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

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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, but upstream drivers of local antibody production in nasal polyps are undetermined.
Objective: We sought to identify upstream drivers and phenotypic properties of local antibody-expressing cells in nasal polyps from subjects with AERD.
Methods: Sinus tissue was obtained from subjects with AERD, chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP), CRS without nasal polyps, and controls without CRS. Tissue antibody levels were quantified via ELISA and immunohistochemistry and were correlated with disease severity. Antibody-expressing cells were profiled with single-cell RNA sequencing, flow cytometry, and immunofluorescence, with IL-5Rα function determined through IL-5 stimulation and subsequent RNA sequencing and quantitative PCR.
Results: Tissue IgE and IgG4 levels were elevated in AERD compared with in controls ($P < .01$ for IgE and $P < .001$ for IgG4 vs CRSwNP). Subjects with AERD whose nasal polyps recurred rapidly had higher IgE levels than did subjects with AERD, with slower regrowth ($P = .005$). Single-cell RNA sequencing revealed increased *IL5RA*, *IGHG4*, and *IGHE* in antibody-expressing cells from patients with AERD compared with antibody-expressing cells from patients with CRSwNP. There were more IL-5Rα$^+$ plasma cells in the polyp tissue from those with AERD than in polyp tissue from those with CRSwNP ($P = .026$). IL-5 stimulation of plasma cells *in vitro* induced changes in a distinct set of transcripts.
Conclusions: Our study identifies an increase in antibody-expressing cells 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 levels are elevated in AERD, and higher IgE levels are associated with faster nasal polyp regrowth. Our findings suggest a role for IL-5Rα$^+$ antibody-expressing cells in facilitating local antibody production and severe nasal polyps in AERD. (J Allergy Clin Immunol 2020;145:1574-84.)

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

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 (CRS) with nasal polyposis (CRSwNP), asthma, and pathognomonic respiratory reactions

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Abbreviations used
AERD: Aspirin-exacerbated respiratory disease
CRS: Chronic rhinosinusitis
CRSsNP: Chronic rhinosinusitis without nasal polyps
CRSwNP: Chronic rhinosinusitis with nasal polyps
$F(ab')_2$: Divalent antibody fragments
PC: Principal component
qPCR: Quantitative PCR
scRNA-seq: Single-cell RNA sequencing
SPRI: Surface plasmon resonance imaging
UMAP: Uniform manifold approximation and projection

to COX-1 inhibitors that involve release of multiple mast cell mediators, including tryptase, leukotriene C_4, and prostaglandin D_2.[4-6] In patients with AERD, nasal polyps are frequently refractory to standard therapy and recur within 2 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 autoantibodies 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 levels and slightly depressed serum IgG1 levels as compared with 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 postoperative course.[20] This suggests a possible role for IgG4 in persistence of sinus disease by as yet unidentified mechanisms.

Nasal polyp tissue contains a variety of cytokines that may drive the B-cell proinflammatory response.[21] Type 2 cytokines, including IL-4, IL-5, IL-13, thymic stromal lymphopoietin, 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 levels in nasal polyp tissue.[27,28]

In the current study, we used 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 whereas elevated polyp IgE concentration 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 whereas 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 it 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, Mass) allergy and immunology clinics and otolaryngology clinics between October 2011 and October 2019 (Table I and see Table E1 in this article's Online Repository at www.jacionline.org). 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, aspirin-tolerant CRSwNP, or CRS without nasal polyps (CRSsNP), with the diagnosis made on the basis of established guidelines.[30] Control patients without CRS 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 the diagnosis later confirmed in all subjects via a physician-observed graded oral challenge to aspirin that 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, including age, sex, number of sinus surgeries, and interval to polyp regrowth following surgery in subjects with recurrent polyposis, were collected from the medical record for patients who donated sinus tissue. Because aspirin desensitization can delay polyp regrowth in subjects with AERD, we included regrowth data only 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 CRSwNP or CRSsNP. For patients without polyps, diseased ethmoid sinus tissue was collected. For both CRSwNP and AERD, polyp tissue originating from the ethmoid sinus was surgically excised and collected. The control subjects without CRS 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, Calif) for RNA extraction, and the remaining tissue was placed in RPMI medium (Corning, Corning, NY) with 10% FBS (ThermoFisher, Waltham, Mass) and 1 U/mL penicillin-streptomycin for transport to the laboratory on ice. Within 2 hours of surgery, the tissue was removed from the RPMI medium 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, Ind) for protein extraction, and the tissue was homogenized with a gentleMACS Dissociator (Miltenyi Biotec, San Diego, Calif). Supernatants were stored at –80°C. One segment

TABLE I. Patient characteristics

Characteristic	AERD	CRSwNP	CRSsNP	Healthy controls
No	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 no. of polypectomies (mean ± SD)	2.4 ± 1.3	1.3 ± 0.4*	N/A	N/A

N/A, Not applicable.
*$P < .0001$ for lifetime number of polyp surgeries for subjects with CRSwNP compared with the lifetime number of polyp surgeries for subjects with AERD.

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 later.

Subjects with AERD, CRSwNP, or CRSsNP who met the inclusion criteria were recruited for scRNA-seq studies. The choice of subjects for the follow-up analyses, including ELISA, qPCR, and immunohistochemistry, was made on the basis of 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 control without CRS), 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 by using a modified version of a previously published protocol.[33] Surgical specimens were collected into 30 mL of cold RPMI medium with 10% FBS and 1 U/mL of penicillin-streptomycin. Specimens were finely minced between 2 scalpel blades and incubated for 15 minutes at 37°C with 600 U/mL of collagenase IV (Worthington, Lakewood, NJ) and 20 μg/mL of DNAse 1 (Roche) in RPMI medium with 10% FBS. After 15 minutes, samples were triturated 5 times by using a syringe with a 16G needle and incubated for another 15 minutes. At the conclusion of the second digestion period, samples were triturated an additional 5 times by 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 500 *g* for 10 minutes followed by a rinse with ice-cold Ca/Mg-free PBS (ThermoFisher). To maintain consistency across patient groups, red blood cells were lysed by using ammonium-chloride-potassium buffer (ThermoFisher) for 3 minutes on ice to remove red blood cells, even if no red blood cell contamination was visible. 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 by using RT^2 SYBR Green qPCR Master Mix (Qiagen) and normalized to glyceraldehyde-3-phosphate dehydrogenase (all primers from Qiagen).

Immunoglobulin quantification

Protein lysate supernatants from sinonasal tissue were collected as already described. Total IgG, IgA, IgE, IgG1, IgG2, IgG3, and IgG4 ELISAs (eBioscience, San Diego, Calif) were performed according to the manufacturer's instructions. Total tissue protein levels were measured with the Pierce BCA Protein Assay kit (ThermoFisher). 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–horseradish peroxidase for mouse primary antibodies (Dako, Carpinteria, Calif). Sections were counterstained with hematoxylin, Gill No. 2. For quantification of IgG4$^+$ cells, the numbers of IgG4$^+$ cells in photomicrographs encompassing at least 3 hpf of subepithelial tissue were counted and expressed per hpf. For immunofluorescence, either sections were blocked with 10% donkey serum and then incubated with both mouse anti-IgG4 and a rabbit polyclonal anti-human IL-5Rα antibody (Sigma PA5-25159) or else rabbit IgG was used in the first step and staining was developed with AF 594 divalent antibody fragments (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 nonspecific binding with a PBS-based blocking with 0.1% Triton X, 0.1% saponin, 3% BSA, and 3% normal donkey serum for 1 hour at room temperature, the slides were incubated overnight at 4°C with a mouse mAb to Syndecan-1 (1 μg/mL, clone B-A38, Abcam, Cambridge, Mass), polyclonal anti-human IL-5Rα Ab (5 μg/mL), or their respective isotype controls, namely, mouse IgG1 (Biolegend, San Diego, Calif) or rabbit polyclonal IgG (Abcam), at the corresponding concentrations. Immunoreactivity was detected with donkey AF 594 F(ab')$_2$, donkey anti-mouse IgG and AF 488 F(ab')$_2$, and donkey anti-rabbit IgG (both from 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 microscope with 20× Zeiss (0.8 numeric aperture) and 63× Zeiss oil (1.4 numeric aperture) objectives.

scRNA-seq analysis

Ethmoid scRNA-seq data were obtained from a previously published study,[34] which is available from the dbGaP database under dbGaP accession number 30434. The unique molecular identifier-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 2520 cells across 12 patient samples. Briefly, a list of the 1902 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) between 0.22 and 7. Principal component analysis was performed over this list of variable genes with the addition of all immunoglobulin isotype heavy chain constant regions, and the 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 by using FindClusters (utilizing a shared nearest neighbor modularity optimization–based clustering algorithm) on the first 8 PCs with a resolution of 0.7. Cells were then graphically displayed by using Uniform Manifold

Approximation and Projection (UMAP) with a minimum distance of 0.75. Pearson correlation with *IL5RA* was evaluated for all detected transcripts by using Seurat.[36]

To determine cytokine sources within AERD polyps, the 4276 cells collected from AERD patient polyps were iteratively clustered in the following fashion. A list of the 1902 most variable genes was generated by using the criteria outlined earlier. After PC analysis had been performed, the first 15 PCs were used for clustering and UMAP display following visual inspection of the PC elbow graph and determination of 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 data set.[34]

Subanalysis of the 282 myeloid cells was conducted on the 2324 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. Subanalysis of the 224 T lymphocytes was conducted on the 2587 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, and CD138 (eBioscience) and 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 $SSC^{high}/CD45^+/CCR3^+$.

Blood from atopic donors without nasal polyposis or CRS 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 already described, 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 and 5% CO_2. RNA was extracted from sorted cells with the RNeasy Micro Kit (Qiagen). For qPCR, RNA from 3 unstimulated/IL-5–stimulated pairs was converted to cDNA by using the RT^2 First Strand Kit (Qiagen). Expression of *CCND2* transcript was examined by using RT^2 SYBR Green qPCR Master Mix (Qiagen) and normalized to glyceraldehyde-3-phosphate dehydrogenase (all primers from Qiagen).

For bulk RNA sequencing, RNA from 2 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 2.2× surface plasmon resonance imaging (SPRI) ratio cleanup by using Agencourt RNAClean XP beads (Beckman Coulter, A63987, Indianapolis, Ind). 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) by using KAPA HiFi PCR Mastermix (Kapa Biosystems KK2602). Sequencing libraries were prepared by using the Nextera XT DNA tagmentation kit (Illumina FC-131-1096, San Diego, Calif) with 250-pg input for each sample. Libraries were pooled after use of the Nextera kit and cleaned by using Agencourt AMPure SPRI beads with successive 0.7× and 0.8× ratio SPRIs and sequenced with an Illumina 75 Cycle NextSeq500/550v2.5 kit (Illumina FC-404-2005) with a loading density at 2.2 pM, with a 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 by using STAR and RSEM.[37,38] After concatenation of the read counts for technical replicates, differential expression analysis was conducted by using the DESeq2 package for R, with patient origin taken into account.[39] Genes with Benjamini-Hochberg–adjusted *P* values corresponding to a false discovery rate less than 0.1 were regarded as differentially expressed.

Statistical analysis

Data are presented as individual points ± SEMs, unless otherwise specified. For the immunoglobulin analyses, comparisons were performed with the Kruskal-Wallis 1-way ANOVA because of non-Gaussian distribution of the data. Binary comparisons were carried out with the Mann-Whitney test. Significance was defined as a 2-tailed *P* value less than .05. The binary polyp regrowth data were analyzed with an unpaired *t* test with Welch 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 antilog 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 by using GraphPad Prism software, version 7.0a (GraphPad Prism, La Jolla, Calif).

For scRNA-seq, data were analyzed with Seurat 2.3.4[35] implemented in RStudio. Disease-of-origin enrichment in clusters was determined in Prism by using the binomial test. All violin plots, which we elected to use on account of sparseness in single-cell data, contain at minimum 292 individual data points in any 1 patient group. Violins were generated through default code implemented in Seurat. Statistical enrichment for genes within clusters and disease states was determined by 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 the Cohen's d, where effect size d = (Mean1 –Mean2)/(SD pooled). For bulk RNA-seq, differential gene expression was evaluated by using DESeq2 implemented in RStudio. Regularized log-transformed expression values for differentially expressed transcripts (false discovery rate <0.1) were visualized by 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 whose cells were studied by scRNA-Seq and by flow cytometry (see Table E1).

RESULTS

Study population and demographics

There were no statistically significant differences in age or sex between subjects with CRSwNP, CRSsNP, and AERD. The control subjects without CRS who had undergone surgical excision of concha bullosa were all female (5 of 5 subjects). The lifetime number of endoscopic sinus surgeries was significantly higher ($P < .0001$) in subjects with AERD than in those with aspirin-tolerant CRSwNP (Table I). All patients with AERD had physician-diagnosed asthma, and their AERD diagnosis had been confirmed with oral aspirin challenge by a physician with expertise in AERD. Of the 27 aspirin-tolerant patients with CRSwNP, 6 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 than did sinonasal tissue from controls without CRS, CRSsNP, or CRSwNP (Fig 1, *A* and *B*). Polyp IgE concentrations were more than 3-fold higher in the AERD samples than in those from CRSwNP samples ($P < .01$) and 14-fold higher in subjects with

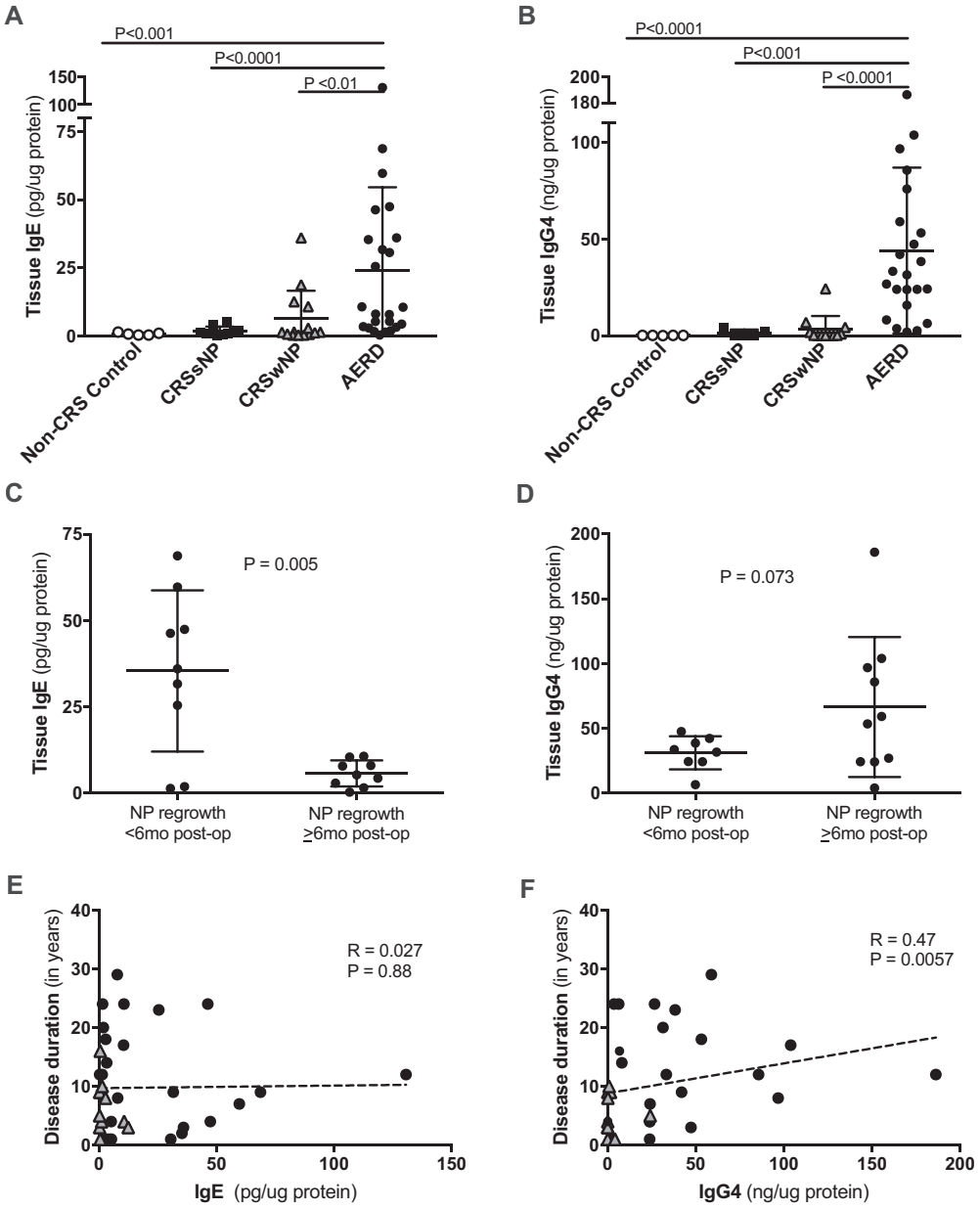

FIG 1. Nasal tissue IgE and IgG4 levels are elevated in AERD and relate to nasal polyp (NP) recurrence. Total tissue levels of IgE **(A)** and IgG4 **(B)** were measured by ELISA from concha bullosa samples of patients without sinus inflammation (non-CRS controls), sinus mucosa of patients with CRSsNP, 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 regrowth (≥6 months). The nasal polyp IgG4 levels, but not the IgE levels, from patients with aspirin-tolerant CRSwNP and AERD correlate with lifetime duration of nasal polyposis (**E** and **F**). **A-D,** Data are means ± SEMs. **E** and **F,** Correlation was calculated by the Spearman test.

AERD than in subjects with CRSsNP ($P < .0001$) (Fig 1, *A*). Nasal polyp IgG4 protein levels were more than 6-fold higher in subjects with AERD than in subjects with CRSwNP ($P < .0001$), 43-fold higher in AERD than in CRSsNP ($P < .001$), and close to 300-fold higher in subjects with AERD than in controls without CRS ($P < .0001$) (Fig 1, *B*). IgG4 as a percent of total IgG was significantly higher in subjects with AERD than in subjects with aspirin-tolerant CRSwNP ($P = .005$) (see Fig E1 in this article's Online Repository at www.jacionline.org), but there was no difference among the 4 phenotypic groups in terms of 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 <6 months) had higher IgE levels than did subjects with slower polyp regrowth ($P = .005$) (Fig 1, *C*), whereas subjects with AERD with slower nasal polyp regrowth had a trend toward higher IgG4 levels (Fig 1, *D*). Across all subjects with nasal polyposis, there was a correlation between IgG4 levels, but not IgE levels, and total lifetime duration of nasal polyposis (Fig 1, *E* and

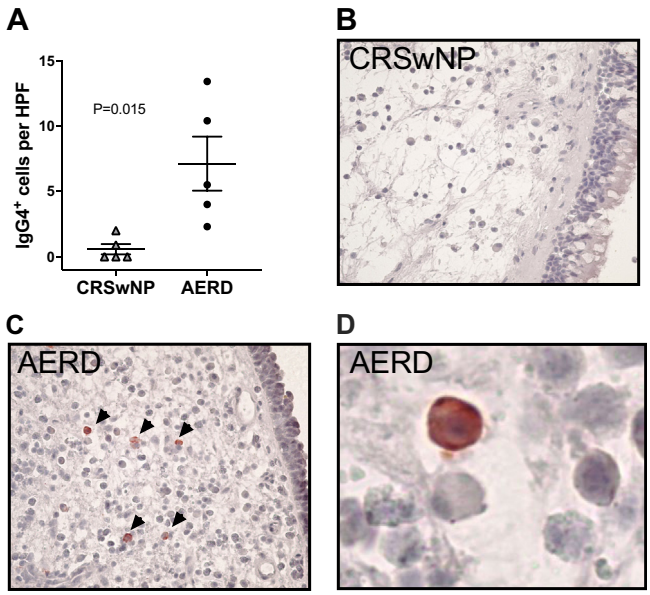

FIG 2. Levels of IgG4$^+$ antibody-expressing cells are specifically elevated in nasal polyps from patients with AERD. **A,** Number of IgG4$^+$ lymphocytes per hpf from nasal polyp tissue of patients with aspirin-tolerant CRSwNP and AERD (n = 5 for each group). **A,** Data are means ± SEMs. **B-D,**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

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-expressing cells. We found that subjects with AERD had more than 5-fold the number of IgG4$^+$ cells than did subjects with CRSwNP (Fig 2, *A-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 than in that of subjects with CRSwNP ($P = .037$), but there were no differences in measured levels of type 2 cytokine mRNA, including the mRNA of *IL4* and *IL13* or other cytokines or growth factors relevant to B-cell function, including *IL6* and *TGFB1* (see Table E2 in this article's Online Repository at www.jacionline.org). 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 data set (data not shown). *IL15* transcript was not detected in the whole polyp, and in the scRNA-seq data set 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 data set of surgically resected and dissociated nasal polyp tissue from a cohort of 3 subjects with AERD, 3 subjects with aspirin-tolerant CRSwNP, and 5 subjects with CRSsNP, specifically focusing on the previously identified antibody-expressing cell clusters.[34] Iterative clustering of these populations yielded 9 clusters (Fig 3, *A*), all of which contained cells derived from at least 8 donors and all 3 disease states (see Fig E2 in this article's Online Repository at www.jacionline.org). The majority of cluster-defining genes encoded immunoglobulin components (see Table E3 in this article's Online Repository at www.jacionline.org). As previously observed,[34] κ and λ light chain use underlies a major division between clusters (see Fig E3, *A* in this article's Online Repository at www.jacionline.org). Little *IGHM* or *IGHD* expression was observed (see Fig E3, *B*), whereas robust expression of IgA and IgG isotype regions informed the remaining clusters, indicating that the majority of antibody-expressing cells detected were class-switched (see Fig E3, *C*). Interestingly, some clusters were associated with disease phenotype (Fig 3, *B*).

To understand the disease-specific differences underlying our clustering, we specifically compared transcript expression between AERD-, CRSwNP-, and CRSsNP-derived antibody-expressing cells (see Table E4 in this article's Online Repository at www.jacionline.org). *IGHG4*, encoding the IgG4 constant region, was significantly increased in AERD relative to CRSwNP and CRSsNP (Fig 3, *C* and see also Fig E3, *C*), confirming a local source for the increased protein levels (Fig 2, *A*). We similarly saw enriched expression for *IGHE*, encoding the IgE constant region (Fig 3, *C* and see also Fig E3, *D*).

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* (Fig 3, *C* and see also Fig E3, *E*), encoding IL-5Rα, and we further observed that this was the sole enriched cytokine receptor (see Table E4). There was no difference between antibody-expressing cell expression of *CSF2RB*, encoding the β-subunit for IL-5R, between patients with AERD and patients with CRSwNP (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 data set. Through this approach, we found that *IGHG4* and *CCND2* displayed the strongest correlation with *IL5RA* ($R = 0.29$ and 0.28, respectively; $P < .0001$) and *IGHE* also correlated with *IL5RA* ($R = 0.18$; $P < .0001$) (see Fig E3, *F*). This is in contrast to *IGHM*, *IGHD*, and *IGHA1/2,* which were all negatively correlated with *IL5RA* in the scRNA-seq data set (data not shown).

To understand the contribution of different cell types to cytokine production in AERD, we utilized our previously

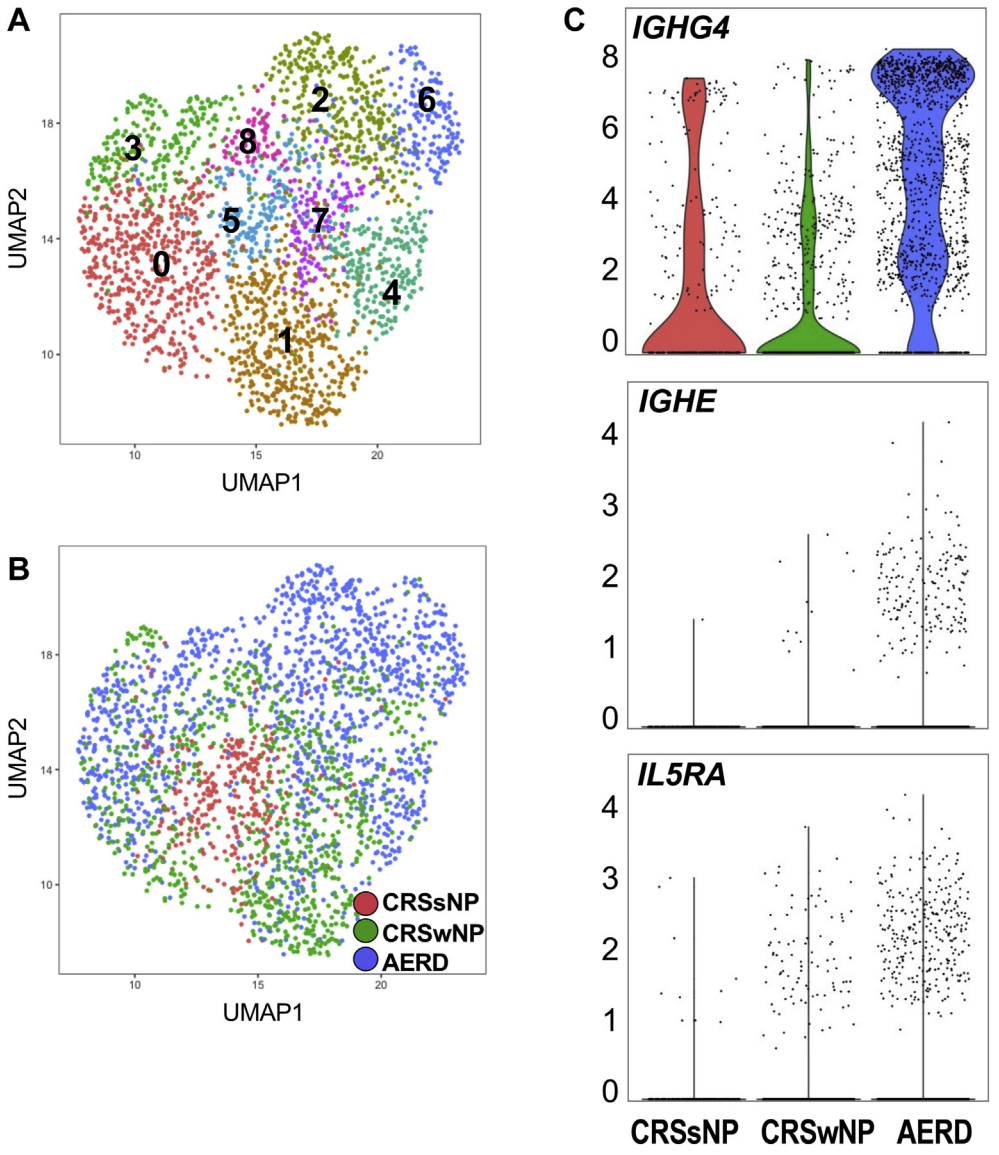

FIG 3. ScRNA-seq of antibody-expressing cell populations from sinus tissue of subjects with CRSsNP (n = 5), CRSwNP (n = 3), and AERD (n = 3). **A,** UMAP plot of 2520 antibody-expressing cells from sinonasal tissue of patients with CRSsNP, CRSwNP, and AERD, indicating 9 clusters identified through a 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 ($P < 1 \times 10^{-15}$), cluster 3 ($P < 1 \times 10^{-15}$), cluster 4 ($P < 2 \times 10^{-12}$), cluster 6 ($P < 1 \times 10^{-15}$), and cluster 7 ($P < 1 \times 10^{-15}$). **C,** Violin plots of select genes significantly enriched in AERD relative to CRSsNP and CRSwNP within sinonasal antibody-expressing cell populations, including *IGHG4* ($P < 1 \times 10^{-201}$), *IGHE* ($P < 2 \times 10^{-34}$), and *IL5RA* ($P < 2 \times 10^{-21}$). Cohen's d effect sizes for AERD relative to CRSwNP are 1.57, 0.50, and 0.37, respectively, for the 3 transcripts.

generated scRNA-seq data set 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 (see Fig E4, *A* in this article's Online Repository at www.jacionline.org). 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 (see Fig E4, *B*). IL-5 expression was restricted to the T-cell cluster, and subanalysis indicated that these T cells coexpressed *IL13* and *HPGDS*,[34] which is suggestive of the recently identified Th2A cell[41] (see Fig E4, *C*).

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 quantified plasma cells via flow cytometry as $CD45^+/CD3^-/CD20^-/CD27^+/CD38^+/CD138^+$ and found that nasal polyp tissue from subjects with AERD has

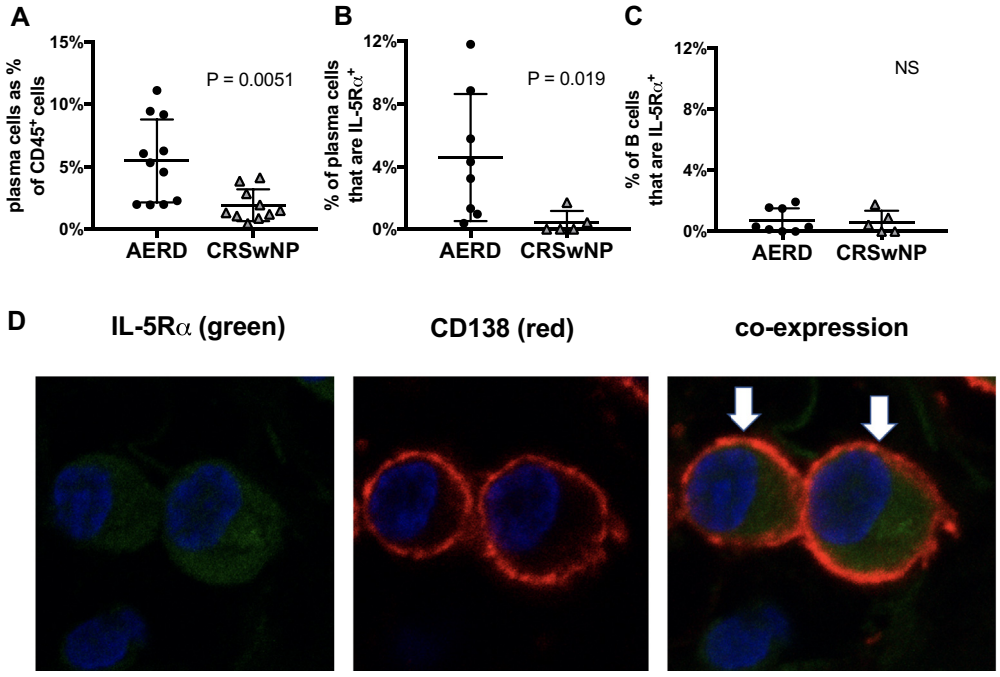

FIG 4. Flow cytometric characterization of plasma cells and immunofluorescence of polyp tissue. Nasal polyp plasma cell frequency as a percentage of $CD45^+$ cells **(A)**, plasma cell surface expression of IL-5Rα **(B)**, B-cell surface expression of IL-5Rα **(C)**, and immunofluorescence staining of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

significantly higher numbers of plasma cells than does tissue from subjects with aspirin-tolerant CRSwNP ($P = .0051$) (Fig 4, *A*). There was no significant difference in the percentage of $CD45^+$ cells that were B cells in subjects with AERD (5.5 ± 1.8%) versus in subjects with CRSwNP (3.5 ± 0.7% [$P = .35$]). The plasma cells in nasal polyps from subjects with AERD also had greater surface expression of IL-5Rα than did sinus tissue plasma cells from subjects with aspirin-tolerant CRSwNP ($P = .019$) (Fig 4, *B* and see also Fig E5 in this article's Online Repository at www.jacionline.org), but there was no difference in B-cell surface expression of IL-5Rα between groups (Fig 4, *C*). Surface expression of IL-5Rα on nasal polyp eosinophils was similar to that on nasal polyp plasma cells (see Fig E6, *A* in this article's Online Repository at www.jacionline.org), and was higher on peripheral blood eosinophils from atopic donors without history of CRS (see Fig E6, *B*). Using immunofluorescence, we examined nasal polyp tissue from 4 patients with AERD and identified co-expression of IL-5Rα and CD138 in plasma cells (Fig 4, *D*; representative sample). We also identified co-expression of IL-5Rα and IgG4 in patients with AERD (see Fig E7 in this article's Online Repository at www.jacionline.org; 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 stimulated in the presence of IL-5, or no cytokines, for 6 hours. Bulk RNA sequencing was done on 2 pairs of IL-5–stimulated/unstimulated plasma cells and identified 28 transcripts that were upregulated following IL-5 stimulation and 28 transcripts that were downregulated (Fig 5, *A*). Upregulated transcripts included several transcripts that correlated highly with *IL5RA* in the scRNA-seq data set, including *CCND2* ($R = 0.28$) and *PTP4A3* ($R = 0.16$).

On the basis of the results of the unbiased bulk RNA sequencing, we assessed for plasma cell *CCND2* transcript with and without stimulation with IL-5 in 3 subjects with AERD (Fig 5, *B*). In the IL-5–stimulated plasma cells, there was a 2.95- to 3.42-fold increase in expression of *CCND2* compared with the levels in unstimulated cells ($P = .0017$).

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. Because of 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 ($R = 0.47$; $P = .0057$), but not IgE, correlated with lifetime disease duration of nasal polyposis (Fig 1, *E-F*). Although all antibody levels tended to be higher in the polyps from subjects with AERD than in the polyps from those with CRSwNP (data not shown), the differences between these 2 groups in total IgE (Fig

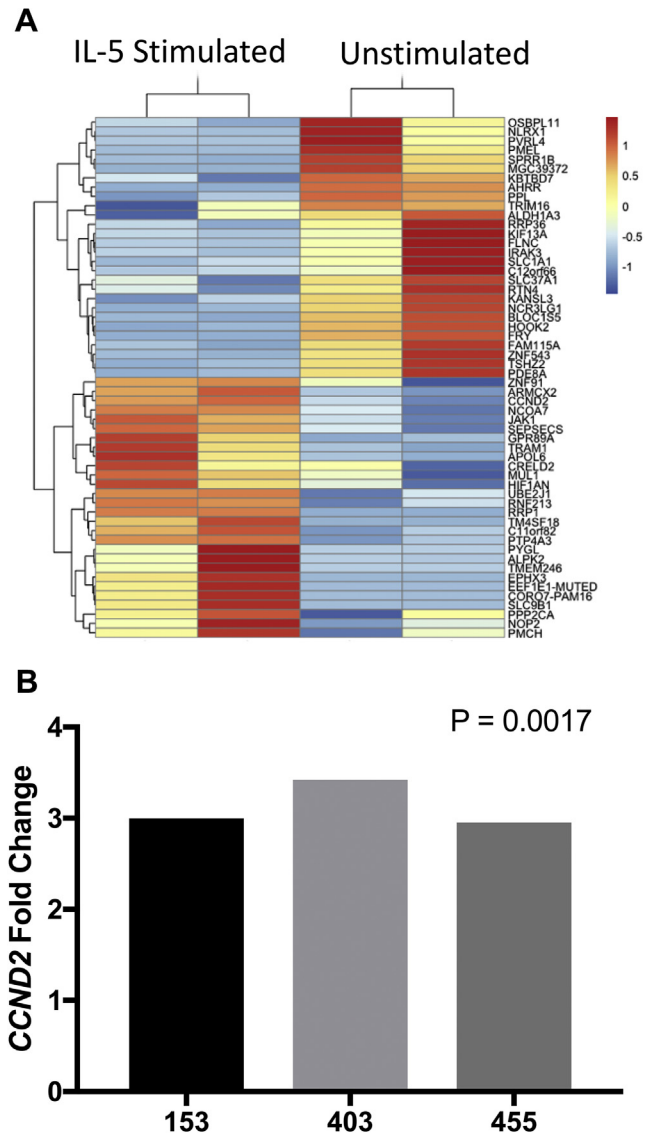

FIG 5. Transcriptional consequence of IL-5 stimulation of human nasal polyp plasma cells. **A,** Heatmap representation of differentially expressed transcripts (false discovery rate <0.1) in plasma cells from 2 subjects with AERD treated with IL-5 (1 ng/mL, 6 hours) relative to vehicle control treatment. 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 3 subjects with AERD, normalized to glyceraldehyde-3-phosphate dehydrogenase; paired *t* test.

1, *A*) and IgG4 levels (Fig 1, *B*) were remarkable. Moreover, polyp IgE levels did not significantly correlate with serum IgE levels from the same subjects, suggesting that IgE was synthesized locally. IgE-producing cells are notoriously difficult to detect owing to very low receptor density compared with that of other isotypes, and to 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; rare IgE-expressing cells could be observed through scRNA-seq analysis (Figs 2, *A-D* and see also Fig E3). These observations support mechanisms that specifically regulate the local production of IgE and IgG4 in nasal polyps and strongly differentiate those subjects with AERD from subjects with 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 mAb against IgE, improved sinonasal symptoms in patients with AERD and also decreased urinary prostaglandin D_2 metabolite and leukotriene E_4 levels (both of which are likely derived from mast cells) by approximately 90%.[44] Therefore, the elevated levels of IgE (Fig 1, *A*) and the association with IgE and aggressive recurrent disease (Fig 1, *C*) could be instrumental to the mast cell activation within the nasal polyp tissue in AERD. Further, our data suggest that the IgG4 production may have a protective effect in preventing nasal polyp regrowth in these patients (Fig 1, *D*). Although 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 (see Fig E1). Our study was limited to retrospective clinical data based on patient recall. Prospective study of the contribution of IgE and IgG4 to polyp severity and chronicity will further elucidate the relationship of these antibodies to disease 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 (see Fig E4). Our finding that AERD polyps express more than 3-fold higher levels of *IL10* mRNA (but not other B-cell–active cytokines) than CRSwNP tissue does (see 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 it is involved in the immune response to invasive parasites.[47] However, its level is also elevated in pathologic conditions, including eosinophilic esophagitis[48] and IgG4-related diseases, a group of fibroinflammatory 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 (Fig 1, *F*), 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 could also possibly modify skin test reactivity in patients with AERD, who are frequently nonatopic, 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 (Fig 3, *B* and *C*). 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 (Fig 4, *B*), and we confirmed the co-expression of IL-5Rα and the plasma cell marker CD138 through immunofluorescence (Fig 4, D). We also found that nasal polyp antibody-expressing cells in subjects with AERD can express both IL-5Rα and IgG4 (see Fig E7). ScRNA-seq analysis of nasal polyp cells from subjects with AERD revealed expression of *IL5* by effector T cells (see Fig E4). Although ILC2 cells are also known to express *IL5*, these cells were not identified in the previous study, likely because of their relative scarcity, as they comprise only 0.01% to 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 it 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]; in addition, 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 data set 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 that is 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 3-fold following stimulation with IL-5 (Fig 5, *B*). 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 (Fig 5, *A*).[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 polyps 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 mAbs 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] Taking these data together with our current findings, we suspect that IL-5– and IL-5Rα–targeting mAbs may alter the survival and function of $IL\text{-}5R\alpha^+$ antibody-expressing cells in addition to having effects on eosinophils, which may contribute to the mechanism of their therapeutic benefit.

Key messages

- **IgG4 and IgE levels are markedly increased in nasal polyp tissue from subjects with AERD compared to nasal polyp tissue from subjects with 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.**

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