids of CRSwNP compared to control patients (Fig. 2A). Unlike NP tissue extracts, levels of MPO were weakly correlated with ECP in nasal lavage fluids in CRSwNP patients ($r = 0.389$, $p = 0.003$, Fig. 2B). We again found that elevation of MPO in nasal lavage fluids was found at significantly higher levels in tissue from patients undergoing a recurrence of CRSwNP and revision surgery (Fig. 2C). Although ECP levels in nasal lavage fluids also showed the tendency of elevation in recurrence CRSwNP patients, it did not reach significance ($p=0.060$, Fig. 2C).

Elevation of activated neutrophils in NPs

We next examined the presence of neutrophils in tissues by flow cytometry. Although we initially gated the $CD16^{high} SSC^{high}$ population as neutrophils and the $CD16^{-} SSC^{high}$ population as eosinophils within the live $CD45^{+}$ cells, we decided to include $CD66b^{16, 17}$ to separate granulocytes better in later samples (Fig. 3A). Importantly, both strategies gave us a similar frequency of eosinophils and neutrophils (Fig. 3A) and mixing results from both strategies did not change the key conclusions. We used inferior turbinate tissues from control patients for flow cytometric analysis due to the fact that only limited suitable control ethmoid and uncinate tissues were available for flow cytometric analysis. We first confirmed our previous findings that both eosinophils and neutrophils were significantly elevated in NPs compared to control tissue (Fig. 3B) and that the levels of neutrophils were not correlated with levels of eosinophils in NPs ($r = 0.144$, $p = 0.533$, $n = 21$, Fig. 3C). We next examined cell surface expression of CD62L (L-selectin)

on peripheral blood (PB), control tissue and NP neutrophils by flow cytometry since down-regulation of CD62L is frequently used as an activation marker in myeloid cells including neutrophils.^{12, 18-20} We found that cell surface expression of CD62L was indeed down-regulated and the frequency of the CD62L negative cell population was up-regulated in NP neutrophils compared to PB neutrophils (Fig. 3D). We also found that neutrophils in control tissues might be activated compared to PB neutrophils but less activated compared to NP neutrophils (Fig. 3D). Taken together, our results suggest that activated neutrophils accumulate in NPs of eosinophilic T2 CRSwNP in the Chicago population we serve.

Identification of up-regulated genes in NP neutrophils

We next sorted neutrophils from peripheral blood and NP tissue and examined whole transcriptome analysis by bulk RNA-Sequencing (RNA-Seq). To reduce the contamination of other cell types, we used more antibodies and sorted neutrophils as the live CD45⁺ CD117⁻ FcεRI⁻ CCR3⁻ CD16^{high} HLA-DR^{low} SSC^{high} cell population (Fig. 4A and Supplementary Fig. E1). The purity of FACS sorted neutrophils was 98.5-99.6% by cytoSpin and flow cytometry (not shown). We were able to obtain sufficient quality of total RNA for RNA-Seq from 15 neutrophil samples (4 control PB, 5 CRSwNP PB and 6 NP). Since RNA-Seq analysis in tissue neutrophils was challenging, we first extracted neutrophil-specific genes from a publicly-available RNA-Seq data set from human peripheral blood cells in the Human Protein Atlas (<http://www.proteinatlas.org>) and compared them to our RNA-Seq results. We found that 20 neutrophil-specific genes, including *CXCR1* and *FCGR3B*, which were reported in the Human Protein Atlas data were also highly detected in both PB and NP neutrophils in our RNA-Seq samples (Fig. 4B). This suggests that we successfully obtained acceptable quality of RNA from neutrophils and that our RNA-Seq results

can be used for whole transcriptome analysis of both PB and NP neutrophils. We next focused on PB neutrophils in control and CRSwNP patients. After filtering by exclusion of undetectable and weakly expressed genes and by selection of protein coding genes, we found that 110 genes were significantly up-regulated >2-fold and 161 genes were down-regulated >2-fold in CRSwNP PB neutrophils compared to control PB neutrophils (Fig. 4C and Supplementary Table E5). However, GO analysis did not identify any specific terms in the up-regulated genes from CRSwNP PB neutrophils (not shown). Therefore we did not further focus on PB neutrophils from CRSwNP in the current study.

To examine whether neutrophils in NPs are truly activated *in vivo*, we next evaluated neutrophils isolated from NP. After filtering, we identified 344 genes that were significantly elevated > 3-fold in NP neutrophils (n=6) compared to total PB neutrophils (PB neutrophils from CRSwNP and control patients, n=9) or control PB neutrophils (n=4) (Fig. 5A and Supplementary Table E6). The cluster and Venn diagram analyses identified a subset of genes that were elevated in both PB and NP neutrophils from patients with CRSwNP compared to control PB neutrophils (Fig. 5A). We examined the function of the upregulated genes in NP neutrophils by GO enrichment analysis and found that they were significantly associated with immune response, adhesion, activation and degranulation (Fig. 5B and Supplementary Table E7). Indeed, when we focused on a GO term, neutrophil activation (GO:0042119), several important molecules in neutrophils, including a neutrophil chemokine receptor *CXCR1*, activation receptors (*C3AR1* and *FCGR2B*), cell surface glycoproteins (*CD44* and *CD177*), an adhesion molecule (*CEACAM3*), protease inhibitors (including serpin family A member 1 [*SERPINA1*, also known as alpha-1 antitrypsin], serpin family B member 6 [*SERPINB6*] and secretory leukocyte protease inhibitor [*SLPI*]), and enzymes (including leukotriene A4 hydrolase [*LTA4H*] and cathepsin B [*CTSB*]) were all

significantly elevated in NP neutrophils (Fig. 5CD and Supplementary Table E6). Furthermore, mRNAs for activation associated proteins including *CD24*, *CD69*, PBX homeobox 1 (*PBX1*) and TNF alpha induced protein 6 (*TNFAIP6*), basic leucine zipper ATF-like transcription factor (*BATF*), early growth response 1 (*EGR1*), *ERG2*, *IL1B* and *IL1RN*²¹⁻²³ were also upregulated in NP neutrophils (Fig. 6AB and Supplementary Table E6). These results strongly suggest that neutrophils are highly activated and raises the important possibility that they exert pro-inflammatory functions in NPs.

It is well known that neutrophils play key roles in innate immunity and express many pattern recognition receptors including toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), C-type lectin receptors and components of the inflammasome, in order to sense and remove or neutralize invading pathogens.²²⁻²⁵ We next sought to examine the up-regulation of innate immune components in NP neutrophils in the RNA-Seq results. Among these genes, we have found that *TLR1*, *TLR5*, formyl peptide receptor 1 (*FPR1*), absent in melanoma-2 (*AIM2*), NLR family CARD domain containing 4 (*NLRC4*), caspase 4 (*CASP4*), C-type lectin domain family 4 member A (*CLEC4A*) and *IL1B* were elevated in NP neutrophils compared to PB neutrophils (Fig. 6C and Supplementary Table E8). This result suggests that up-regulation and activation of pattern recognition receptors and inflammasome components should be considered as a potential mechanism that enhances the production of proinflammatory cytokines including IL-1 β and reactive oxygen species in neutrophils in NPs.

DISCUSSION

Although it is becoming clearer that neutrophilic inflammation is highly associated with many important features of asthma, including severity, steroid insensitivity and asthma exacerbations, and that combined eosinophilic (T2) and neutrophilic (non-T2) inflammation induces the most severe forms of asthma,²⁶⁻²⁸ the presence and importance of neutrophils in western T2 NPs has not been well studied. Our group first reported that the epithelial barrier-disrupting cytokine oncostatin M (OSM) is elevated in T2 eosinophilic NPs in the US, and that neutrophils are a major source of OSM in NPs.^{29, 30} This suggests the elevation and importance of neutrophils in eosinophilic NPs. A Belgian group recently reported direct evidence that neutrophils are elevated in eosinophilic NPs as assessed by immunohistochemistry.¹² However, the accumulation of neutrophils in T2 CRSwNP has only begun to be studied by histological examination and the occurrence and reproducibility of neutrophilia in T2 CRSwNP in the US was unclear. In the current study, we utilized three approaches to examine the presence of neutrophils in NPs in Chicago and found elevations in CRSwNP of neutrophil markers, namely NE in tissue extracts and MPO in nasal lavage fluids, and tissue neutrophil numbers as detected by flow cytometry (Fig. 1-3). We next examined the cell surface expression of L-selectin (CD62L) on neutrophils, a marker of activation of adhesion and transendothelial migration.^{12, 18-20} We confirmed findings by Delemarre et al. in the Belgian population¹² that CD62L is down-regulated on NP neutrophils and the frequency of CD62L negative activated neutrophils is elevated in NPs (Fig. 3). These studies suggest that neutrophils are reproducibly elevated in T2 NPs and that neutrophils that accumulate in NPs are highly activated in the US as has been shown to be the case in Europe.

To obtain direct evidence of neutrophil activation in NPs, we next performed a RNA-Seq study in sorted neutrophils from PB and NPs. The GO analysis identifying >3-fold and significantly up-regulated genes in NP neutrophils also confirmed that neutrophils in NPs are highly activated (Fig. 5). We also found the up-regulation of important neutrophil enzymes including *LTA4H* and *CTSB* (Fig. 5). Leukotriene B4 (LTB4) is one of the most potent chemoattractants and activators of leukocytes produced from neutrophils. LTB4 is a lipid mediator that is not stored in cells but is synthesized de novo from arachidonic acid upon stimulation.^{31, 32} After activation, arachidonic acid is oxidized by the 5-lipoxygenase pathway and converted to an unstable intermediate leukotriene A4 (LTA4). LTA4 is further metabolized to either LTB4 by *LTA4H* or LTC4 by *LTC4 synthase*. Therefore *LTA4H* is a rate limiting enzyme controlling synthesis of LTB4 in neutrophils and up-regulation of *LTA4H* can enhance the capability to produce LTB4 upon activation. *CTSB* belongs to a family of cysteine proteases and exhibits both endopeptidase and exopeptidase activity. *CTSB* degrades extracellular matrix and induces tissue damage and inflammation.^{33, 34} Future studies will be required to specifically investigate the elevation and importance of LTB4 and *CTSB* in CRSwNP.

We also discovered an up-regulation of several innate immune receptors including IgG receptors, complement receptors, formyl peptide receptors and TLRs. It was of particular interest that genes that are associated with inflammasome cascades³⁵ were clearly elevated and detected in NP neutrophils (Fig. 6 and Supplementary Table E8). We found that IL-1 β , which is the key downstream molecular product of the priming step of inflammasomes, as well as two inflammasome sensors, *AIM2* and *NLRC4*, and a component of the non-canonical inflammasome pathway molecule *CASP4* were all significantly elevated in NP neutrophils (Fig. 6). Furthermore, other key inflammasome components³⁵, including *TLR4*, *CD14*, *NOD2*, NLR family pyrin domain

containing 3 (*NLRP3*), *CASP1*, PYD and CARD domain containing (*PYCARD*, also known as ASC), interferon gamma inducible protein 16 (*IFI16*), gasdermin D (*GSDMD*) and the Familial Mediterranean Fever Gene (*MEFV*, also known as pyrin) were highly detected but not elevated in NP neutrophils (Supplementary Table E8). These results suggest that neutrophils in NPs are likely to be sensitized to pathogens and strongly activate both canonical and non-canonical inflammasome pathways to contribute to inflammation in NPs.

Our results clearly demonstrate that neutrophils are highly elevated in NP tissue in the US and that neutrophils, but not eosinophils, are key immune effector cells that distinguish between NP tissue and other inflamed areas surrounding the ostiomeatal complex of CRSwNP patients (Fig. 1A). Furthermore, we found that neutrophils are significantly higher in NPs that were obtained from patients undergoing revision surgery, an important indicium of severity (Fig. 1). This implies that neutrophils play an important role in the formation of NPs and are associated with disease severity and recurrence. Although we found that neutrophil markers were present in NPs at significantly higher levels in CRSwNP patients who required revision surgery, only a subset of revision cases showed actual elevation of neutrophil markers. Furthermore, neutrophil markers were also elevated in NPs from a subset of primary surgery cases. It is possible that neutrophil marker high patients may carry a risk of disease recurrence in both primary and revision surgery cases and a follow up study will be required to examine whether neutrophils and their markers can be biomarkers to predict outcome after surgery.

However, it is well known that the disease severity and recurrence rate are lower in the purely neutrophilic NPs that are mainly found in east Asia than in eosinophilic CRSwNP.³⁶⁻³⁸ This suggests that neutrophilia alone does not explain the severity and higher recurrence rate in T2 CRSwNP and that neutrophils may have different states of activation and distinct roles between

T2 and non-T2 CRSwNP. It is possible that the interaction between neutrophils and T2 immune cells including eosinophils is necessary to control the severity of polypoid disease in T2 CRSwNP. Indeed, Succar et al. found that CRSwNP patients with mixed eosinophilic and neutrophilic inflammation showed the highest disease symptom scores in their US patient populations.¹¹ Further study will be required to understand the specific roles of neutrophils in the T2 condition in CRSwNP.

Although we found evidence supporting the importance of neutrophils in T2 CRSwNP and have presented the first whole transcriptome study in neutrophils in CRS, our present study has certain limitations. Since we were not able to sort sufficient numbers of neutrophils for RNA-Seq study from control sinus tissue, we could not separate between tissue resident neutrophil associated genes and NP specific neutrophil genes. Furthermore, we also recognized that some of the identified up-regulated genes in NP neutrophils might be coming from contaminating cells including epithelial cells. Indeed, several epithelial associated keratin genes (such as *KRT5* and *KRT19*) were included in the up-regulated genes in NP neutrophils (Supplementary Table E6). Although we isolated neutrophils by cell sorting, the purity of neutrophils was 98.5-99.6%. In addition, it is known that neutrophils contain less RNA than lymphocytes and structural cells on a per cell basis. Indeed, we found that neutrophils have less than 20% of the total mRNA per cell compared to epithelial cells in NPs (not shown). This indicates that genes from less than 1 % contaminating cells may influence the result of transcriptome analysis if these genes are highly expressed in the contaminating cells but not in neutrophils. To reduce the risk of observing gene expression effects resulting from contaminating cells, we also excluded genes that were undetected in more than 50% of PB neutrophils. We identified 281 genes (Supplementary Table E9) and used these genes for GO analysis. After the above filtering efforts, we still found that up-regulated

neutrophil genes were significantly associated with activation, degranulation and adhesion (Supplementary Table E10). This suggests that our key conclusion that accumulated neutrophils in NPs are activated is supported by this analysis. Although exclusion of undetected genes in PB neutrophils reduces the potential of effects by contaminant cells, it is also possible that this approach excludes important genes that are only expressed in NP neutrophils. To identify clear NP neutrophil-associated genes, single cell RNA-Seq is probably the best current approach since this can separate neutrophils from contaminating cells by gene expression profiles. However, it is also well known that there is a technical difficulty to detect tissue neutrophils and eosinophils by single cell RNA-Seq. Indeed, at least two groups, including ours, published results of single cell RNA-Seq in NP tissue and showed that neutrophils and eosinophils were almost absent in NPs in the single cell RNA-Seq data set, even though up to 30 and 60% of CD45⁺ cells in NPs are neutrophils and eosinophils, respectively.^{39, 40} Future study will be required to optimize single cell RNA-Seq technology for tissue granulocytes.

In conclusion, we report that neutrophils are highly elevated in NP tissue of eosinophilic CRSwNP and that elevated neutrophils in NPs are highly activated and are associated with NP recurrence. The identification of mechanisms for neutrophil recruitment and activation in T2 CRSwNP may help in developing new therapeutic targets to target these cells in severe CRSwNP.

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Figure Legends

Figure 1. Elevation of a neutrophil marker in nasal polyps.

ECP and NE in uncinate tissues (UT) from control (n=32), CRSsNP (n=42) and CRSwNP (n=38) and NP tissues (n=61) were determined by ELISA. NP samples were further divided into no prior (n=47) and revision (n=14) surgery cases (C). The Spearman rank correlations were assessed by matched NP tissue samples (B, n=61). Results are shown as mean $\pm$ SEM. * $p<0.05$, *** $p<0.001$, **** $p<0.0001$ by one-way ANOVA (A) and the Mann-Whitney test (C).

Figure 2. Elevation of a neutrophil marker in nasal lavage fluids from CRSwNP.

ECP and MPO in nasal lavage fluids from control (n=29) and CRSwNP (n=57) were determined by ELISA. CRSwNP samples were further divided into no prior (n=33) and revision (n=24) surgery cases (C). The Spearman rank correlations were assessed by matched CRSwNP nasal lavage samples (B, n=57). Results are shown as mean $\pm$ SEM. * $p<0.05$, **** $p<0.0001$ by the Mann-Whitney test (A and C).

Figure 3. Neutrophils are elevated and activated in NPs.

A representative gating strategy for eosinophils and neutrophils within the live CD45⁺ population is shown in A. We collected inferior turbinate tissues from control patients (Control, n=12) and NP tissues from patients with CRSwNP (NP, n=21) and the numbers of eosinophils and neutrophils in sinus tissues were normalized by mg of tissue (B). The Spearman rank correlation was assessed by matched NP tissue samples (C, n=21). Representative histograms of flow cytometric plots for CD62L, the levels of CD62L on neutrophils by gMFI (geometric mean fluorescence intensity)

ratio between CD62L and fluorescence minus one (FMO) control and the frequency of CD62 negative neutrophils in peripheral blood (PB, n=16), control inferior turbinate (Control, n=8) and NPs (n=7) were shown in D. Results are shown as mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by the Mann-Whitney test (B) and one-way ANOVA (D).

Figure 4. Bulk RNA-Seq in neutrophils.

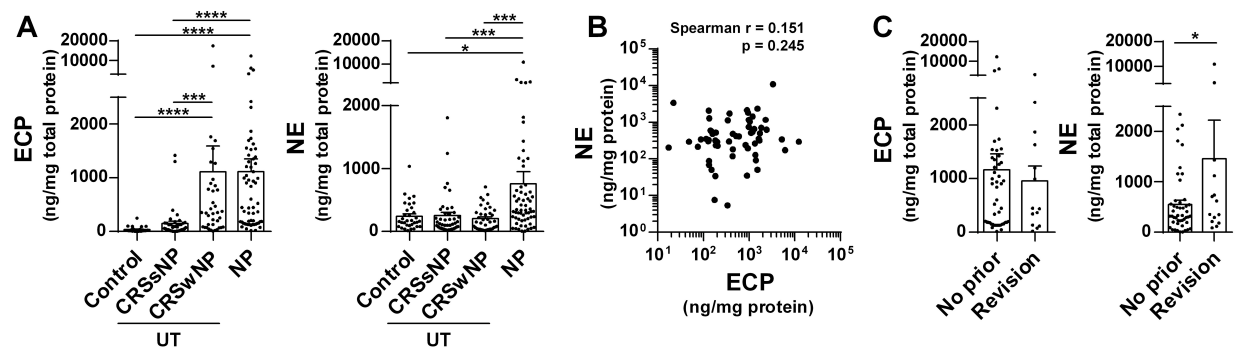
A representative cell sorting strategy for neutrophils within the live $CD45^+CD117^+Fc\epsilon R1^+$ population is shown in A. The hierarchical clustering was performed using 20 neutrophil specific genes in the RNA-Seq result of human peripheral blood cells from the Human Protein Atlas (<http://www.proteinatlas.org>) with RNA-Seq result of our neutrophil samples (9 PB and 6 NP) (B). The hierarchical clustering was performed using >2-fold significantly up-regulated (110 genes) or down-regulated (161 genes) genes in CRSwNP PB neutrophils (n=5) compared to control PB neutrophils (n=2) in the bulk RNA-Seq data (C).

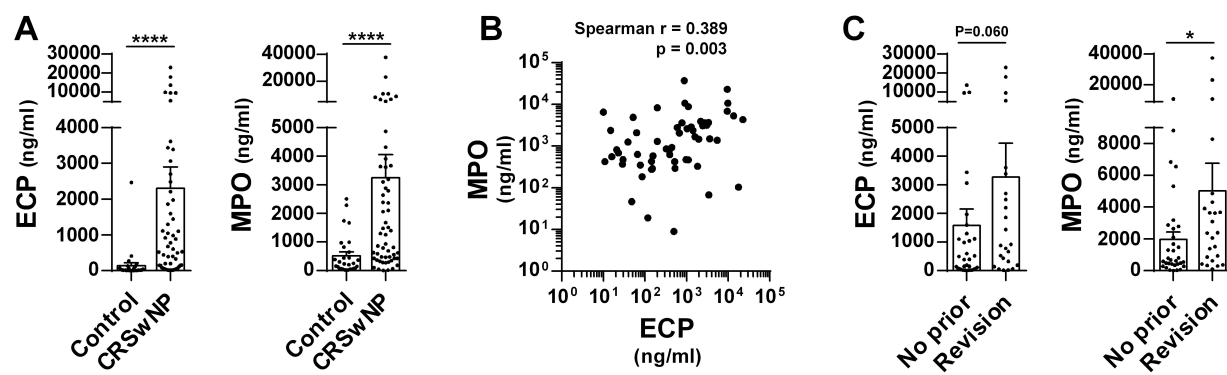
Figure 5. Elevated genes in NP neutrophils are associated with activation.

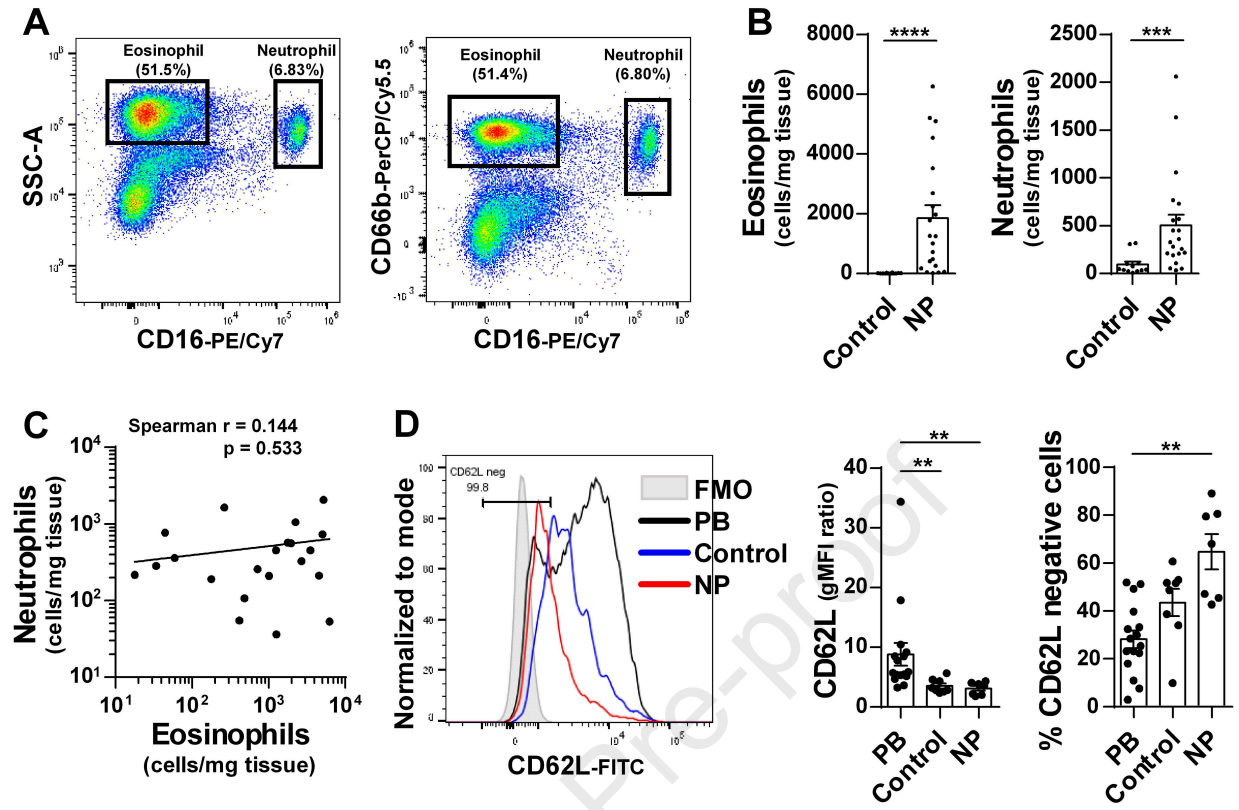
A Venn diagram identified that 261 and 265 genes were >3-fold significantly elevated in NP neutrophils compared to all peripheral blood (PB) neutrophils (control PB neutrophils + CRSwNP PB neutrophils) and control PB neutrophils, respectively, and hierarchical clustering was performed using these 344 genes in the RNA-Seq data (A). The top 10 GO biological processes associated with elevated genes in NP neutrophils are shown (B). The hierarchical clustering was performed using elevated genes in NP neutrophils associated with a GO term, neutrophil activation (GO:0042119) (C). The expression levels were shown by TPM (transcripts per million) in RNA-Seq data and results are shown as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA (D).

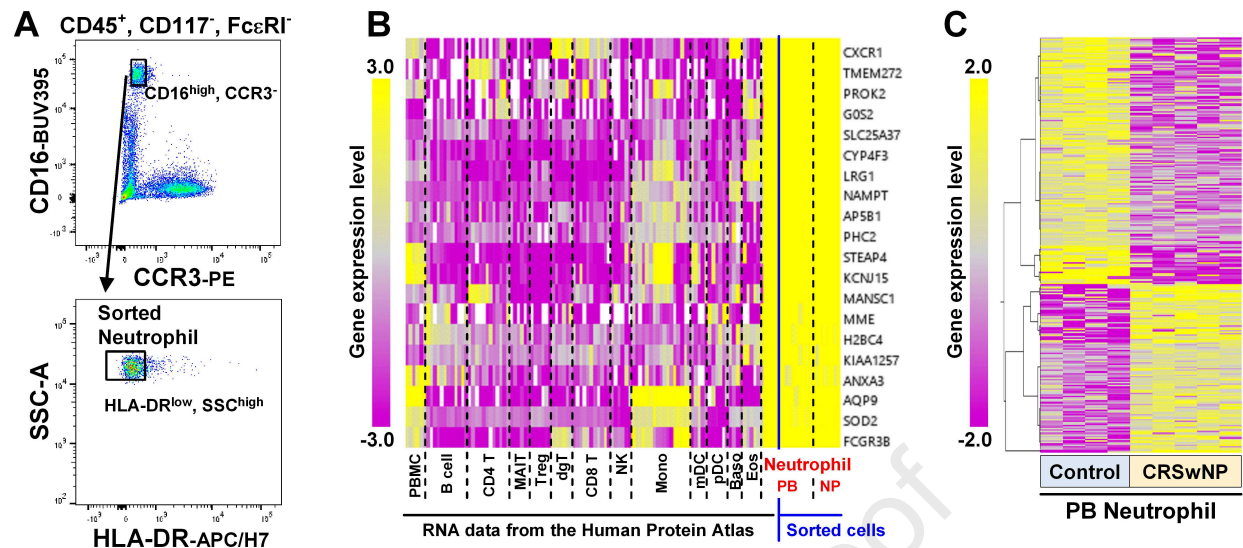
Figure 6. Elevation of activation markers and innate receptors in NP neutrophils.

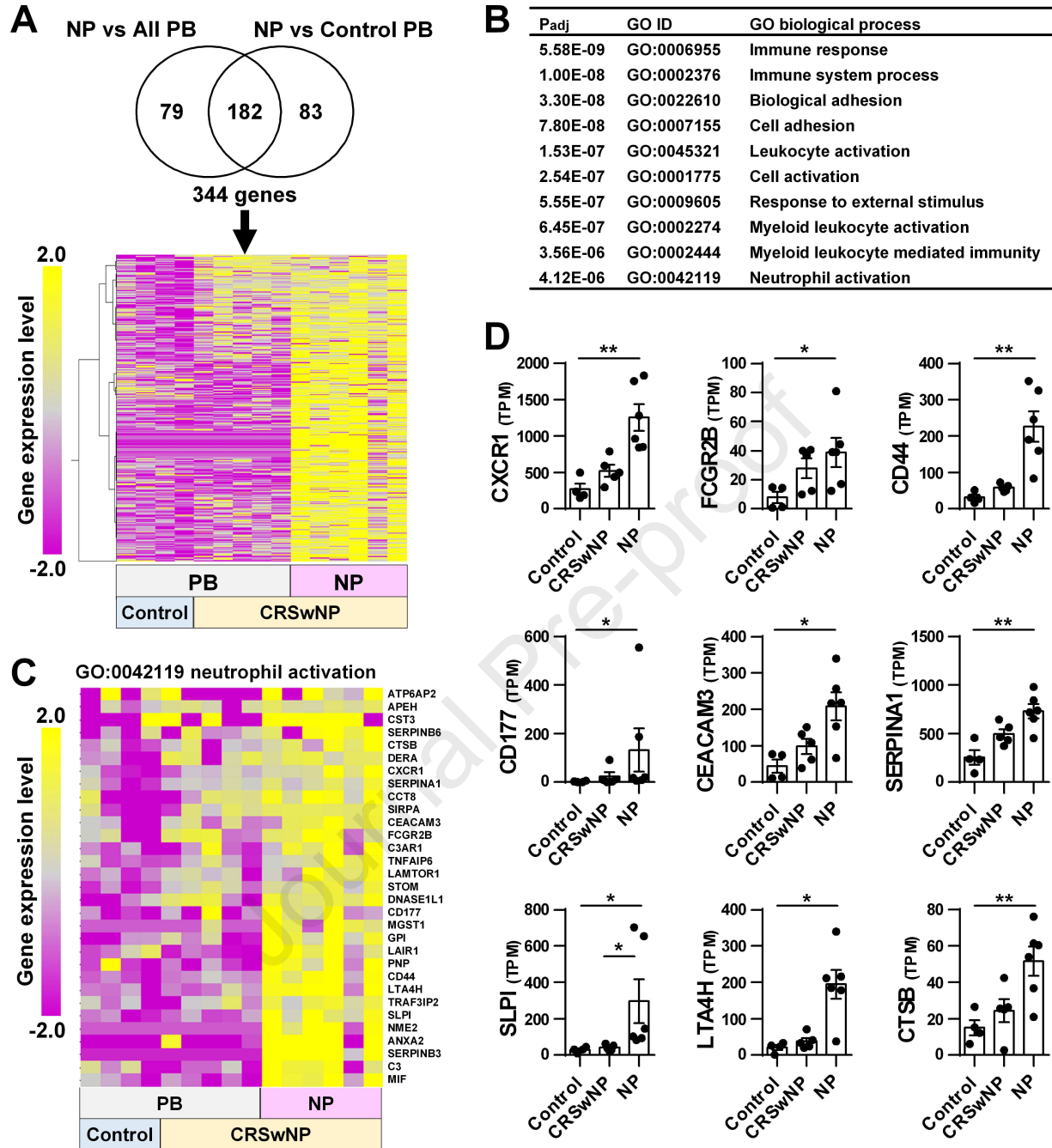
The expression of activation markers (A, B) and innate pattern recognition receptors and inflammasome components (C) in control PB neutrophils (Control, n=4), CRSwNP PB neutrophils (CRSwNP, n=5) and NP neutrophils (NP, n=6) was determined by RNA-Seq. We also compared total PB neutrophils (PB, n=9 [4 control PB + 5 CRSwNP PB]) and NP neutrophils in B. The expression levels were shown by TPM (transcripts per million) and results are shown as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA (A, C) and the Mann-Whitney test (B).

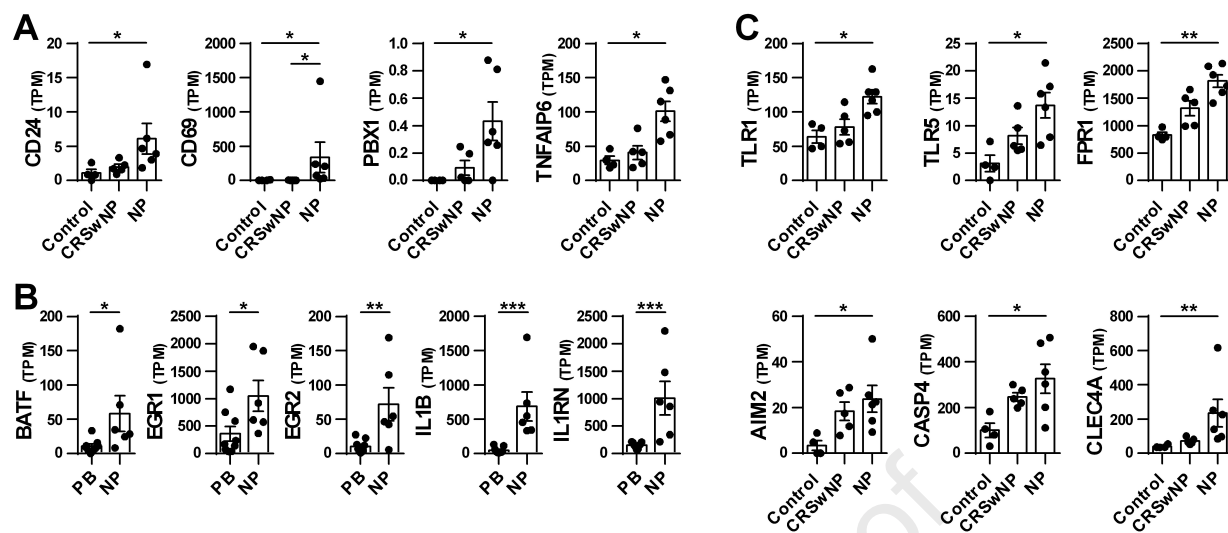












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Elevation of activated neutrophils in chronic rhinosinusitis with nasal polyps

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17 **Supplementary Table E1. Patient characteristics in tissue extract study**

	Control UT (n=32)	CRSsNP UT (n=42)	CRSwNP UR (n=38)	CRSwNP NP (n=61)
Age (y), median (range)	48 (20-73)	40 (19-69)	41 (20-71)	46 (20-71)
Sex (female), yes (%)	50.0	52.4	44.7	37.7
Atopy, yes (%)	6.3	26.2	39.5	37.7
Asthma, yes (%)	0.0	28.6	47.4	54.1
NSAID sensitivity, yes (%)	0.0	0.0	5.3	14.8
Current smoker yes (%)	0.0	0.0	7.9	6.6
Nasal steroid, yes (%)	3.1	21.4	18.4	19.7
Inhaled steroid, yes (%)	3.1	16.7	21.1	21.3
Oral steroid, yes (%)	9.4	2.4	23.7	26.2

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21 **Supplementary Table E2. Patient characteristics in nasal lavage fluid study**

	Control (n=29)	CRSwNP (n=57)
Age (y), median (range)	38 (18-67)	47 (19-79)
Sex (female), yes (%)	31.0	28.6
Atopy, yes (%)	13.8	66.7
Asthma, yes (%)	0.0	36.8
NSAID sensitivity, yes (%)	0.0	1.8
Current smoker yes (%)	0.0	5.3
Nasal steroid, yes (%)	0.0	29.8
Inhaled steroid, yes (%)	0.0	21.1
Oral steroid, yes (%)	0.0	22.8

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25 **Supplementary Table E3. Patient characteristics in flow cytometry study**

	Control (n=12)	CRSwNP (n=21)
Age (y), median (range)	41 (19-73)	46 (18-67)
Sex (female), yes (%)	50.0	42.9
Atopy, yes (%)	41.7	38.1
Asthma, yes (%)	0.0	33.3
NSAID sensitivity, yes (%)	0.0	14.3
Current smoker yes (%)	0.0	9.5
Nasal steroid, yes (%)	0.0	33.3
Inhaled steroid, yes (%)	0.0	9.5
Oral steroid, yes (%)	8.3	23.8

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27 **Supplementary Table 4 Patient characteristics in RNA-Seq study**

	Control PB (n=4)	CRSwNP PB (n=5)	CRSwNP NP (n=6)
Age (y), median (range)	38 (31-58)	49 (29-64)	43 (29-64)
Sex (female), yes (%)	25.0	0.0	16.7
Atopy, yes (%)	50.0	60.0	33.3
Asthma, yes (%)	0.0	40.0	33.3
NSAID sensitivity, yes (%)	0.0	40.0	16.7
Current smoker yes (%)	0.0	0.0	0.0
Nasal steroid, yes (%)	20.0	20.0	16.7
Inhaled steroid, yes (%)	0.0	20.0	0.0
Oral steroid, yes (%)	0.0	0.0	0.0

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Figure Legends

Supplementary Figure E1. Gating strategy to sort neutrophils.

Representative flow cytometric plots for neutrophil sort in peripheral blood (A) and NP (B) are shown. We sorted neutrophils as the Aqua⁻ CD45⁺ CD117⁻ FcεRI⁻ CCR3⁻ CD16^{high} HLA-DR^{low} SSC^{high} population. Representative cytopsin images for sorted neutrophils are shown (200x).

Supplementary Figure E2. The effect of asthmatic, atopic status, smoking status and steroid treatment on the levels of ECP and NE in NPs.

ECP and NE in NP tissues (n=61) were determined by ELISA. We compared levels of ECP and NE in NP tissues between non-asthmatics (n=28) and asthmatics (n=33) (A), between non-atopic (n=11), atopic (n=23) and non-tested (n=27) patients (B), between non-smoker (n=57) and smoker (n=4) (C), and between steroid untreated patients (n=31) with nasal steroid treated (n=12, D), inhaled steroid treated (n=11, E) or oral steroid treated (n=16, F) patients. Results are shown as mean ± SEM. ** p<0.01 by the Mann-Whitney test (A, C-F) and one-way ANOVA (B).