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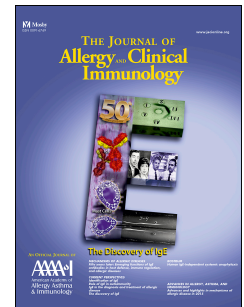
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Large scale gene expression profiling reveals distinct type 2 inflammatory patterns in chronic rhinosinusitis subtypes

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Capsule Summary: In an attempt to better classify molecular profiles in chronic rhinosinusitis (CRS) subtypes and promote endotyping, we characterize the expression of relevant type 2 inflammatory markers in several clinical subtypes of CRS.

Key Words: rhinosinusitis; microarray; expression profiling; nasal polyps; allergic fungal sinusitis; type 2 inflammation; IL1RL1; ST2

To the Editor:

Chronic rhinosinusitis (CRS) represents a heterogeneous disease comprising several different subtypes which are grouped together by common criteria lasting at least 12 weeks. The classification of CRS subtypes remains largely clinical and carries little prognostic value. At present, clinicians possess limited capability in predicting a patient's disease course and anticipating response to available therapies. More recently, efforts have focused on categorizing CRS subtypes into *endotypes*. Endotypes organize disease subtypes according to molecular patterns believed to underlie the expression of different clinical phenotypes¹. The push for disease endotyping in CRS derives from the successful application of this method in asthma, a disease sharing many pathophysiologic features with CRS.

Indeed, the presence of type 2 inflammation represents an important point of disease stratification, both in asthma and CRS. In asthma, increased blood and tissue eosinophilia, IgE levels, and expression of type 2 inflammatory biomarkers, such as IL-4, IL-5, IL-13, and periostin, have been leveraged to endotype disease, develop novel biologic therapies and monitor response to treatment².

In this study, we characterized the expression patterns of several type 2 inflammatory cytokines of interest in different clinical subtypes of CRS. To this end, we employ a top-down approach in the form of large-scale microarray gene profiling techniques in 130 patients representing different clinical subtypes of CRS: AERD, AFRS, HC, CRSsNP, and CRSwNP. We believe that a study of this design is well-suited to elucidate patterns of differential gene expression between subtypes that may serve to advance disease endotyping in CRS. To date, this is the largest microarray study of its type in CRS.

Supplementary Table 1 (Online Repository Table E1) summarizes the different disease subtypes enrolled in each group and relevant clinical characteristics.

We first performed hierarchical cluster analysis using microarray data from 130 patients with different subtypes of CRS. This resulted in the identification of 500 distinct gene expression clusters

which are illustrated in a heat map (**Online Repository Figure E1**). Many gene clusters had differential expression patterns characterized by either highly upregulated (red signal) or highly downregulated (green signal) gene expression. Polyp subtypes AERD, AFRS, and CRSwNP demonstrated similar patterns of increased or decreased cluster expression and segregated from CRSsNP and HC based on their differential cluster expression, supporting a clear molecular delineation between polyp and non-polyp CRS phenotypes.

Clusters containing the most differentially expressed sequences between HC and disease, or those of particular interest, were examined for differential expression between subtypes. In addition, data was analyzed to identify correlative and reciprocal relationships between these clusters, and with other biological pathways of interest, especially those involved in type 2 inflammation. Sequences with the highest differential expression between groups were between HC and AFRS or HC and AERD. The single highest differential signal was from a sequence specific to the soluble form of IgE (**Figure 1A**). This expression was significantly upregulated in all subtypes when compared to HC, with AERD and AFRS demonstrating increased local IgE levels when compared to other disease subtypes. IgE expression was higher in CRSwNP patients with asthma in comparison to CRSwNP patients without asthma ($p < 0.001$), while other subtypes showed no difference in tissue IgE expression when comparing those with and without asthma.

We next analyzed the expression of several canonical type 2 inflammatory cytokines (IL-4, IL-5, IL-13) and IFN γ by digital droplet quantitative PCR (**Figure 1B**). We also measured the expression of type 2 cytokine gene clusters that commonly act downstream of these cytokines (CCL13+CCL18 clusters, CCL26+Periostin clusters, **Figure 1C and 1D**). The cytokines CCL13, -18, -26 and periostin are of particular interest in CRS, as they have been shown to mediate localized eosinophilic inflammation^{3,4}. No subtypes demonstrated increased expression of IFN γ , a canonical type 1

inflammatory cytokine, which is consistent with other studies showing lack of increased IFN γ in CRS subtypes, including CRSsNP, in Japanese, Chinese, and American populations³. All subtypes demonstrated relative increased expression of IL-13. Similarly, all subtypes exhibited increased expression of IL-5 when compared to HC. Only AFRS and CRSwNP subtypes demonstrated significant increases in IL-4 gene expression. Despite no differences detected between the mean copy of IL-13 and IL-4 among CRSwNP, AFRS and CRSsNP samples, CRSwNP and AFRS had more samples expressing higher copies of IL-13 and IL-4 as compared to samples from CRSsNP patients. Gene cluster expression analysis of CCL13+CCL18 and CCL26+periostin revealed overexpression in comparison to HC of these gene clusters in all CRS subtypes. Taken together, with the exception of IL-4, the expression of the above type 2 inflammatory markers did not separate clinical phenotypes but rather highlighted subgroups (possible endotypes) among the clinical phenotypes with different expression levels of these type 2 inflammatory markers when compared to HC.

Because we could not readily delineate subtypes of CRS disease based on the relative expression of markers chosen in Figure 1, we explored additional components of type 2 inflammation that might be driving disease in CRS. The mast cell axis represents a central *node* of type 2 inflammation, capable of responding to both innate and adaptive immune arms to promote a type 2 response. Studies originating from the work of the senior author have found that mast cells are present at increased levels, independent of atopy, in polyp mucosa of patients with CRSwNP⁵. We have also shown that inflamed mucosa of CRSwNP patients harbor type 2 innate lymphoid cells (ILC2s) expressing IL1RL1 (also known as ST2), a receptor for IL-33, and that these ILC2s secrete IL-13 in response to stimulation by IL-33⁶. We wished to determine if there existed a correlation between the expression of IL1RL1 and genes associated with mast cell activity, and furthermore, assess if these genes were exclusive to certain disease subtypes.

We found that IL1RL1 transcripts were significantly overexpressed in polyp subtypes, AFRS and CRSwNP, when compared to CRSsNP and HC (**Figure 2A**). Additionally, the expression of IL1RL1 correlated with a cluster enriched for mast cell-related genes (tryptase cluster, **Figure 2B**). We also found that IL1RL1 expression correlated with the expression of 21 genes associated with mast cell and eosinophil activity (**Online Repository, Table E2**). We performed an immunohistochemical analysis in a representative collection of surgically removed CRSwNP polyp tissue (**Figure 2C and 2D**). IL-33 expression was evident in the epithelial cells and around vessels in all polyp samples (**Figure 2C**). Immunohistochemistry indicated the presence of abundant eosinophils and mast cells, both known IL1RL1-expressing cells (**Figure 2D**). Taken together, these results implicate an IL1RL1-mast cell signaling axis as a potential endotype marker and target for therapeutic intervention.

Discussion (See Online Repository)

Molecular pathways that drive disease in different CRS clinical phenotypes – so-called *endotypes* – need further clarification to facilitate the application of personalized therapies in CRS. Our top down, microarray-based analysis allowed us to characterize the relative expression of several type 2 inflammatory gene clusters of interest in well-defined clinical subtypes of CRS, many of which take into account eosinophilic inflammation, comorbid asthma, aspirin sensitivity, fungal colonization, and atopy. Admittedly, we do not define specific endotypes in this study; however, we evaluated key type 2 inflammatory markers as a means of endotyping CRS patients. We found that there were trends of elevated expression of certain type 2 inflammatory markers in clinical phenotypes but also found notable variation of expression within a given phenotype. One such marker, local IgE expression, was elevated in subgroups of AERD, AFRS, and CRSwNP patients with asthma, clinical subtypes that characteristically exhibit increased local sinonasal eosinophilia. Our observations support previous

studies which indicate a correlation between local sinonasal IgE expression and increased tissue eosinophilia.^{7,8,9,E1}

Interestingly, with the exception of IL-4, all CRS subtypes demonstrated increased expression of several canonical type 2 inflammatory markers (Figure 1). The observed increase in these measured type 2 inflammatory markers in CRSsNP may in part be due to the high prevalence of co-morbid allergic rhinitis in this patient population; however, multiple studies have suggested that atopic sinonasal inflammation represents a distinct process from that observed in CRS.^{E2, E3, E4} Our findings complement those of Tomassen et al., which showed the presence of type 2 inflammation in both polyp and non-polyp clinical subtypes, and highlight the complexity of the inflammatory milieu in CRS mucosa. Together, these findings prompt a need to investigate additional type 2 inflammatory pathways that better separate disease subtypes.

Studies in asthma and CRS have highlighted multiple different mechanisms that can promote type 2 inflammation, including an adaptive immune component and a recently identified *innate* type 2 inflammatory component. Epithelial-derived cytokines, like IL-25, IL-33, thymic stromal lymphopoietin and mast cells may play important roles in mediating type 2 inflammatory disease independent of adaptive immunity. Indeed, elevation of the IL-33 receptor, IL1RL1, in CRSwNP is not a novel finding; nonetheless, our results add credence to preceding studies which have shown the importance of this pathway in the pathogenesis of CRSwNP^{6,E4,E5,E6}. Additionally, to the best of our knowledge, we present novel findings correlating IL1RL1 expression with mast cell activity in polyp subtypes and we extend these findings to the AFRS clinical subtype. We noted a trend towards increased IL1RL1 expression in the AERD clinical subtype (Figure 2A); however, the small sample size of this group likely precluded achieving statistical significance. Taken together, our results implicate an

IL1RL1-mast cell signaling axis as a potential disease marker, a mediator of type 2 inflammation and a potential target for therapeutic intervention.

The study presented herein illustrates the complexity of the inflammatory makeup in CRS. While features of type 2 inflammation appear active in many clinical subtypes of CRS, they may culminate in a type 2 inflammatory response via different mechanisms, including infection, barrier disruption, allergy, etc. Additionally, the *degree* of type 2 inflammatory activation may be an important factor in differentiating disease endotypes. As clinical trials that test the efficacy of novel therapies in CRS progress, a better understanding of the specific molecular pathways that drive a patient's disease will aid in identifying those who will benefit most from these novel treatment strategies.

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194 **Figure 1.** Expression of canonical type 2 inflammatory markers in CRS subtypes. **(A)** Expression of
195 IgE. Samples from each disease subtype are stratified according to presence or absence of asthma
196 phenotype. **(B)** Relative expression of several canonical upstream regulators of type 1 (IFNG) or type 2
197 inflammation (IL-4, IL-5, IL-13) as measured by droplet digital PCR. **(C and D)** Relative expression
198 of important downstream type 2 inflammatory gene clusters. †p<0.05, * p<0.01, ** p<0.001 for
199 comparisons of HC vs each subtype. When no annotation is indicated, the comparison was not
200 significant. POSTN - periostin

201 **Figure 2.** IL1RL1 expression and mast cell activity in CRS. **(A)** Box and whisker plot of differential
202 expression of IL1RL1 by microarray analysis. **(B)** Correlation of IL1RL1 and tryptase gene cluster. r^2
203 = 0.46, p<0.001. **(C and D)** IL-33 expression, mast cell and eosinophil infiltration in representative
204 CRSwNP tissue. **(C)** Representative photomicrograph illustrating IL-33 expression located in the nasal
205 surface epithelium (black arrow) as well as the stroma (blue arrow) containing inflammatory infiltrate.
206 **(D)** Photomicrographs demonstrating expression of IL-33 associated with varying levels of eosinophil
207 and mast cell infiltration. MBP: major basic protein. ** p<0.001 for comparisons of HC vs each
208 subtype. When no annotation is indicated, the comparison was not significant.

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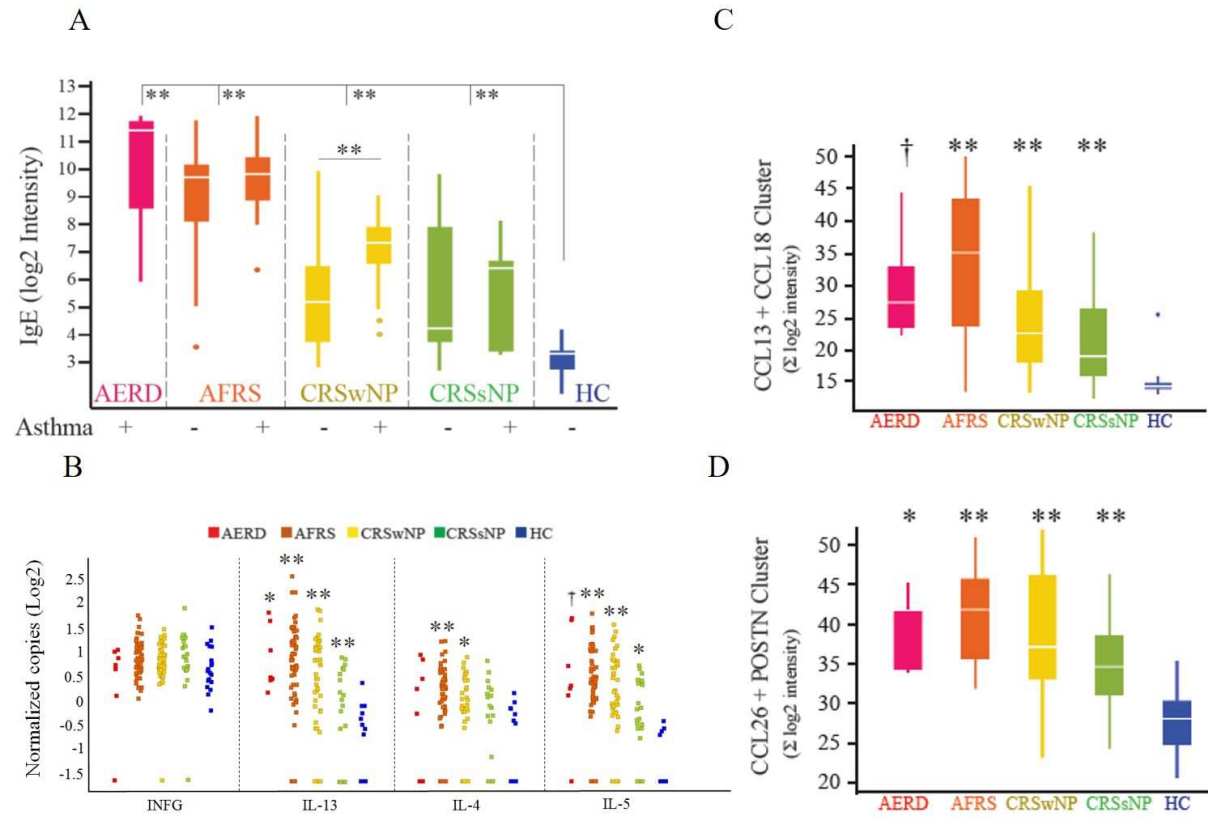
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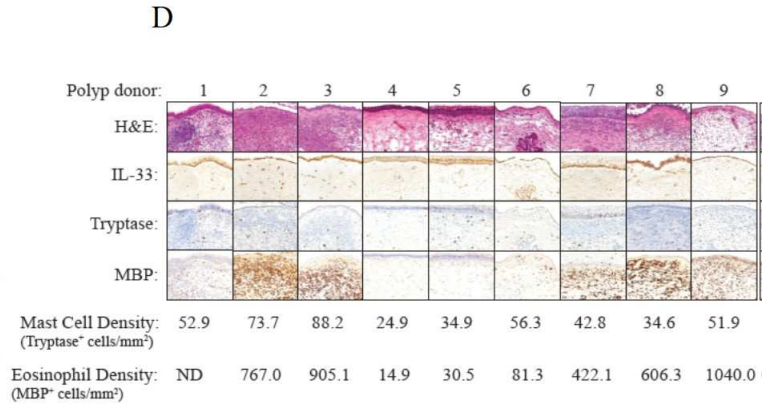
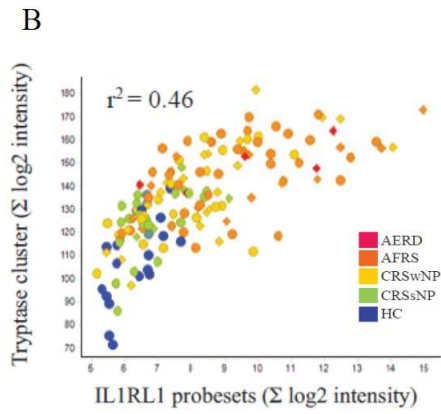
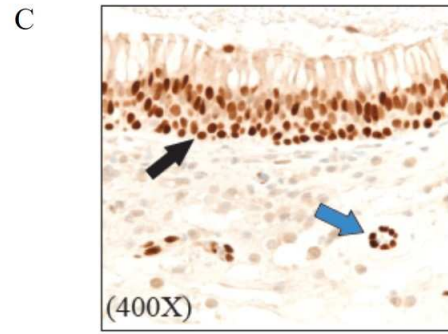
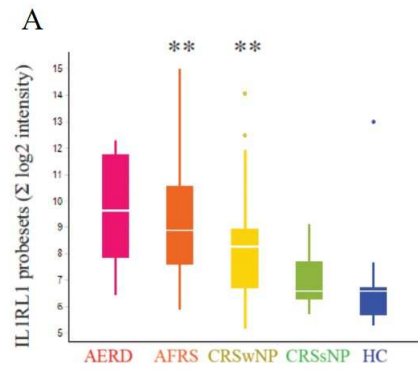
Authorship: CBR, DES, and AL jointly conceived the study, its design and implemented the analysis. MAT, XH and SA assisted with the analysis. MAT, CBR, DES, and AL wrote the manuscript, and CJP, MJC, and SF edited the manuscript and provided conceptual advice.

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Methods (See Online Repository)

Patients

Patients undergoing medically indicated functional endoscopic sinus surgery (FESS) were consented to having sinonasal tissue, which was removed as a standard of their surgery, collected and analyzed for gene expression. The Institutional Review Board at the University of Texas Health Science Center at Houston approved the study protocol. Patients were grouped into CRSwNP, CRS without nasal polyps (CRSsNP), aspirin exacerbated respiratory disease (AERD) or allergic fungal rhinosinusitis (AFRS) according to criteria set forth in the European Position Paper on Rhinosinusitis and Nasal Polyps^{E7}. Patients were grouped into CRSwNP or CRSsNP based on presence or absence of polyps on nasal endoscopy. Polyps documented by an ENT physician on nasal endoscopy at any time categorized a patient as CRSwNP subtype. CRS patients with nasal polyps were diagnosed with AFRS if there was evidence of eosinophil-rich mucus with noninvasive fungal hyphae, hypersensitivity to fungi, and characteristic radiographic findings^{E8}. AERD was characterized by the presence of asthma, the presence or documented history of nasal polyps, and history of intake of aspirin or non-steroid anti-inflammatory (NSAID) inciting worsening respiratory symptoms. Patients classified as asthmatic had a prior diagnosis of asthma by a pulmonologist, allergist and/or positive pulmonary function testing. In this study, CRSwNP excludes patients with AERD and AFRS. Healthy controls were defined as patients with no history of atopy or asthma symptoms. These patients were undergoing sinus surgery as a component of surgical approach to the skull base for removal of benign pituitary lesion or repair of anterior skull base cerebrospinal fluid leak. A table delineating the number of patients enrolled according to each clinical subtype, along with relevant clinical factors, has been presented in **Online Repository Table E1**. No clinical subtype was treated in an exclusive

manner, in that they did not receive any specific medical or surgical therapy that deviated from the standard of care delivered to other subtypes. Per study protocol, all topical and systemic corticosteroids were withheld at least 4 weeks prior to surgery when tissue was harvested.

RNA extraction and analysis

A fragment of inflamed ethmoid mucosa was removed during endoscopic sinus surgery, immediately wrapped in aluminum foil and flash frozen in liquid nitrogen. Frozen biopsies were placed in lysis buffer and homogenized with a Qiagen TissueRupter (Qiagen, Valencia, CA). In instances where a substantial amount of mucosa was harvested during surgery, tissue was separated and treated as independent samples. For microarray analysis, this was the case in a select group of patients: AFRS (45 patients yielded 51 samples), CRSwNP (38 patients, 45 samples) and HC (17 pts, 22 samples). For droplet digital PCR (described below), we included RNA from subsequent enrollees in addition to the index (n=130, 147 samples) patient set: AERD (6 patients, 6 samples), AFRS (49 patients, 55 samples), HC (17 patients, 23 samples). RNA was prepared using the mirVana miRNA isolation kit (Applied Biosystems, Carlsbad, CA), modified to include an on-column treatment with RNase-free DNase (Qiagen). RNA quality was assessed by RIN values from the BioAnalyzer 2100 (Agilent, Palo Alto, CA).

Fifty ng total RNA was amplified using the Ovation® RNA Amplification System V2 and WB reagent (Nugen, Inc., San Carlos, CA). Of the amplified cDNA, 4.4µg was labeled using the FL Ovation™ cDNA Biotin Module V2 (Nugen, Inc.) according to the manufacturer's recommendations. The labeled cDNA was hybridized onto Affymetrix human genome HG-U133_Plus_2 arrays (Affymetrix, Santa Clara, CA) and processed according to Affymetrix technical protocols. The average intensity of each array was scaled to a target intensity of 500.

Single genes were analyzed by droplet digital PCR on the BioRad (Hercules, CA) QX100 system, in duplex mode. Target gene probe sets were labeled with FAM, and the RBM22 reference normalizer probe set was labeled with VIC. All probe sets were standard Applied Biosystems (Grand Island, NY) catalog probe sets (IL-4: Hs00174122_m1, IL-5: Hs01548712_g1, IL-13: Hs00174379_m1, IFNG: Hs00989291_m1)

Microarray data analysis

Raw Affymetrix .CEL files were pre-processed in Array Studio (OmicSoft, Cary, NC, USA, <http://www.omicsoft.com>) using its default procedures including data normalization by RMA (Robust Multi-array Average) approach. Microarray data were analyzed for different patterns of gene expression by hierarchical clustering analysis. To identify sets of genes with highly correlated expression patterns, a set of 32,719 sequences with coefficients of variation greater than 0.03 across all the samples was used. This method was used to identify (dis)similarities between datasets based on a measurement of Euclidean distance. These (dis)similarities between data sets were organized into discrete clusters that are visualized as a dendrogram (**Online Repository Figure E1**). We used Ward's linkage method, which uses within-cluster sum of squares computations to group clusters of gene expression. Using these methods, we identified 500 clusters of differential gene expression and evaluated the separation of different subtypes qualitatively in **Figure E1**. Clusters containing the most differentially expressed sequences, or some of previously identified biological significance, were then examined for differential expression between the disease groups and their relationships with each other (**Figure 1**).

Immunohistochemistry

Tissue sections from formalin-fixed, paraffin embedded human nasal polyps from CRS patients undergoing routine surgical removal (n = 9) were cut at 5 microns, baked at 65°C for 30-60 minutes, deparaffinized in xylene and rehydrated through graded ethanol solutions. Serial sections were stained with H&E following standard methodology and specific immunohistochemical staining was performed. Antigen retrieval for tissue sections stained for IL-33 and mast cell tryptase was accomplished by steaming the slides in DIVA buffer (Biocare Medical #DV2004, Concord, CA) for 60 minutes. Endogenous protein was blocked with Background Sniper (Biocare Medical, #BS966, Concord, CA), then endogenous avidin and biotin were blocked (Biocare Medical #AB972). Antigen retrieval for tissue sections stained for eosinophil major basic protein was accomplished by incubating the sections with Pepsin for 30 minutes (Biocare Medical, Carezyme II # PEP956, Concord, CA). Slides were then incubated with anti-IL-33 (Goat polyclonal, R&D systems #AF3625), anti-mast cell tryptase (Rabbit polyclonal, Abcam #ab35118, Cambridge, MA), or anti-eosinophil major basic protein (Mouse monoclonal, AbD Serotec #MCA 5751, Raleigh, NC) diluted in DaVinci Green diluent (Biocare Medical #PD900, Concord CA). Next, endogenous peroxidase was quenched (Biocare Medical #PX968). Slides stained for IL-33 were incubated with biotinylated anti-Goat IgG (Vector PK6105, Vector Labs Burlingame, CA) followed by ABC-HRP (Vector PK6105, Vector Labs Burlingame, CA). Slides stained with mast cell tryptase were detected with goat anti-rabbit HRP-labeled polymer (Biocare Medical #RHRP520, Concord, CA). Primary antibody against eosinophil major basic protein was detected using a goat anti-mouse HRP-labeled polymer (Biocare Medical #RMRP520). Slides were then developed with DAB chromogen (Biocare Medical #DCB859), rinsed in water, counterstained in hematoxylin, dehydrated, cleared and mounted.

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Online Repository Table E1

CRS Subtype (n)	HC (17)	AERD (5)	AFRS (45)	CRSwNP (38)	CRSsNP (25)
Age (median, yrs)	53	43	26	50	48
Male/Female	10/7	1/4	25/20	23/15	15/10
Asthma (%)	0/17 (0)	5 /5 (100)	12/45 (27)	21/38 (55)	5/25 (20)
Inhalant Allergy (%)	0 (0)	3 (60)	45 (100)	25 (66)	12 (52)
ASA Sensitivity (%)	0 (0)	5 (100)	0 (0)	0 (0)	0 (0)

Online Repository Table E2

Correlation	Rank	Gene	Description	Probe
0.885292	2	IL1RL1	ST2	242809_PM_at
0.723186	3	IL1RL1	ST2	207526_PM_s_at
0.685895	4	IL10	IL10	207433_PM_at
0.676026	5	SAMSN1	(Nash1, mast cell nuclear adaptor protein)	1555638_PM_a_at
0.670993	6	SOCS1	SOCS1	213337_PM_s_at
0.662589	7	CCL23	CCL23 (eosinophil-enriched chemokine)	210549_PM_s_at
0.654215	9	LYVE1	Lymphatic vessel endothelial hyaluronan receptor 1	220037_PM_s_at
0.653251	10	IL18R1	IL18 receptor	206618_PM_at
0.648994	11	SRGN	Serglycin (Mast cell protease-associated proteoglycan)	201858_PM_s_at
0.648871	12	ADORA3	Adenosine A3 receptor (expressed by eosinophils and macrophages)	206171_PM_at
0.63764	14	IGHA1	Immunoglobulin heavy locus	217469_PM_at
0.630232	16	ALOX5AP	Arachidonate 5-lipoxygenase-activating protein (FLAP)	204174_PM_at
0.626879	18	C3AR1	Complement component 3a receptor 1 (mast cells?)	209906_PM_at
0.625483	19	SOCS1	SOCS1	210001_PM_s_at
0.619688	20	CCL18	CCL18	209924_PM_at
0.619456	21	IL1R1	IL1 receptor	202948_PM_at
0.618864	22	HPGDS	prostaglandin D synthase (Mast cells)	206726_PM_at
0.616832	23	PMCH	Pro-melanin-concentrating hormone	206942_PM_s_at
0.614175	24	CCND2	Cyclin D2	200951_PM_s_at
0.613632	25	NFIL3	Nuclear factor, IL-3 regulated (IL4-induced, regulates IgE)	203574_PM_at
0.608201	27	SRGN	Serglycin (Mast cell protease-associated proteoglycan)	201859_PM_at
0.607935	28	CTSG	Cathepsin G (Mast cell protease)	205653_PM_at
0.606948	30	PIM1	Pim-1 oncogene	209193_PM_at
0.602255	32	PTGDR2	Prostaglandin D2 receptor 2 (CRTH2) (expressed by eosinophils and ILC2)	206361_PM_at

Table and Figure Legend

Online Repository Table E1. Clinical phenotypes of CRS patients and healthy controls enrolled in study.

Online Repository Figure E1. Hierarchical gene expression cluster analysis in CRS subtypes. Ward's linkage analysis was employed to organize the 32,719 sequences into 500 clusters which are represented by the dendrogram with arbitrarily colored columns across the top of the heat map. Changes with $\log_2(\text{FC}) \geq 2$ are bright red (highly upregulated), and those with $\log_2(\text{FC}) \leq -2$ are bright green (highly downregulated). The separation of CRS subtypes according to their differential gene cluster expression is illustrated on the right of the heat map.

Online Repository Table E2. Mast cell- and eosinophil-related gene cluster and its correlation with IL1RL1 (ST2) expression. The genes listed above were identified as highly correlated with one another as well as with the expression of IL1RL1. Data shown was generated from pooled expression values from all subtypes of CRS. Correlation coefficients (leftmost column) were generated using a Spearman's rank test and the IL1RL1 probe (234066_at). For all correlations, $p < 0.001$.

