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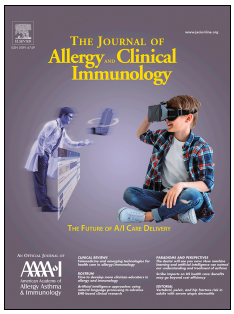

IL-5Rα marks nasal polyp IgG4 and IgE-expressing cells in aspirin-exacerbated respiratory disease

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Title: IL-5Rα marks nasal polyp IgG4 and IgE-expressing cells in aspirin-exacerbated respiratory disease

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Abstract:

Background: The cause of severe nasal polyposis in aspirin-exacerbated respiratory disease (AERD) is unknown. Elevated antibody levels have been associated with disease severity in nasal polyps (NPs), but upstream drivers of local antibody production in NPs are undetermined.

Objective: We sought to identify upstream drivers and phenotypic properties of local antibody-expressing cells (AECs) in NPs from AERD subjects.

Methods: Sinus tissue was obtained from subjects with AERD, chronic rhinosinusitis with NPs (CRSwNP), CRS without NPs (CRSsNP), and non-CRS controls. Tissue antibody levels were quantified via ELISA and immunohistochemistry, and were correlated with disease severity. AECs were profiled with single-cell RNA-sequencing (scRNA-seq), flow cytometry and immunofluorescence, with IL-5Rα function determined through IL-5 stimulation and subsequent RNA-seq and qPCR.

Results: Tissue IgE and IgG4 were elevated in AERD compared to controls ($P<0.01$ for IgE and $P<0.001$ for IgG4, vs. CRSwNP). AERD subjects whose NPs recurred rapidly had higher IgE levels than AERD subjects with slower regrowth ($P=0.005$). ScRNA-seq revealed increased *IL5RA*, *IGHG4*, and *IGHE* in AECs from AERD compared to CRSwNP. There were more IL-5Rα$^+$ plasma cells in the polyp tissue from AERD than CRSwNP ($P=0.026$). IL-5 stimulation of plasma cells in vitro induced changes in a distinct set of transcripts.

Conclusions: Our study identifies an increase in AECs in AERD defined by transcript enrichment of *IL5RA* and *IGHG4* or *IGHE*, with confirmed surface expression of IL-5Rα, and functional IL-5 signaling. Tissue IgE and IgG4 are elevated in AERD and higher IgE levels are associated with faster NP regrowth. Our findings suggest a role for IL-5Rα$^+$ AECs in facilitating local antibody production and severe NPs in AERD.

Key Messages:

- **IgG4 and IgE levels are markedly increased in nasal polyp tissue from subjects with AERD compared to aspirin-tolerant CRSwNP.**
- **High nasal polyp IgE levels are associated with more rapid nasal polyp recurrence.**
- **Tissue IgG4 levels positively correlate with disease duration.**
- **IL-5Rα transcript and protein surface expression is elevated in antibody-expressing cells from subjects with AERD, and may play a role in facilitating survival of antibody-expressing cells.**

Capsule Summary: Our study identified a plasma cell population enriched in the nasal polyps of patients with aspirin-exacerbated respiratory disease, defined by transcript enrichment of *IL5RA*, *IGHG4* and *IGHE*, surface expression of IL-5Rα, and a functional IL-5 signaling pathway.

Key Words:
Aspirin-exacerbated respiratory disease, chronic rhinosinusitis, nasal polyposis, plasma cell, interleukin-5, IgG4, IgE, IL-5Rα

Abbreviations:

AERD	**Aspirin-exacerbated respiratory disease**
CRSwNP	**Chronic rhinosinusitis with nasal polyps**
CRSsNP	**Chronic rhinosinusitis without nasal polyps**
COX	**Cyclooxygenase**
FDR	**False discovery rate**
Ig	**Immunoglobulin**
IL	**Interleukin**
LT	**Leukotriene**
PC	**Principal component**
PCA	**Principal component analysis**
PG	**Prostaglandin**
scRNA-seq	**Single-cell RNA-sequencing**
SNN	**Shared nearest neighbor**
UMAP	**Uniform Manifold Approximation and Projection**

Introduction:

Nasal polyps are inflammatory outgrowths of sinonasal mucosa that cause nasal obstruction and anosmia, frequently require surgical excision, and are associated with significant medical resource consumption.[1-3] Nasal polyps are particularly severe and recurrent in aspirin-exacerbated respiratory disease (AERD) – a distinct, adult-onset respiratory syndrome consisting of eosinophilic chronic rhinosinusitis with nasal polyposis (CRSwNP), asthma, and pathognomonic respiratory reactions to cyclooxygenase (COX)-1 inhibitors that involve release of multiple mast cell mediators, including tryptase, leukotriene (LT)C_4 and prostaglandin (PG)D_2.[4-6] In patients with AERD, nasal polyps are frequently refractory to standard therapy and recur within two years after surgical excision in 85 percent of patients.[7] The factors contributing to the severity and recalcitrance of the mucosal pathology in this severe phenotype of CRSwNP remain largely unknown.

Activated B cells and antibody-secreting cells are present in nasal polyps and generate antibodies locally. Subjects with recurrent nasal polyposis have elevated total nasal polyp IgA, IgG, and IgE levels.[8-12] Potential mechanisms by which local nasal tissue immunoglobulins may contribute to nasal polyp severity include IgE- and free light chain-induced activation of polyp mast cells,[13] IgA-enhanced eosinophil survival,[14] and IgG-directed local complement activation.[15, 16] IgE antibodies to staphylococcal enterotoxins[12, 17] and nasal bacteria such as Staphylococcus aureus, Strepococcus pyogenes, and Haemophilus influenzae[18] have been linked to nasal polyp pathogenesis, and a role for auto-antibodies in nasal polyp pathogenesis has been proposed,[8] but no single antigen has been consistently linked to recurrent nasal polyposis in general or to AERD in particular. A previous study reported that patients with AERD have elevated serum IgG4 and slightly depressed serum IgG1 as compared to healthy controls, independent of corticosteroid exposure or IgE levels.[19] More recently, IgG4 was identified in nasal polyp tissue from subjects with CRS and AERD and was correlated with a poor post-operative course.[20] This suggests a possible role for IgG4 in sinus disease persistence by as yet unidentified mechanisms.

Nasal polyp tissue contains a variety of cytokines that may drive the B cell pro-inflammatory response.[21] Type 2 cytokines, including IL-4, IL-5, IL-13, TSLP, and IL-33, as well as IL-10, are abundant in eosinophilic nasal polyps.[6, 12, 22-24] Some of these cytokines have been shown to influence B cell differentiation, activation and class switching, and can drive immunoglobulin production in other settings.[25, 26] In a study of inflammatory endotypes in CRS, the group with the highest IL-5 levels also demonstrated the highest concentration of IgE and asthma prevalence, reflecting a severe endotype of CRSwNP.[24] Other studies of CRSwNP have shown elevated soluble IL-5Rα in nasal polyp tissue.[27, 28]

In the current study, we use massively parallel single-cell RNA-sequencing (scRNA-seq) and flow cytometry to identify a population of antibody-expressing cells enriched in nasal polyps from patients with severe nasal polyposis and AERD. These antibody-expressing cells express *IL5RA* and a functional IL-5 receptor alpha subunit (IL-5Rα), along with *IGHG4* and *IGHE*, encoding for the IgG4 and IgE heavy chains, respectively. Both IgE and IgG4 concentrations are selectively elevated in the nasal polyp

tissue of subjects with AERD. However, we find that while elevated polyp IgE is associated with fast polyp regrowth, polyp IgG4 is associated with disease persistence. Taken together, our findings indicate that class switching to IgE and to IgG4 in the nasal polyp environment may reflect disease severity and chronicity, respectively. Furthermore, they suggest that while IgE may be pathogenic and driven in part by the effect of local T cell-derived IL-5 on antibody-expressing cells in the nasal polyp tissue, increased IgG4 may be a compensatory mechanism reflecting chronic antigen exposure and the influence of IL-10 from myeloid cells. Finally, our data suggest that, in addition to its established role in controlling tissue eosinophilia, IL-5 may also influence the activation state of antibody-expressing cells[29] and their antibody production, and may be amenable to modification with IL-5-neutralizing biologic therapies.

Methods:

Patient characterization

Subjects between the ages of 18 and 75 years were recruited from the Brigham and Women's Hospital (Boston, MA) Allergy and Immunology clinics and Otolaryngology clinics between October 2011 and October 2019 **(Table 1 and Table E1)**. The local Institutional Review Board approved the study and all subjects provided written informed consent. Sinus tissue was collected at the time of elective endoscopic sinus surgery from patients with physician-diagnosed AERD, and aspirin-tolerant CRS with and without nasal polyps with the diagnosis made based on established guidelines.[30] Non-CRS control patients were undergoing sinus surgery to correct anatomic abnormalities by removal of concha bullosa. Patients were suspected of having AERD if they had asthma, nasal polyposis, and a history of respiratory reaction on ingestion of a COX-1 inhibitor, with diagnosis later confirmed in all subjects via a physician-observed graded oral challenge to aspirin which induced objectively-defined upper and/or lower respiratory symptoms including nasal congestion, rhinorrhea, sneezing, ocular pruritus, conjunctival injection, wheezing, dyspnea, and/or fall in FEV_1. Subjects with known cystic fibrosis, allergic fungal rhinosinusitis and unilateral polyps were excluded from the study.

Retrospective data was collected from the medical record for patients that donated sinus tissue including age, gender, number of sinus surgeries, and interval to polyp regrowth following surgery in subjects with recurrent polyposis. Because aspirin desensitization can delay polyp regrowth in subjects with AERD, we only included regrowth data from subjects who were not desensitized to aspirin after surgery.[31, 32]

Sinus tissue procurement and preparation

Sinus tissue was collected at the time of elective endoscopic sinus surgery from patients with physician-diagnosed CRS with and without nasal polyps. For non-polyp patients, diseased ethmoid sinus tissue was collected. For both CRSwNP and AERD, polyp tissue, originating from the ethmoid sinus, was surgically excised and collected. Non-CRS control subjects had no known history of CRS or nasal polyposis and were undergoing sinus surgery for removal of concha bullosa. One tissue segment was

immediately preserved in RNAlater (Qiagen, Valencia, CA) for RNA extraction, and the remaining tissue was placed in RPMI (Corning, Corning, NY) with 10% fetal bovine serum (ThermoFisher, Waltham, MA) and 1 U/mL penicillin-streptomycin for transport to the laboratory on ice. Within 2 hours of surgery, the tissue was removed from RPMI and divided into segments. One segment was transferred into Cell Lytic M Cell Lysis Reagent (Sigma-Aldrich, St Louis, MO) with 2% protease inhibitor (Roche, Indianapolis, IN) for protein extraction, and the tissue was homogenized with a gentleMACS Dissociator (Miltenyi Biotec, San Diego, CA). Supernatants were stored at -80°C. One segment was fixed in 4% paraformaldehyde, embedded in paraffin, and kept at -80°C until sectioning. For some patients, a tissue segment was also digested into a single-cell suspension for flow cytometric studies as described below.

Subjects with AERD, CRSwNP and CRSsNP who met inclusion criteria were recruited for scRNA-seq studies. The choice of subjects for the follow-up analyses including the ELISA data, qPCR and immunohistochemistry was made based on samples that had been previously banked and were available for confirmatory analyses by an investigator who was blinded to the subjects included in the scRNA-seq analysis. To guard against potential experimental bias other than disease phenotype (AERD, CRSwNP, CRSsNP, or non-CRS control), the investigators and research technicians were blinded to clinical markers of disease severity and markers of type 2 immunity when the banked samples were selected for the confirmatory analyses, and no exclusions were made after analyses were complete.

Tissue Digestion

Single-cell suspensions from surgical specimens were obtained using a modified version of a previously published protocol.[33] Surgical specimens were collected into 30 mL of cold RPMI with 10% fetal bovine serum and 1 U/mL penicillin-streptomycin. Specimens were finely minced between two scalpel blades and incubated for 15 minutes at 37°C with 600 U/mL collagenase IV (Worthington, Lakewood, NJ) and 20 ug/mL DNAse 1 (Roche, Indianapolis, IN) in RPMI with 10% fetal bovine serum. After 15 minutes, samples were triturated five times using a syringe with a 16G needle and incubated for another 15 minutes. At the conclusion of the second digest period, samples were triturated an additional five times using a syringe with a 16G needle. Samples were typically fully dissociated at this step and were filtered through a 70 µm cell strainer and spun down at 500G for 10 minutes followed by a rinse with ice-cold Ca/Mg free PBS (ThermoFisher, Waltham MA). Red blood cells were lysed using ACK buffer (ThermoFisher) for three minutes on ice to remove red blood cells, even if no red blood cell contamination was visibly seen in order to maintain consistency across patient groups. Some single-cell suspensions were cryopreserved in CryoStor CS10 (Sigma) for batched flow cytometric analyses.

Quantitative PCR

RNA was extracted from the whole nasal tissue specimens with Tri Reagent (Qiagen) and converted to cDNA by using the RT^2 First Strand Kit (Qiagen). Expression

of *IL4*, *IL5*, *IL6*, *IL7*, *IL10*, *IL13*, *IL21*, *IL23*, *TGFB1*, *IFNA1*, *CXCL12*, *CXCL13*, *PRDM1*, and *TNFSF13B* transcripts was examined using RT2 SYBR Green qPCR Master Mix (Qiagen), and normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; all primers from Qiagen).

Immunoglobulin quantification

Protein lysate supernatants from sinonasal tissue were collected as described above. Total IgG, IgA, IgE, IgG1, IgG2, IgG3, and IgG4 ELISAs (eBioscience, San Diego CA) were performed according to the manufacturer's instructions. Total tissue protein levels were measured with the Pierce® BCA Protein Assay kit (Thermo Scientific). Tissue immunoglobulin levels were normalized to total protein levels.

Immunohistochemistry and Immunofluorescence

Tissue segments were fixed in 4% paraformaldehyde, embedded in paraffin or frozen in optimal cutting temperature compound, and 5 μm sections were prepared. Tissue sections were incubated with a mouse anti-human IgG4 mAb (clone MRQ-44; Sigma) or isotype control. For immunohistochemistry, staining was developed with the EnVision System-HRP for mouse primary antibodies (Dako, Carpinteria, CA). Sections were counterstained with hematoxylin, Gill no. 2. For quantification of $IgG4^+$ cells, numbers of IgG4-positive cells in photomicrographs encompassing at least 3 high power fields of subepithelial tissue were counted and expressed per high power field. For immunofluorescence, sections were either blocked with 10% donkey serum, then were incubated with both mouse anti-IgG4 and a rabbit polyclonal anti-human IL-5Rα Ab (Sigma PA5-25159) or rabbit IgG in the first step, and staining was developed with AF 594 $F(ab')_2$, donkey anti-mouse IgG and AF 488 $F(ab')_2$, donkey anti-rabbit IgG.

Syndecan-1 (CD138) immunoreactivity was assessed in fresh frozen tissue slides. After elimination of non-specific binding with a PBS-based blocking with 0.1% Triton X, 0.1% saponin, 3% bovine serum albumin and 3% normal donkey serum for 1 hour at room temperature, the slides were incubated overnight at 4ºC with a mouse monoclonal antibody to Syndecan-1 (1 μg/ml, clone B-A38, Abcam, Cambridge, MA), polyclonal anti-human IL-5Rα Ab (5 μg/ml) or their respective isotype controls: mouse IgG1 (Biolegend) or rabbit polyclonal IgG (Abcam) at the corresponding concentrations. Immunoreactivity was detected with donkey anti-mouse secondary antibody, Alexa Fluor 594 and donkey anti-rabbit secondary antibody, Alexa Fluor 488 (both Life Technologies) applied for 2 hours at room temperature. Nuclear staining was performed with Hoechst 33342 nuclear stain (Sigma). Images were acquired at the Brigham and Women's Confocal Microscopy Core Facility with a Zeiss LSM 800 with Airyscan confocal system on a Zeiss Axio Observer Z1 inverted microcope with a 20 x Zeiss, 0.8NA and a 63 x Zeiss oil, 1.4NA objectives.

scRNA-Seq Analysis

Ethmoid scRNA-seq data was obtained from a previously published study,[34] available from the dbGaP database under dbGaP accession 30434. The UMI-collapsed cells-by-genes matrix was input into Seurat[35] and scaled, centered and log normalized through default code implemented in Seurat. Clustering was conducted as previously

described.[34] Iterative clustering was conducted on the previously defined plasma cell cluster, consisting of 2,520 cells across 12 patient samples. Briefly, a list of the 1,902 most variable genes among these cells was generated by including genes with an average log-normalized and scaled expression value greater than 0.22 and with a dispersion (variance/mean) of between 0.22 and 7. Principal component analysis (PCA) was performed over this list of variable genes with the addition of all immunoglobulin isotype heavy chain constant regions and first 8 principal components (PCs) were selected for further analysis based on visual identification of the "elbow" in a plot of the percent variance explained per PC. Clusters were determined using FindClusters (utilizing a shared nearest neighbor (SNN) modularity optimization-based clustering algorithm) on the first 8 principal components with a resolution of 0.7. Cells were then graphically displayed using Uniform Manifold Approximation and Projection (UMAP) with a minimum distance of 0.75. Pearson correlation with *IL5RA* was evaluated for all detected transcripts using Seurat.[36]

To determine cytokine sources within AERD polyps, the 4,276 cells collected from AERD patient polyps were iteratively clustered in the following fashion. A list of the 1,902 most variable genes was generated using the criteria outlined above. After performing PCA, the first 15 PCs were used for clustering and UMAP display following visual inspection of the principal component elbow graph and determining the inflection point. We note that this number of PCs separated all previously identified cell types. Cellular identities were retained from previous analysis of this dataset.[34]

Sub-analysis of the 282 myeloid cells was conducted on the 2,324 most variable genes, determined as previously mentioned. The first 5 PCs were utilized for clustering and UMAP following visual inspection of the PC elbow plot, and clustering was performed with a resolution of 0.6. Sub-analysis of the 224 T lymphocytes was conducted on the 2,587 most variable genes, determined as previously mentioned. The first 6 PCs were utilized for clustering and UMAP following visual inspection of the PC elbow plot, and clustering was performed with a resolution of 1.0.

Flow cytometry

Cells from the digested nasal polyp single cell suspension were stained with mAbs against CD45, CD3, CD4, CCR3, CD27, CD38, CD138 (eBiosciences), IL-5Rα and CD20 (BD Biosciences, Franklin Lakes NJ) to identify plasma cells/plasmablasts, B cells, eosinophils and expression of the IL-5Rα. Plasma cells were defined as $CD45^+/CD3^-/CD20^-/CD27^+/CD38^+/CD138^+$, B cells were defined as $CD45^+/CD3^-/CD20^+$, and eosinophils were defined as $SSC^{Hi}/CD45^+/CCR3^+$.

Blood from atopic donors without nasal polyposis or chronic rhinosinusitis was obtained and stained with CD45, CCR3 and IL-5Rα to determine the level of IL-5Rα expression by peripheral eosinophils.

IL-5 stimulation of sorted plasma cells

Cells from freshly digested nasal polyp tissue were stained with mAbs against CD45, CD3, CD4, CD27, CD38, CD138, and CD20 as above and plasma cells, defined as $CD45^+/CD3^-/CD20^-/CD27^+/CD38^+/CD138^+$ were purified with a BD FACSAria Fusion Cell Sorter. Purified plasma cells were stimulated with or without IL-5 (1 ng/mL;

PeproTech, Rocky Hill, NJ) for 6 hours at 37°C, 5% CO2. RNA was extracted from sorted cells with the RNeasy Micro Kit (Qiagen). For qPCR, RNA from three unstimulated/IL-5 stimulated pairs was converted to cDNA by using the RT^2 First Strand Kit (Qiagen). Expression of *CCND2* transcript was examined using RT^2 SYBR Green qPCR Master Mix (Qiagen), and normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; all primers from Qiagen).

For bulk RNA sequencing, RNA from two patients (each with unstimulated and IL-5 1 ng/mL stimulated conditions evaluated in duplicate) was normalized to 10 ng as the input amount for a 2.2X SPRI ratio cleanup using Agencourt RNAClean XP beads (Beckman Coulter, A63987). After oligo-dT priming, Maxima H Minus Reverse Transcriptase (ThermoFisher EP0753) was used to synthesize cDNA with an elongation step at 52 °C before PCR amplification (18 cycles for sorted plasma cells) using KAPA HiFi PCR Mastermix (Kapa Biosystems KK2602). Sequencing libraries were prepared using the Nextera XT DNA tagmentation kit (Illumina FC-131-1096) with 250 pg input for each sample. Libraries were pooled post-Nextera and cleaned using Agencourt AMPure SPRI beads with successive 0.7X and 0.8X ratio SPRIs and sequenced with an Illumina 75 Cycle NextSeq500/550v2.5 kit (Illumina FC-404-2005) with loading density at 2.2 pM, with paired end 35 cycle read structure. Tissue samples were sequenced at an average read depth of 12.9 million reads per sample. Sorted plasma cell samples were aligned to the Hg19 genome and transcriptome using STAR and RSEM.[37, 38] After concatinating read counts for technical replicates, differential expression analysis was conducted using the DESeq2 package for R taking patient origin into account.[39] Genes with Benjamini-Hochberg adjusted p-values corresponding to a false discovery rate (FDR) <0.1 were regarded as differentially expressed.

Statistical analysis

Data are presented as individual points plus standard error (SEM), unless otherwise specified. For the immunoglobulin analyses, comparisons were performed with the Kruskal-Wallis one-way ANOVA due to non-Gaussian distribution of the data. Binary comparisons were carried out with the Mann-Whitney test. Significance was defined as a two-tailed *P*-value of less than 0.05. The binary polyp regrowth data were analyzed with an unpaired t-test with Welch's correction. For the whole polyp mRNA cytokine analyses, comparisons were performed with an unpaired, 2-tailed *t*-test. The IL-5 stimulated plasma cell mRNA analysis was analyzed with a paired *t*-test on log2 transformed data and the fold-change was calculated as the anti-log of log2 stimulated-log2 unstimulated. For the IL-5Rα surface expression analysis, comparisons were carried out with the Mann-Whitney test. Linear dependence was measured with the Spearman correlation coefficient. Statistical analyses were performed using GraphPad Prism v7.0a (GraphPad Prism, La Jolla, CA).

For scRNA-seq, data was analyzed with Seurat 2.3.4[35] implemented in RStudio. Disease-of-origin enrichment in clusters was determined in Prism using the binomial test. All violin plots, which we elected to use due to zero inflation in single-cell data, contain at minimum 292 individual data points in any one patient group. Violins were generated

through default code implemented in Seurat. Statistical enrichment for genes within clusters and disease states was determined using the Tobit test for differential gene analysis.[40] For scores in single-cell data, we report effect sizes in addition to statistical significance as an additional metric for the magnitude of the effect observed. The calculation was performed as Cohen's d where: effect size d = (Mean1-Mean2)/(S.D. pooled). For bulk RNA-seq, differential gene expression was evaluated using DESeq2, implemented in RStudio. Regularized log transformed expression values for differentially expressed transcripts (FDR<0.1) were visualized using the pheatmap package, implemented in RStudio.

Of note, there was not sufficient tissue from each subject for every analysis. There was very little overlap between the subjects whose sinus tissue was studied for ELISAs, qPCR, immunohistochemistry, and immunofluorescence, and there was no overlap between the subjects who cells were studied by scRNA-Seq and by flow cytometry **(Supplemental Table E1)**.

Results:

Study population and demographics

There were no statistically significant differences in age or sex between subjects with CRSwNP, CRSsNP, and AERD. Non-CRS control subjects with surgical excision of concha bullosae were all female (5 of 5 subjects). The lifetime number of endoscopic sinus surgeries was significantly higher ($P < 0.0001$) in AERD subjects compared to aspirin-tolerant CRSwNP (**Table I**). All patients with AERD had physician-diagnosed asthma and their AERD diagnosis had been confirmed with an oral aspirin challenge by a physician with expertise in AERD. Six of 27 aspirin-tolerant CRSwNP patients had a diagnosis of asthma.

Nasal polyp IgE and IgG4 levels are elevated in AERD

Polyp tissue lysates from subjects with AERD contained significantly higher concentrations of IgE and IgG4 compared to sinonasal tissue from non-CRS controls, CRSsNP, and CRSwNP **(Figure 1 A and B)**. Polyp IgE concentrations were more than 3-fold higher in the AERD samples than in those from CRSwNP samples ($P <0.01$), and 14-fold higher in subjects with AERD than with CRSsNP ($P < 0.0001$) **(Figure 1 A)**. Nasal polyp IgG4 protein levels were more than 6-fold higher in subjects with AERD than with CRSwNP ($P < 0.0001$), 43-fold higher in AERD compared to CRSsNP ($P < 0.001$) and close to 300-fold higher in AERD compared to non-CRS controls ($P < 0.0001$) **(Figure 1B)**. IgG4 as a percent of total IgG was significantly higher in subjects with AERD as compared to aspirin-tolerant CRSwNP ($P = 0.005$) **(Figure S1)**, but there was no difference among the four phenotypic groups in their levels of IgG1, IgG2, and IgG3 as a percentage of total IgG (data not shown). Notably, nasal polyp IgG4 levels did not correlate with IgE levels in the same samples (data not shown).

The subjects with AERD who had the most rapidly recurrent nasal polyps (within less than 6 months) had higher IgE levels than subjects with slower polyp regrowth ($P = 0.005$) **(Figure 1C)**, whereas AERD subjects with slower nasal polyp regrowth had a trend toward higher IgG4 levels **(Figure 1D)**. Across all subjects with nasal polyposis, there was a correlation between IgG4, but not IgE levels, and total lifetime duration of

nasal polyposis **(Figure 1 E and F)**. There was no association between rate of polyp regrowth and nasal polyp IgA levels (data not shown). Total antibody levels did not correlate with subject age (data not shown). There was not a significant correlation between serum IgE and polyp IgE levels in the samples from the 22 patients for whom both serum and polyp IgE levels were available (data not shown).

To further confirm our findings, we immunohistochemically evaluated nasal polyp tissue for $IgG4^+$ antibody-associated cells. We found that subjects with AERD had over 5-fold more $IgG4^+$ cells compared to subjects with CRSwNP **(Figure 2A-D)**.

Type 2 cytokine and B cell function-related mRNA expression in nasal polyp subsets

To determine the factors driving local IgE and IgG4 production in the nasal polyp tissue of subjects with AERD, we used qPCR to measure mRNA for a number of cytokines potentially involved in immunoglobulin production and class switch recombination in the nasal polyp tissue of subjects with AERD and CRSwNP. There was significantly more *IL10* mRNA present in the whole nasal polyp tissue of subjects with AERD compared to CRSwNP ($P = 0.037$), but no differences in type 2 cytokine mRNA levels measured, including *IL4* and *IL13*, or in other cytokines or growth factors relevant to B cell function, including *IL6* and *TGFB1* **(Supplemental Table E2)**. We could not detect *IL21* transcript in a sufficient number of samples to make a comparison between groups and *IL21* was not detected in the scRNA-seq dataset (data not shown). *IL15* transcript was not detected in the whole polyp, and in the scRNA-seq dataset there was no difference in *IL15* expression between AERD and CRSwNP. IL-5 protein was below the limit of ELISA detection in most of our samples (data not shown).

ScRNA-seq identifies a transcriptionally distinct antibody-expressing cell cluster increased in subjects with AERD

To extend our primary observations and identify the cellular sources of class switch-associated cytokines in an unbiased fashion, we utilized a previously generated scRNA-seq dataset of surgically-resected and dissociated nasal polyp tissue from a cohort of three subjects with AERD, three subjects with aspirin-tolerant CRSwNP, and five subjects with CRSsNP, specifically focusing on the previously identified antibody-expressing cell clusters.[34] Iterative clustering of these populations yielded 9 clusters **(Figure 3A)**, all of which contained cells derived from at least eight donors and all three disease states **(Figure S2)**. The majority of cluster-defining genes encoded immunoglobulin components **(Supplemental Table E3)**. As previously observed,[34] kappa and lambda light chain usage underlies a major division between clusters **(Figure S3A)**. Little *IGHM* or *IGHD* expression was observed **(Figure S3B),** while robust expression of IgA and IgG isotype regions informed the remaining clusters, indicating that the majority of antibody-expressing cells detected were class-switched **(Figure S3C)**. Interestingly, some clusters were associated with disease phenotype **(Figure 3B)**.

To understand the disease-specific differences underlying our clustering, we specifically compared transcript expression between AERD, CRSwNP and CRSsNP-derived antibody-expressing cells **(Supplemental Table E4)**. *IGHG4*, encoding the IgG4 constant region, was significantly increased in AERD relative to CRSwNP and CRSsNP **(Figure 3C**, **Figure S3C)**, confirming a local source for the increased protein

levels **(Figure 2A)**. We similarly saw enriched expression for *IGHE*, encoding the IgE constant region **(Figure 3C, Figure S3D)**.

To gain additional insights into potential mechanisms regulating these AERD-enriched antibody-expressing cell clusters, we further analyzed the underlying gene lists to look for unique cell-surface receptor expression. Despite not identifying significant differences in *IL5* mRNA levels in bulk tissue, we found that AERD-derived antibody-expressing cells were significantly enriched for *IL5RA* **(Figure 3C, Figure S3E)**, encoding IL-5Rα, and further observed that this was the sole enriched cytokine receptor **(Supplemental Table E4)**. There was no difference between antibody-expressing cell expression of *CSF2RB*, encoding the beta subunit for IL-5R, between AERD and CRSwNP patients (data not shown). To evaluate the possible contribution of *IL5RA* to antibody-expressing cell biology, we evaluated transcripts correlating with *IL5RA* in our scRNA-seq dataset. Through this approach, we found that *IGHG4* and *CCND2* displayed the strongest correlation with *IL5RA* ($R = 0.29$ and 0.28 respectively, $P < 0.0001$) and *IGHE* also correlated with *IL5RA* ($R = 0.18$, $P < 0.0001$) **(Figure S3F)**. This is in contrast to *IGHM*, *IGHD*, and *IGHA1/2* which were all negatively correlated with *IL5RA* in the scRNA-seq dataset (data not shown).

To understand the contribution of different cell types to cytokine production in AERD, we utilized our previously generated scRNA-seq dataset of dissociated nasal polyp cells from subjects with AERD.[34] ScRNA-seq of all polyp cells revealed the cellular identity of respiratory epithelial, stromal and immune cell types in the nasal polyp tissue **(Figure S4A)**. We examined the transcripts of each cell type to identify the potential cell-of-origin for type 2 cytokines possibly involved in class switching to IgE and IgG4 in AERD. Myeloid cells were the dominant source of *IL10*, with IL-10 expression specifically mapping to the previously identified *S100A8*-expressing inflammatory DC-3 and *C1Q*-expressing macrophages[34] within the myeloid cluster **(Figure S4B)**. IL-5 expression was restricted to the T cell cluster, and sub-analysis indicated that these T cells co-expressed *IL13* and *HPGDS*,[34] suggestive of the recently identified Th2A cell[41] **(Figure S4C)**.

Surface expression of IL-5Rα on antibody-expressing cells from nasal polyps

To further evaluate differences in antibody-expressing cells between subjects with AERD and CRSwNP, we examined plasma cells in the nasal polyp single-cell suspensions from subjects with AERD and CRSwNP. We flow cytometrically quantified plasma cells as $CD45^+/CD3^-/CD20^-/CD27^+/CD38^+/CD138^+$ and found that subjects with AERD have significantly higher numbers of plasma cells within their nasal polyps compared to tissue from subjects with aspirin-tolerant CRSwNP ($P = 0.0051$) **(Figure 4A)**. There was no significant difference in the percentage of $CD45^+$ cells that were B cells in subjects with AERD ($5.5 \pm 1.8\%$) vs. CRSwNP ($3.5 \pm 0.7\%$, $P = 0.35$). The plasma cells in nasal polyps from subjects with AERD also had greater surface expression of IL-5Rα compared to tissue from subjects with aspirin-tolerant CRSwNP ($P = 0.019$) **(Figure 4B, C and Figure S5)**, but there was no difference in B cell surface expression of IL-5Rα between groups **(Figure 4C)**. Surface expression of IL-5Rα on nasal polyp eosinophils was similar to that on nasal polyp plasma cells **(Figure S6A)**, and was higher on peripheral blood eosinophils from atopic donors without history of chronic rhinosinusitis **(Figure S6B)**. Using immunofluorescence, we examined nasal polyp tissue

from four patients with AERD and identified co-expression of IL-5Rα and CD138 in plasma cells (**Figure 4D,** representative sample). We also identified co-expression of IL-5Rα and IgG4 in patients with AERD (**Figure S7**, representative sample).

Functional IL-5 signaling on sorted plasma cells from nasal polyps in AERD

To assess the function of IL-5Rα, plasma cells were purified flow cytometrically from subjects with AERD and were stimulated in the presence of IL-5, or no cytokines, for 6 hours. Bulk RNA sequencing was done on two IL-5 stimulated/unstimulated plasma cells pairs, and identified 28 transcripts that were upregulated following IL-5 stimulation and 28 transcripts that were downregulated (**Figure 5A**). Upregulated transcripts included several transcripts that correlated highly with *IL5RA* in the scRNA-seq dataset, including *CCND2* (R = 0.28) and *PTP4A3* (R = 0.16).

Based on the results of the unbiased bulk RNA sequencing, we assessed for plasma cell *CCND2* transcript before and after stimulation with IL-5 in three subjects with AERD (**Figure 5B**). In the IL-5-stimulated plasma cells, there was a 2.95- to 3.42-fold increase in expression of *CCND2* compared to the pre-stimulation levels (P = 0.0017).

Discussion:

Neither the regulatory factors nor the direct consequences of local antibody production in nasal polyp tissue are known. Furthermore, differences in antibody production levels between subjects with aspirin-tolerant CRSwNP and subjects with more severe polyposis and AERD had not previously been recognized. Due to the potential importance of IgE and IgG4 in AERD pathogenesis and the potential for additional antibody-driven effector mechanisms, we sought to characterize local antibody production in nasal polyp tissue in subjects with AERD and identify factors that influence the relevant antibody-expressing cells.

We tested whole nasal polyp extracts from patients with AERD, aspirin-tolerant CRSwNP, and controls with CRSsNP and concha bullosa tissue (as a surrogate non-CRS control tissue) for concentrations of discrete antibody isotypes. As anticipated, polyps contained all antibody isotypes at higher concentrations than in non-polyp control tissue. Furthermore, total polyp IgG4 (R=0.47, P=0.0057), but not IgE, correlated with lifetime disease duration of nasal polyposis **(Figure 1E-F)**. While all antibody levels tended to be higher in the polyps from AERD subjects than those from CRSwNP (data not shown), the differences between these two groups in total IgE **(Figure 1A)** and IgG4 **(Figure 1B)** were remarkable. Moreover, polyp IgE levels did not significantly correlate with serum IgE from the same subjects, suggesting that IgE was synthesized locally. IgE-producing cells are notoriously difficult to detect due to very low receptor density compared with other isotypes, and their ephemeral nature in the memory B cell pool of blood and secondary lymphoid organs.[42] However, $IgG4^+$ cells were readily detectable in the AERD polyps, and were far more numerous than in the aspirin-tolerant control polyps, and rare IgE-expressing cells could be observed through scRNA-seq analysis **(Figures 2A-D, 3, S3)**. These observations support mechanisms that specifically regulate the local productions of IgE and IgG4 in nasal polyps, and that strongly differentiate the subjects

with more severe polyposis who have AERD from aspirin-tolerant CRSwNP. It is suspected that local tissue mast cell activation contributes to nasal tissue inflammation in AERD, though the underlying mechanisms that lead to chronic mast cell activation in the tissue have not been elucidated. Although many subjects with AERD lack classic atopy,[43] they do tend to have elevated serum IgE levels.[43] A recent study reported that treatment with omalizumab, a monoclonal antibody against IgE, improved sinonasal symptoms in patients with AERD and also decreased urinary PGD_2 metabolite and leukotriene E_4 levels, both of which are likely derived from mast cells, by ~90%.[44] Therefore, the elevated levels of IgE **(Figure 1A)** and the association with IgE and aggressive recurrent disease **(Figure 1C)** could be instrumental to the mast cell activation within the nasal polyp tissue in AERD. Further, our data suggests that the IgG4 production may have a protective effect in preventing nasal polyp regrowth in these patients **(Figure 1D)**. While the tissue has many plasma cells expressing transcript for all IgG isotypes, IgG4 is the only isotype higher in AERD as a percentage of total IgG (**Figure S1**). Our study was limited to retrospective clinical data based on patient recall. Prospective study of contribution of IgE and IgG4 to polyp severity and chronicity will further elucidate the relationship of these antibodies to polyp severity.

Whereas locally-generated IgE may permit mast cells, basophils, and other FcεRI-bearing effector cells to respond to cryptic or microbial antigens, the pathophysiologic significance of IgG4 is not clear. Like IgE production, IgG4 production by B cells is regulated by IL-4/IL-13 signaling, but the balance toward IgG4 is controlled by the regulatory cytokine IL-10.[45] ScRNA-seq analysis of nasal polyp cells from subjects with AERD revealed expression of *IL10* by macrophages and inflammatory DC3, with a minor contribution from the T cell compartment **(Figure S4**). Our finding that AERD polyps express more than three-fold higher levels of *IL10* mRNA (but not other B cell active cytokines) than CRSwNP tissue **(Supplemental Table E2)** is consistent with regulatory T cells or myeloid cells driving IgG4 production in response to chronic antigen exposure. IgG4 may have an immunoregulatory role in patients with allergic sensitization[46] and is involved in the immune response to invasive parasites.[47] However, it is also elevated in pathologic conditions including eosinophilic esophagitis[48] and IgG4-related diseases, a group of fibro-inflammatory disorders involving multiple organ systems.[49] It is possible that in the AERD polyp environment and in eosinophilic esophagitis, high IL-5 levels may facilitate IgG4-producing plasma cells. We see that IgG4 levels are highest in patients with the longest duration of nasal polyposis **(Figure 1F)** possibly reflecting chronic antigenic exposure or a failed compensatory response. Given that IgG4 can potentially block antigen binding to IgE in nasal polyp tissue,[50] it is also possible that it could modify skin test reactivity in patients with AERD, who are frequently non-atopic, as it may in subjects with eosinophilic esophagitis who respond clinically to food protein withdrawal even without evidence for IgE sensitization.[51]

We then sought to identify cell type-intrinsic factors that might favor the production of IgE and IgG4 over other isotypes in AERD polyps. Massively parallel scRNA-seq can reveal cell-type and disease-specific differences in mRNA expression profiles by revealing the most strongly differentially expressed transcripts. Accordingly, we identified distinct clusters of antibody-expressing cells that were enriched in AERD

notable for their strong expressions of *IGHG4* and *IGHE* and also distinguished by *IL5RA* expression **(Figure 3B and 3C)**. We verified through flow cytometry that AERD polyps contained substantially greater numbers and percentages of plasma cells bearing surface IL-5Rα than did CRSwNP control polyps **(Figure 4B)**, and confirmed the co-expression of IL-5Rα and the plasma cell marker CD138 through immunofluorescence **(Figure 4D)**. We also found that nasal polyp antibody-expressing cells in subjects with AERD can express both IL-5Rα and IgG4 **(Figure S7).** ScRNA-seq analysis of nasal polyp cells from subjects with AERD revealed expression of *IL5* by effector T cells **(Figure S4)**. While ILC2 cells are also known to express *IL5*, these cells were not identified in the previous study likely due to their relative scarcity, comprising only 0.01%-0.1% of $CD45^+$ nasal polyp cells.[52]

Though best known for its survival-sustaining effects on eosinophils, IL-5 was originally described as a factor required for the activation, proliferation, and differentiation of mouse B cells into antibody-secreting plasma cells,[53-55] and acts as a strong survival factor for mouse plasma cells.[56] Further, IL-5 has been shown to act synergistically with IL-4 to increase lymphocyte production of IgE from human lymphocytes *in vitro*[57] and IL-5 is known to be associated with IgE levels in humans *in vivo*.[58] To evaluate the possible contribution of *IL5RA* to antibody-expressing cell biology in the nasal polyp tissue, we evaluated transcripts correlating with *IL5RA* in our scRNA-seq dataset and found that *IGHG4* and *CCND2* displayed the strongest correlation with *IL5RA* (R = 0.29 and 0.28 respectively). *CCND2*, which encodes for cyclin D2, is a cell cycle gene known to be involved in the development of murine lymphocytes[59] and was previously identified as a murine antibody-secreting cell transcript upregulated following IL-5 stimulation[36] To explore this further, we assessed *CCND2* expression in unstimulated/IL-5 stimulated human nasal polyp plasma cell pairs with qPCR, and found that *CCND2* expression increases three-fold following stimulation with IL-5 (**Figure 5A**). To confirm that the IL-5Rα on these cells was functional and biologically relevant, we investigated the transcriptional consequences of IL-5 stimulation *in vitro* and determined that stimulation with IL-5 leads to upregulation of multiple transcripts involved in cell cycle and proliferation (**Figure 5B**).[59-61]

IL-5Rα is not expressed on resting B cells.[55] However, when B cells are activated, IL-5Rα is induced through a STAT6-dependent pathway.[29] Thus, our findings confirm the presence of locally activated B cells in nasal polyp and support a potential role for IL-5 signaling in B cell differentiation, proliferation, and survival, that could lead to increased generation of antibodies within the inflamed tissue.

Humanized monoclonal antibodies against IL-5 and IL-5Rα show efficacy in the treatment of eosinophilic asthma and nasal polyposis.[62, 63] A Phase 2 trial of IL-5 inhibition with mepolizumab in patients with nasal polyposis showed a therapeutic effect with a reduction in both polyp size and patient symptoms.[64] Furthermore, we recently demonstrated that mepolizumab improved upper respiratory symptoms and asthma control in subjects with AERD.[65] However, another recent study of dexpramipexole, an experimental drug that depletes nearly all eosinophils from within the nasal polyp tissue, failed to show any symptomatic improvement or any reduction in nasal polyp size.[66]

Taken together with our current findings, we suspect that IL-5 and IL-5Rα-targeting monoclonal antibodies may alter the survival and function of IL-5Rα^{+} antibody-expressing cells in addition to their effects on eosinophils, which may contribute to the mechanism of their therapeutic benefit.

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858

Figure Legends:

Table 1. Patient characteristics.

Figure 1. Nasal tissue IgE and IgG4 levels are elevated in AERD and relate to nasal polyp recurrence. Total tissue levels of **(A)** IgE and **(B)** IgG4 were measured by ELISA from concha bullosa samples of patients without sinus inflammation (non-CRS controls), sinus mucosa of patients with CRSsNP, and nasal polyp tissue from patients with aspirin-tolerant CRSwNP and AERD. Nasal polyp IgE **(C)** and IgG4 **(D)** levels in AERD patients with rapid nasal polyp regrowth (< 6 months) or slower nasal polyp regrown (≥ 6 months). The nasal polyp IgG4, but not IgE levels, from patients with aspirin-tolerant CRSwNP and AERD correlate with lifetime duration of nasal polyposis **(E-F)**. Data in A - D are mean ±SEM, correlation in E and F was calculated by Spearman.

Figure 2. $IgG4^+$ antibody-expressing cells are specifically elevated in nasal polyps from patients with AERD. (A) Number of $IgG4^+$ lymphocytes per HPF from nasal polyp tissue of patients with aspirin-tolerant CRSwNP and AERD, n=5 for each group. Data in A are mean ±SEM. **(B - D)** Representative samples (CRSwNP, **B** and AERD, **C,** magnified in **D**) of nasal polyp tissue stained with anti-IgG4. Black arrows identify $IgG4^+$ cells.

Figure 3. ScRNA-seq of antibody-expressing cell populations from sinus tissue of subjects with CRSsNP (n=5), CRSwNP (n=3) and AERD (n=3). (**A)** UMAP plot of 2,520 antibody-expressing cells from sinonasal tissue of CRSsNP, CRSwNP and AERD patients, indicating 9 clusters identified through a shared nearest neighbor analysis. (**B)** UMAP plot of sinonasal antibody-expressing cells, colored by disease of origin. Statistical enrichment for AERD disease-of-origin was observed for cluster 2 ($P<1x10^{-15}$), cluster 3 ($P<1x10^{-15}$), cluster 4 ($P<2x10^{-12}$), cluster 6 ($P<1x10^{-15}$), and cluster 7 ($P<1x10^{-15}$) (**C)** Violin plots of select genes significantly enriched in AERD relative to CRSsNP and CRSwNP within sinonasal antibody-expressing cell populations, including *IGHG4* ($P<1x10^{-201}$), *IGHE* ($P<2x10^{-34}$), and *IL5RA* ($P<2x10^{-21}$). Cohen's d effect size for AERD relative to CRSwNP is 1.57, 0.50, and 0.37, respectively for the 3 transcripts.

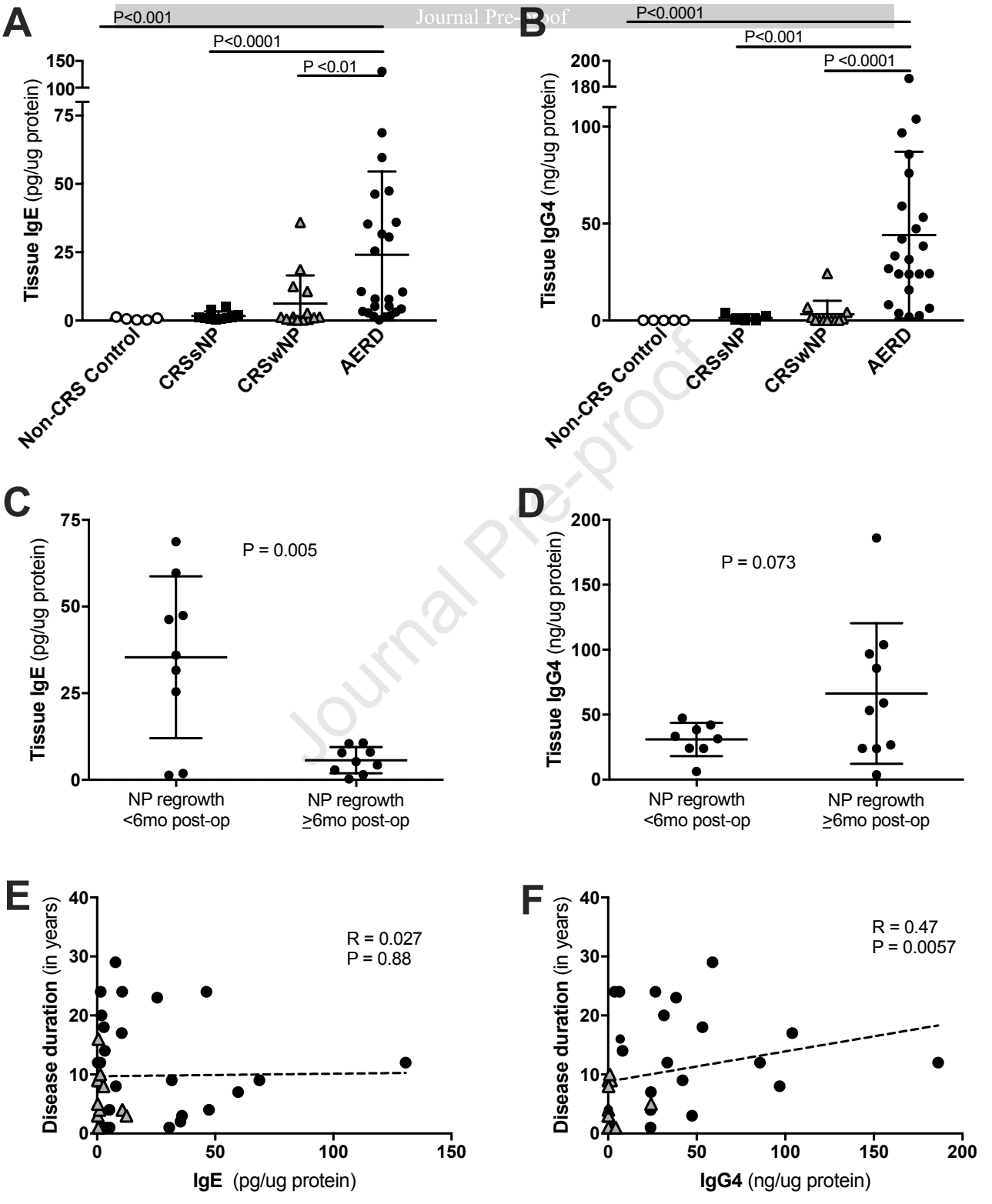
Figure 4. Flow cytometric characterization of plasma cells and immunofluorescence of polyp tissue. (A) Nasal polyp plasma cell frequency as a percentage of $CD45^+$ cells, **(B)** plasma cell surface expression of IL-5Rα, **(C)** B cell surface expression of IL-5Rα, and **(D)** immunofluorescence staining of nasal polyp tissue for plasma cells (white arrows) co-expressing IL-5Rα (green) and CD138 (red).

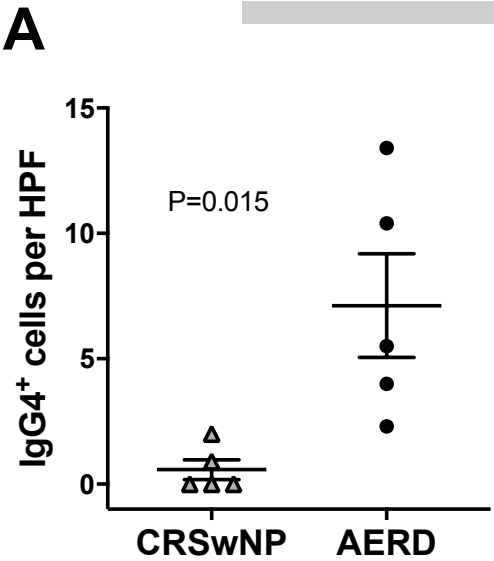
Figure 5. Transcriptional consequence of IL-5 stimulation of human nasal polyp plasma cells. (A) Heatmap representation of differentially expressed transcripts (FDR < 0.1) in plasma cells from two subjects with AERD treated with IL-5 (1 ng/mL, 6 hours) relative to vehicle control treatment. Scale bar indicates Z-score scaled by row. **(B)** *CCND2* fold change with IL-5 stimulation (1 ng/mL, 6 hours) in nasal polyp plasma cells from three subjects with AERD, normalized to glyceraldehyde-3-phosphate dehydrogenase; paired t-test.

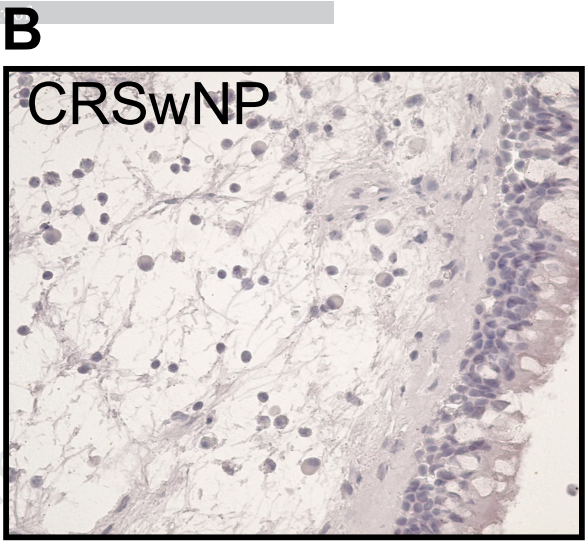
Table 1. Patient characteristics.

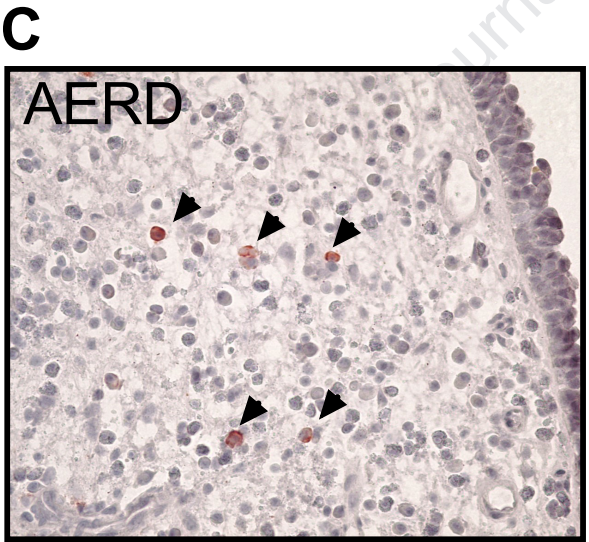
	AERD	CRSwNP	CRSsNP	Healthy Controls
Number	N = 42	N = 27	N = 14	N = 5
Sex (male:female)	19:23	16:11	4:10	0:5
Median age (y) [range]	47.5 [20-77]	52 [20-74]	31 [23-67]	41 [31-67]
Asthma (%)	100%	22%	36%	0
Lifetime number of polypectomies (mean ±SD)	2.4 (± 1.3)	1.3 (± 0.4)*	N/A	N/A

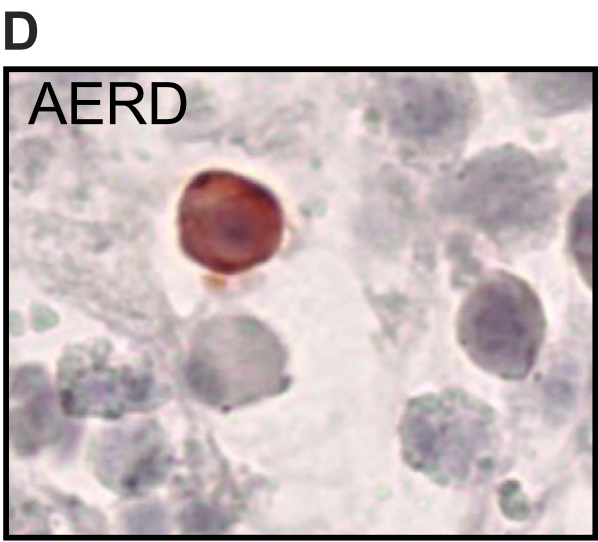
* This is $P < 0.0001$ for CRSwNP lifetime number of polyp surgeries compared to the AERD lifetime number of polyp surgeries

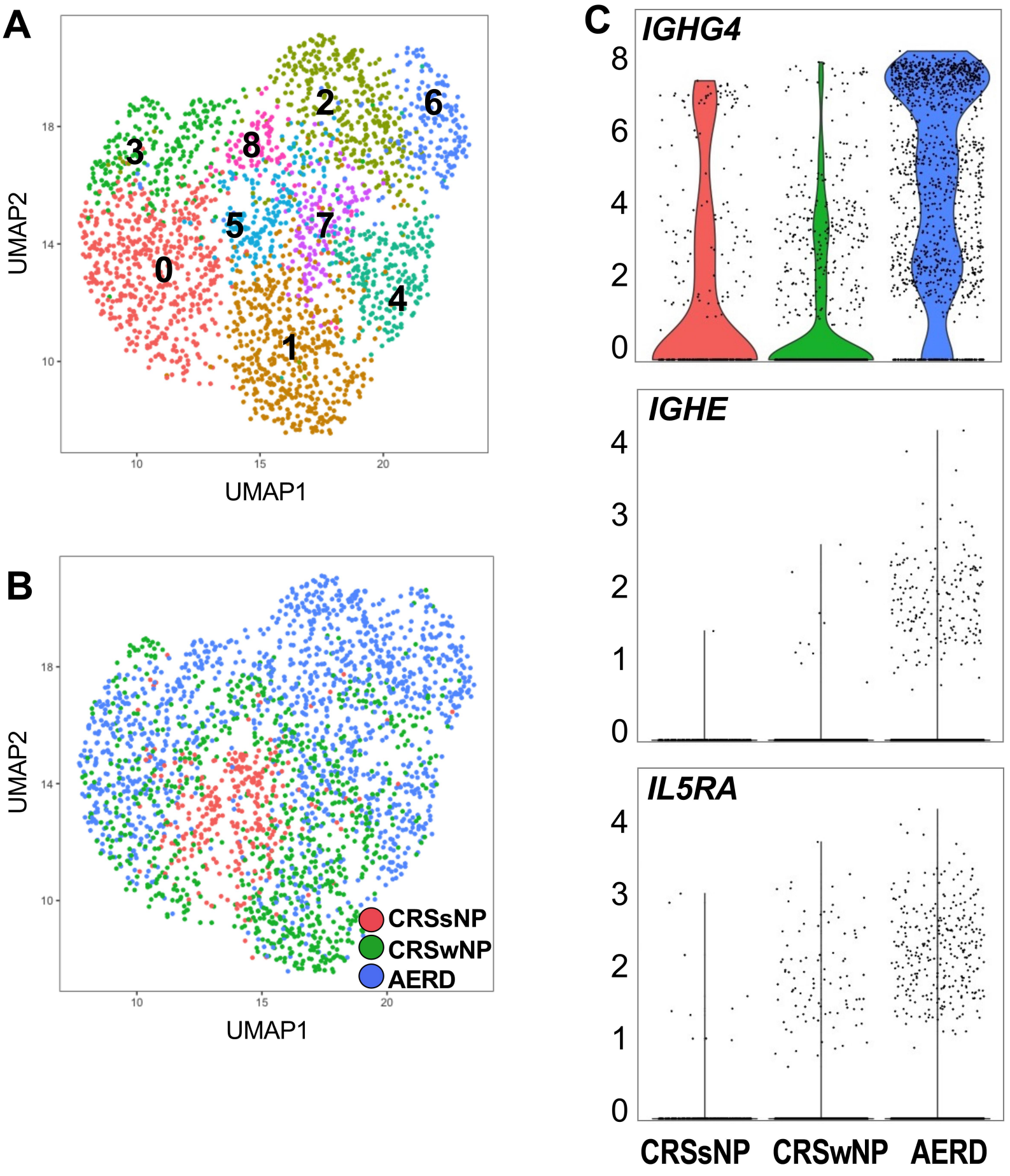


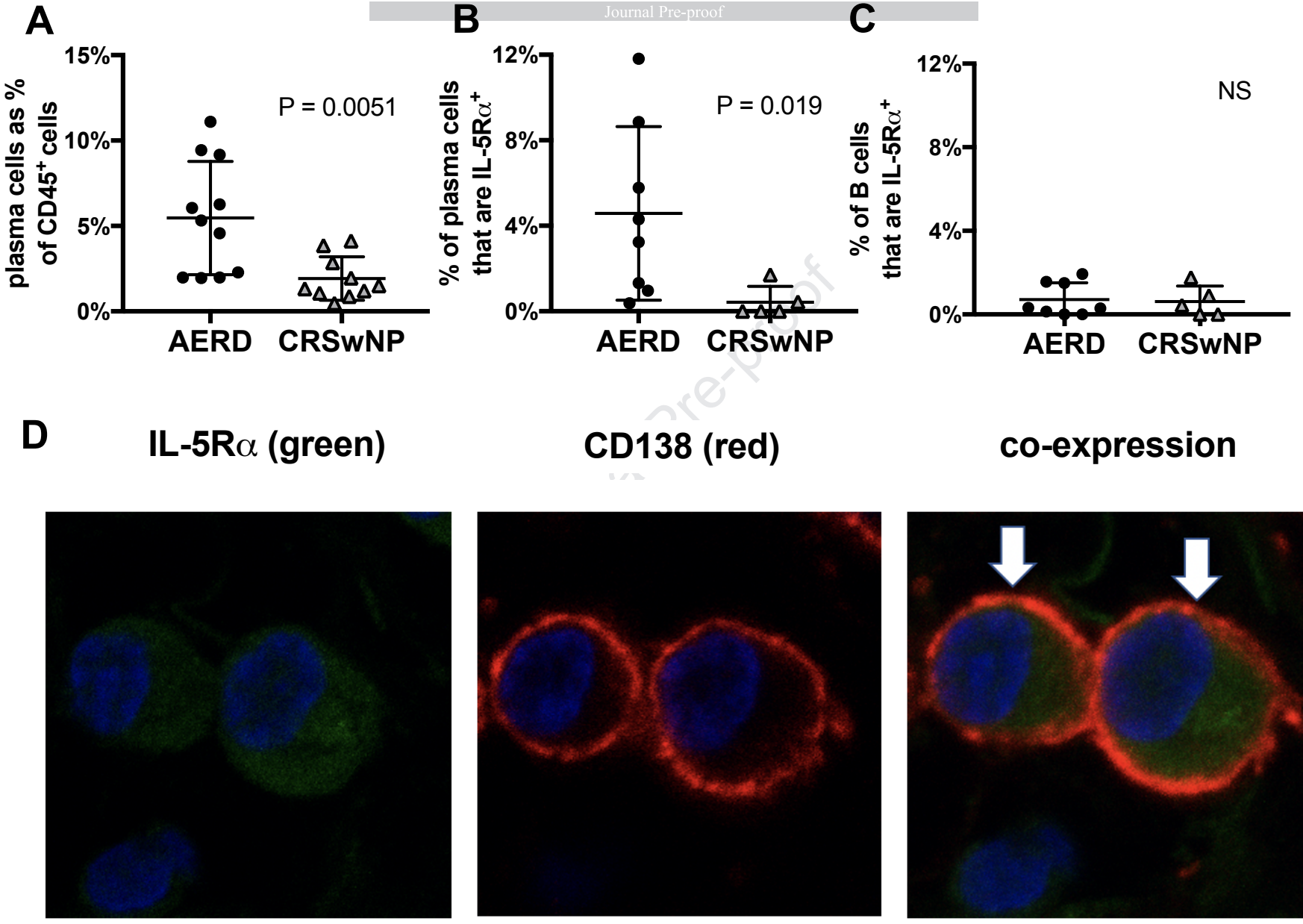


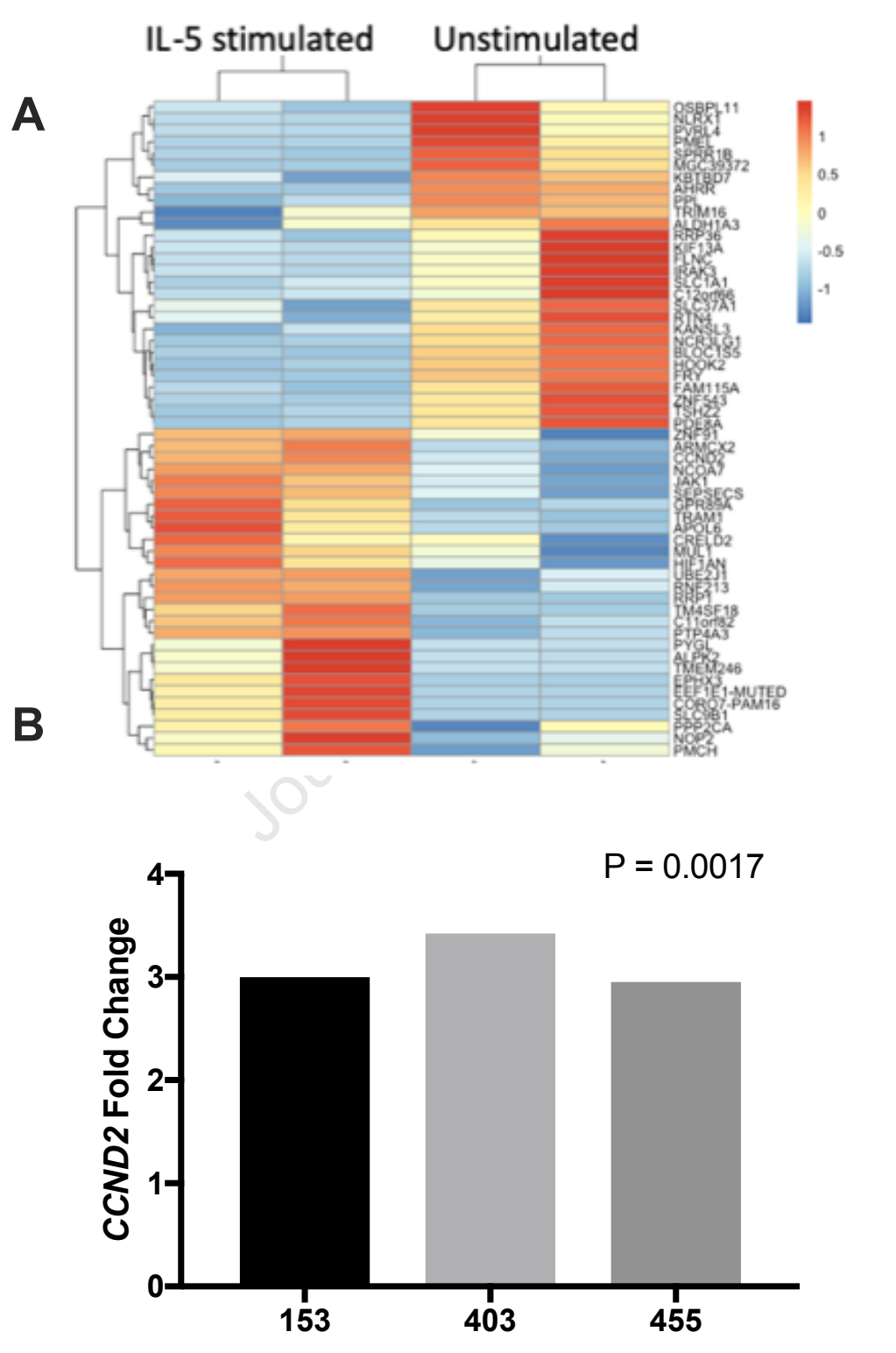







A
UMAP2
UMAP1
0
1
2
3
4
5
6
7
8
B
CRSsNP
CRSwNP
AERD
C
IGHG4
IGHE
IL5RA
CRSsNP
CRSwNP
AERD

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Unstimulated
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PVRL4
PMEL
SPRR1B
MGC39372
KBTBD7
AHRR
PPL
TRIM16
ALDH1A3
RRP36
KIF13A
FLNC
IRAK3
SLC1A1
C12orf66
SLC37A1
RYK
KANSL3
NCRNLG1
BLOC1S5
HOOK2
FRY
FAM115A
ZNF543
TSHZ2
PDE8A
ZNF91
ARMCX2
CCND2
NCOA7
JAK1
SEPSECS
GPR89A
TBAM1
APOL6
CRELD2
MUT
HIFTAN
UBE2J1
RNF213
RRP1
TM4SF18
C11orf82
PTP4A3
PYGL
ALPK2
TMEM246
EPHX3
EEF1E1-MUTED
CORO7-PAM16
SLC9B1
PPP2CA
NOP2
PMCH
1
0.5
0
-0.5
-1
B
P = 0.0017
CCND2 Fold Change
4
3
2
1
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403
455