

Group 2 innate lymphoid cells are recruited to the nasal mucosa in patients with aspirin-exacerbated respiratory disease

Jacqueline J. Eastman, MD,^a Kellen J. Cavagnero, BS,^a Adam S. Deconde, MD,^b Alex S. Kim, MD,^a Maya R. Karta, PhD,^a David H. Broide, MB, ChB,^a Bruce L. Zuraw, MD,^a Andrew A. White, MD,^c Sandra C. Christiansen, MD,^a and Taylor A. Doherty, MD^a San Diego and La Jolla, Calif

Background: Aspirin-exacerbated respiratory disease (AERD) is characterized by tissue eosinophilia and mast cell activation, including abundant production of prostaglandin D₂ (PGD₂). Group 2 innate lymphoid cells (ILC2s), which promote tissue eosinophilia and mast cell responses, undergo chemotaxis and cytokine production in response to PGD₂, but it is unknown whether ILC2s are active in patients with AERD.

Objective: We sought to determine whether ILC2 numbers change in peripheral blood and the nasal mucosa during COX-1 inhibitor-induced reactions in patients with AERD.

Methods: Blood and nasal scrapings were collected at baseline, during reactions, and after completion of ketorolac/aspirin challenge/desensitization in 12 patients with AERD. ILC2s and eosinophils were quantitated by means of flow cytometry. Urine was also collected, and quantification of PGD₂ metabolite and leukotriene E₄ levels was done by using ELISA. Baseline and nonsteroidal anti-inflammatory drug reaction clinical data were correlated with cell changes.

Results: ILC2 numbers significantly increased in nasal mucosal samples and decreased in blood at the time of COX-1 inhibitor reactions in 12 patients with AERD. These changes were not observed in 2 patients without AERD. Furthermore, eosinophil numbers decreased in blood concurrently with significant increases in urinary PGD₂ metabolite and leukotriene E₄ levels. The magnitude of increases in nasal mucosal ILC2 numbers positively correlated with maximum symptom scores during challenges. Furthermore, blood ILC2 numbers during the reaction correlated with time for the reaction to resolve, possibly reflecting reaction severity.

Conclusions: ILC2s are recruited to the nasal mucosa during COX-1 inhibitor-induced reactions in patients with AERD, correlating with enhanced production of prostaglandins and leukotrienes. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: Group 2 innate lymphoid cells, aspirin-exacerbated respiratory disease

Aspirin-exacerbated respiratory disease (AERD) is clinically characterized by a relentless progression of nasal congestion, anosmia, formation of nasal polyps, and, typically, asthma. Naso-ocular and lower respiratory tract symptoms after ingestion of COX-1 inhibitors are pathognomonic for AERD. AERD is present in about 7% of adult asthmatic patients and 14% of patients with severe asthma¹ and can develop in patients without a prior history of atopy, asthma, or rhinitis.² Nasal polyps of patients with AERD are intensely eosinophilic and contain activated mast cells.^{3,4} The disease is characterized by release of large amounts of cysteinyl leukotrienes (CysLTs) and mast cell mediators, particularly prostaglandin D₂ (PGD₂), after COX-1 inhibitor ingestion.^{5,6} Recently, thymic stromal lymphopoietin (TSLP) and IL-33 have also been implicated in AERD pathogenesis.^{7,8} These 2 cytokines, in addition to PGD₂ and CysLTs, are known to promote group 2 innate lymphoid cell (ILC2) activation,⁹⁻¹² and their presence suggests that ILC2s might be active in patients with AERD.

ILC2s are a recently described lymphocyte population that lack antigen specificity but are capable of producing large amounts of type 2 cytokines (IL-4, IL-5, IL-6, IL-9, and IL-13) and have been shown to promote type 2 inflammation.¹³ In human subjects ILC2 numbers are increased in affected tissues from patients with atopic dermatitis, allergic asthma, eosinophilic esophagitis, and allergic rhinitis.¹⁴⁻¹⁸ Importantly, ILC2 numbers are increased in nasal polyps and levels correlate with the degree of eosinophilia.¹⁹ ILC2s are identified as lymphocytes that are lineage negative (ie, lacking the markers of B, T, or natural killer cells) and express chemoattractant receptor-homologous molecule expressed on T_H2 cells (CRTH2) and CD161.³ CRTH2 is a G protein-coupled receptor that binds to PGD₂, and PGD₂ has been shown to promote ILC2 chemotaxis and cytokine production *in vitro*.^{10,20} Thus these suggest a potential role for ILC2s in patients with AERD.

Our aim for this study was to determine ILC2 changes in the blood and nasal mucosa of patients with AERD during COX-1 inhibitor challenges. To our knowledge, there are no reports focused on a potential role for ILC2s in AERD-related nonsteroidal anti-inflammatory drug (NSAID)-induced reactions. Importantly, we found that ILC2 numbers were significantly increased in nasal scrapings and decreased in peripheral blood during the ketorolac-induced reactions in patients with AERD. These patients also had a corresponding decrease in blood

From ^athe Department of Medicine, University of California, San Diego; ^bthe Department of Surgery, University of California-San Diego, La Jolla; and ^cthe Division of Allergy, Asthma and Immunology, Scripps Clinic, La Jolla.

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Corresponding author: Taylor A. Doherty, MD, Department of Medicine, University of California-San Diego, Biomedical Sciences Building, Room 5090, 9500 Gilman Dr, La Jolla, CA 92093-0635. E-mail: tdoherty@ucsd.edu.

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Abbreviations used

AERD:	Aspirin-exacerbated respiratory disease
CRT2:	Chemoattractant receptor-homologous molecule expressed on T _H 2 cells
CysLT:	Cysteinyl leukotriene
ILC2:	Group 2 innate lymphoid cell
LTE ₄ :	Leukotriene E ₄
NSAID:	Nonsteroidal anti-inflammatory drug
PGD ₂ :	Prostaglandin D ₂
11β-PGF ₂ α:	11β Prostaglandin F ₂ α
SNOT-22:	Sino-Nasal Outcome Test 22
TSLP:	Thymic stromal lymphopoietin

eosinophil numbers, indicating that these processes happen simultaneously. Concomitantly, patients showed an increase in levels of 11β prostaglandin F₂α (11β-PGF₂α; a stable metabolite of PGD₂),²¹ as well as the terminal CysLT leukotriene E₄ (LTE₄). Overall, this study implicates ILC2s as an additional cell type apart from mast cells and eosinophils that can contribute to AERD pathogenesis.

METHODS**Study subjects with AERD**

Patients (n = 14) undergoing aspirin desensitization for AERD at the Allergy Divisions of the University of California, San Diego and Scripps Clinic were recruited after institutional review board approval at both institutions. Informed consent was obtained from all patients before starting the study. All patients were suspected of having a diagnosis of AERD based on a history of nasal polyps and asthma and a compelling history of reactions to aspirin/NSAIDs within 2 to 3 hours. Demographic and clinical data were collected from retrospective chart review and included age, sex, ethnicity, asthma, and rhinoconjunctivitis characteristics; nasal polyp surgical details and clinical response; aspirin/NSAID reaction characteristics; current medications; and laboratory data. Symptom scores and spirometry were assessed at baseline. The modified AERD symptom scoring system included 8 symptoms (nasal congestion, runny nose, sneezing, itchy nose, itchy/watery eyes, tight throat, wheezing, and chest tightness), which the patients rated on a scale of 0 to 3 (range of possible scores, 0–24; see Fig E1 in this article's Online Repository at www.jacionline.org). Sino-Nasal Outcome Test 22 (SNOT-22) scores were recorded either that day or from a recent clinic visit.

Intranasal ketorolac and aspirin challenge/desensitizations

Intranasal ketorolac and aspirin challenge/desensitizations were done per the standard protocol at each institution (see Fig E2 in this article's Online Repository at www.jacionline.org, which is adapted from Lee et al²²). Reactions were identified as any upper and/or lower respiratory tract signs or symptoms consistent with conjunctivitis, rhinitis, laryngospasm, and bronchospasm or decrease in FEV₁.

Nasal scrapings, blood draws, and urine samples

Nasal scraping was done with a Rhino-probe (Arlington Scientific, Springville, Utah) and involved 1 to 3 passes over the inferior turbinate on one side. Nasal scrapings were transported in RPMI for processing for flow cytometry to detect ILC2s. Blood was collected in BD vacutainers (BD Biosciences, San Diego, Calif) to quantitate ILC2 and eosinophil numbers. Samples, including nasal scrapings, blood, and urine, were collected at baseline. At the time of the first clinician-confirmed reaction, blood and a nasal scraping were again collected, and urine was collected 1 hour later. A third set of samples was collected at the end of the desensitization before patient

discharge. Some patients were not able to provide all 3 samples. Symptom scores (per the system noted above), FEV₁, and medications required were recorded at each time point.

Identification of ILC2s and eosinophils in peripheral blood and nasal scrapings

PBMCs and granulocytes were separated with Histopaque 1119 and Histopaque 1077 (Sigma-Aldrich, St Louis, Mo) per the manufacturer's protocol. Nasal scrapings were processed through a strainer and washed before staining. Cells were first incubated with Fc Receptor Blocking Reagent (Miltenyi Biotec, San Diego, Calif). Granulocytes were then stained with peridinin-chlorophyll-protein complex-conjugated CD45, fluorescein isothiocyanate-conjugated FcεRIα, and allophycocyanin-conjugated CCR3 (BioLegend, San Diego, Calif). Eosinophils were defined as the percentage of CD45⁺ granulocytes that were CCR3⁺FcεRIα⁺. PBMCs and nasal scrapings were stained with peridinin-chlorophyll-protein complex-conjugated CD45 (BioLegend), fluorescein isothiocyanate-conjugated lineage markers (CD235a, FcεRIα, T-cell receptor γ/δ, and CD4 [BioLegend] and BD Lineage Cocktail 1 [CD3, CD14, CD16, CD19, CD20, and CD56]) and phycoerythrin-conjugated CRT2 (Miltenyi Biotec). PBMCs were also stained for allophycocyanin-conjugated CD161 (BioLegend). Blood ILC2s were defined as percent CD45⁺Lin[−]CD161⁺CRT2⁺ lymphocytes of all cells. Percentages of ILC2s are reported as percentages of enriched PBMCs. Nasal scraping ILC2s were defined as CD45⁺Lin[−]CRT2⁺ lymphocytes. Percentages of ILC2s are reported as percentages of total cells in nasal scraping samples. Flow cytometry was performed with a NovoCyt Cytometer (Acua Biosciences, San Diego, Calif). Data were further analyzed with FlowJo software (FlowJo, Ashland, Ore).

Urine 11β-PGF₂α and LTE₄

Urine samples were stored at −80°C. PGD₂ is metabolized to 11β-PGF₂α, a stable active metabolite that is measurable in urine.²¹ 11β-PGF₂α levels were analyzed by means of ELISA, according to the manufacturer's protocol (Cayman Chemical, Ann Arbor, Mich). Urinary LTE₄ levels were analyzed by means of ELISA, according to the manufacturer's protocol (Cayman Chemical). Values were corrected for specific gravity, as previously described.²³

Nasal polyps

Nasal polyps were collected from 2 subjects undergoing endoscopic sinus surgery at the University of California, San Diego who ultimately underwent desensitization for AERD and are included in that analysis. Tissue samples were transported in RPMI and processed for flow cytometry the same day. Nasal polyp tissue was digested with Collagenase Type 4 (Worthington, Lakewood, NJ) and DNase I (200 μg/mL; Roche, Indianapolis, Ind) and then filtered into a single-cell suspension. ILC2s were identified per above. GATA-3⁺ cells were identified as CD45⁺Lin[−]GATA-3⁺ lymphocytes. The cells were initially stained for cell-surface CD45 and lineage markers per above. The cells then underwent fixation and permeabilization (eBioscience, San Diego, Calif) and were stained for phycoerythrin-conjugated GATA-3 (eBioscience). Flow cytometry and analysis were done as indicated above.

Statistical analyses

Statistical analysis was performed with GraphPad Prism software (GraphPad Software, La Jolla, Calif). The paired *t* test or Pearson correlation test were used, where indicated. A *P* value of less than .05 was considered statistically significant.

RESULTS**Demographics of patients with AERD and NSAID reaction characteristics**

A total of 14 suspected patients with AERD were enrolled in the study. Baseline characteristics of patients are summarized in

TABLE I. Patients' characteristics

No. of patients	14
Sex (male/female [no.])	5/9
Median age (range [y])	54.5 (40-66)
Race (no.)	
White	13
African American	1
Personal history of reaction to aspirin/NSAID (%)	100
Leukotriene modifier drug use (%)	92.8
Montelukast (%)	85.7
Zileuton (%)	7.1
Asthma (%)	92.8
Baseline FEV ₁ % (range)	82 (67-96)
Daily inhaled corticosteroid use (%)	85.7
Daily oral corticosteroid use (%)	14.3
Omalizumab treatment (%)	21.4
Personal history of sinus surgery (%)	100
Median number of sinus surgeries (range [no.])	4 (1-7)
SNOT-22 score (range)	30 (0-73)
Nasal corticosteroid use (%)	100
Nasal budesonide rinse use (%)	28.6
Personal history of atopy (%)	64.2
Median total IgE (range [IU/mL])	52 (9-354)
Median blood eosinophils/mm ³ (range)	519 (100-1200)
Reaction	
No. of positive ketorolac challenges	11
No. of silent desensitizations	1
No. considered aspirin tolerant	2
Provocative dose of ketorolac (no.)	
2.53 mg	1
5.05 mg	6
7.58 mg	4
Median decrease in FEV ₁ % (range)	9.45 (0-43.9)
Abdominal symptoms (no.)	4
Treatment requiring corticosteroids (no.)	6

Table I. At the time of desensitization, 13 patients were receiving a leukotriene modifier for a minimum of 1 week; 1 patient was not receiving a leukotriene modifier because of adverse effects. Two (14%) patients were receiving daily oral corticosteroids. Eleven patients had typical COX-1 inhibitor-induced reactions, including bronchoconstriction and/or nasocular symptoms, during ketorolac challenge. Three patients did not have reactions to ketorolac or aspirin challenge. One of the 3 patients had a history of recurrent aggressive nasal polyps and severe asthma and was receiving omalizumab, which could possibly avert COX-1 inhibitor-induced exacerbations²⁴; this patient was determined to nonetheless have AERD (a "silent reaction"). The 2 other patients had less consistent histories of reactions to COX-1 inhibitors, were not receiving omalizumab, and had no clinical response to aspirin. These 2 patients were determined not to have AERD and served as control subjects. One of these control subjects was the sole patient taking zileuton. "Reaction" samples for these 3 were taken after the final dose of ketorolac.

Identification of ILC2s in nasal scrapings and blood

Nasal polyp ILC2s have been previously reported as CD45⁺Lin⁻ lymphocytes that express CRTH2.^{3,19} To confirm our ILC2 gating strategy for nasal scrapings, we first stained nasal

polyps from 2 patients with AERD, along with additional staining for the master T_H2 cytokine transcription factor GATA-3, as proof of concept that ILC2s are indeed identifiable in these patients (see Fig E3 in this article's Online Repository at www.jacionline.org). Nasal scraping ILC2s were identified as CD45⁺Lin⁻CRTH2⁺ lymphocytes (Fig 1, A). We did not use CD161 for tissue ILC2 staining to maintain consistency with what we had previously performed on nasal polyps.¹⁹ Blood samples were separated into PBMCs and granulocytes, as described in the Methods section. ILC2s were obtained from the enriched PBMCs and identified as CD45⁺Lin⁻ lymphocytes that expressed CD161 and CRTH2 (Fig 1, B). Isotype controls were done for all CRTH2 gating. Overall, we were able to identify ILC2s in blood and nasal scrapings of patients with AERD.

ILC2 numbers increase in nasal mucosa and decrease in blood from baseline values during COX-1 inhibitor challenge

The percentage of ILC2s of all cells in nasal scrapings increased significantly from baseline to those during COX-1 inhibitor-induced reaction ($P = .001$; Fig 2, A). There was also a subsequent significant decrease in nasal scraping ILC2s from reaction to post-aspirin desensitization samples ($P = .004$). Furthermore, blood ILC2 numbers decreased significantly from baseline to reaction samples ($P = .031$; Fig 2, B). Representative baseline, reaction, and postdesensitization blood ILC2 plots are shown in Fig 1, C. The trend for blood ILC2 numbers to increase from reaction to postdesensitization samples approached but did not reach significance ($P = .084$). Changes in nasal scraping and blood ILC2 numbers were not seen in 2 control subject (Fig 2, C). The decrease in blood ILC2 numbers occurred concurrently with a significant decrease in blood eosinophil numbers from baseline to the time of reaction ($P = .044$; Fig 2, D). Because of low nasal scraping cell numbers, we were able to identify eosinophils in only 2 patients. These 2 samples showed a 1.5- and 7.9-fold increase in nasal scraping eosinophils from baseline to reaction (3.34% to 5.01% reaction and 0.34% to 2.68%, respectively).

As expected, there was an increase in levels of the PGD₂ metabolite 11 β -PGF₂ α and LTE₄ measured in urine during the COX-1 inhibitor-induced reaction (11 β -PGF₂ α : $P = .008$ and LTE₄: $P = .017$, difference between baseline and reaction samples; Fig 2, D and E). The increases in urinary 11 β -PGF₂ α and LTE₄ levels were observed at the same time as the increase in nasal scraping ILC2 numbers and decrease in blood ILC2 and eosinophil numbers. Baseline nasal scraping ILC2 numbers did not correlate with reaction levels of 11 β -PGF₂ α ($P = .12$) or LTE₄ ($P = .16$). The magnitude of increase in nasal scraping ILC2 numbers did not correlate with the magnitude of increase in 11 β -PGF₂ α ($P = .105$) or LTE₄ ($P = .470$) levels or the decrease in blood ILC2 ($P = .713$) or eosinophil ($P = .281$) numbers. Baseline blood ILC2 numbers negatively correlated with levels of reaction-associated urine 11 β -PGF₂ α ($R^2 = 0.559$, $P = .033$) and LTE₄ ($R^2 = 0.506$, $P = .048$). The magnitude of the decrease in blood ILC2 numbers did not directly correlate with the magnitude of the decrease in blood eosinophil numbers ($P = .536$) or increase in 11 β -PGF₂ α ($P = .327$) or LTE₄ ($P = .064$) levels. Thus, ILC2 numbers increase in the nasal mucosa and decrease in the blood during COX-1 inhibitor-induced reactions in patients with AERD.

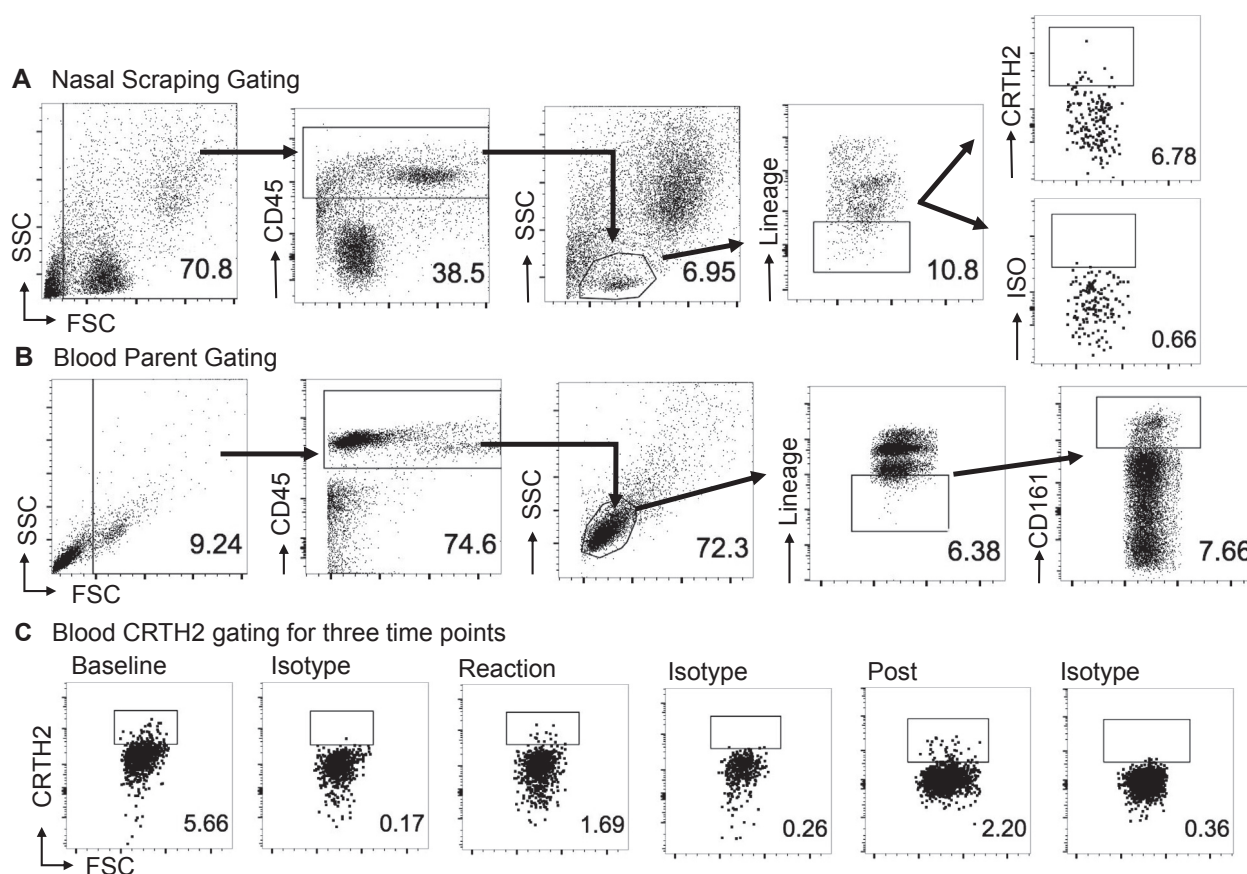


FIG 1. Gating strategy for nasal scrapings and blood ILC2s. The nasal scraping gating strategy was derived from polyp data (see Fig E3). **A**, Representative nasal sample ILC2 gating from a postdesensitization sample in a patient with AERD. ILC2s were identified as $CD45^{+}Lin^{-}$ lymphocytes that expressed CRTH2. **B**, Representative parent gating for blood ILC2s. Blood ILC2s were identified as $CD45^{+}Lin^{-}$ lymphocytes that expressed both $CD161^{+}$ and $CRTH2^{+}$. **C**, Representative data of blood CRTH2 plots of baseline, reaction, and postdesensitization samples along with their respective isotypes. FSC, Forward scatter; SSC, side scatter.

Nasal ILC2 numbers correlate with maximum symptom scores

Symptom scores (evaluating upper and lower airway symptoms, as noted in the Methods section) were assessed at regular intervals during desensitization. Baseline SNOT-22 scores positively correlated with maximum symptom scores during desensitization (Pearson correlation coefficient, 2-tailed $R^2 = 0.490$, $P = .036$; Fig 3, A). Baseline nasal scraping ILC2 levels were assigned a value of 1, and fold change was calculated from there. Interestingly, fold change from baseline to reaction in nasal ILC2 numbers positively correlated with maximum symptom scores ($R^2 = 0.472$, $P = .028$; Fig 3, B). The percentage of ILC2s in blood negatively correlated with the maximum symptom score, although this fell short of significance (Pearson correlation coefficient, 2-tailed $R^2 = 0.396$, $P = .051$). Baseline nasal scraping and blood ILC2 numbers did not correlate with maximum symptom score ($P = .95$ and $P = .21$, respectively).

ILC2 numbers in the blood at the time of the reaction negatively correlated with the time it took for the reaction to subside (Pearson correlation coefficient, 2-tailed $R^2 = 0.395$, $P = .038$; Fig 3, C). This might reflect that the more severe the reaction, the more ILC2s respond to stimuli to move out of the blood

and into the tissues. Baseline nasal scraping and blood ILC2 numbers did not correlate with time for reaction to subside ($P = .31$ and $.25$, respectively). In those patients with symptoms on the final day of desensitization (skin, gastrointestinal, or respiratory) who required treatment, the average number of ILC2s in the blood was lower than in those without symptoms (although this trend fell short of significance; 0.027% vs 0.11% ; $P = .061$, 2-tailed unpaired t test). Neither postdesensitization blood ILC2 nor eosinophil numbers correlated with time since reaction on day 1 or final dose of aspirin. ILC2 numbers on the last day did not differ if the patient received systemic corticosteroids during the reaction on day 1. We did not find a correlation between blood ILC2 numbers and the decrease in FEV₁ ($P = .52$). Reaction-associated nasal scraping or blood ILC2 numbers did not differ based on sex, age, atopic status, severity of asthma, use of nasal steroids, number of sinus surgeries, or time since last surgery.

DISCUSSION

Although intense investigation has recently characterized ILC2s in patients with asthma, allergic rhinitis, and nasal polyposis, there are no reports evaluating changes in ILC2

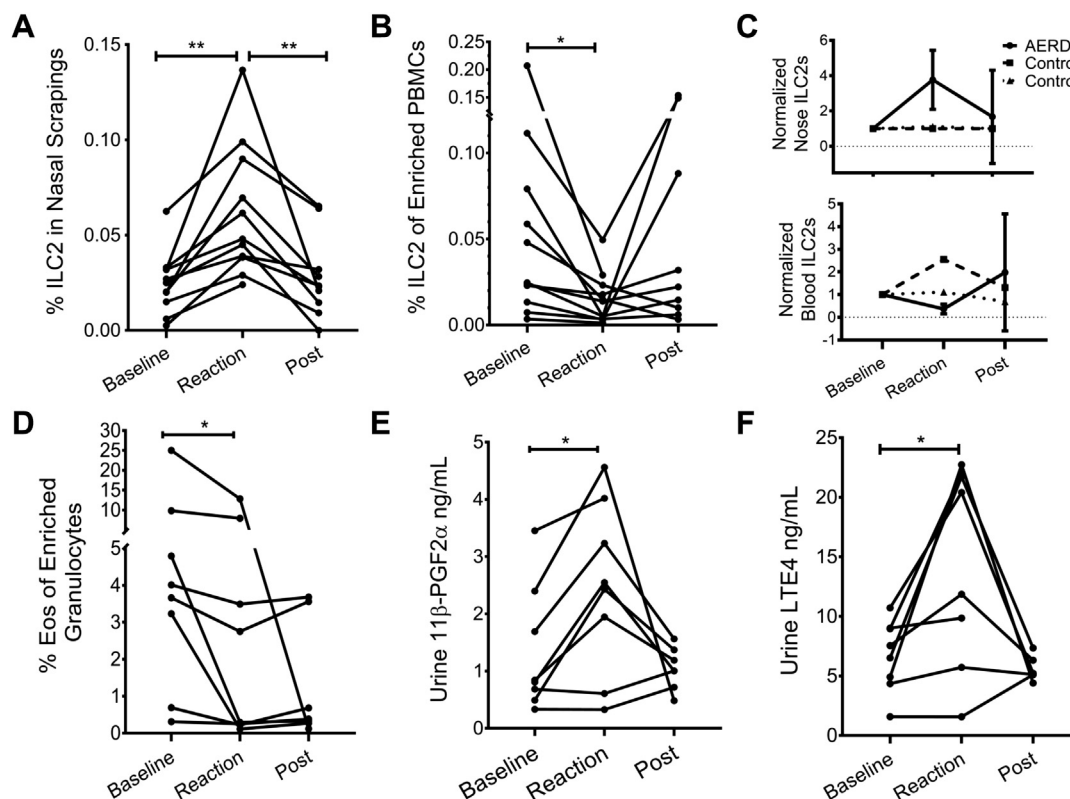


FIG 2. Changes in ILC2 and eosinophil numbers and urinary 11β -PGF $_2\alpha$ and LTE $_4$ levels during aspirin desensitization. **A**, Change in percentage of ILC2s as percentage of all cells in nasal scrapings before challenge, during reaction, and after desensitization was complete (*post*). Change between baseline and reaction samples was significant ($P = .001$, paired *t* test), as well as between reaction and postdesensitization samples ($P = .004$, paired *t* test). **B**, Change in percentage of ILC2s from enriched PBMCs before challenge, during reaction, and after desensitization was complete (*post*). Change between baseline and reaction samples was significant ($P = .031$, paired *t* test). **C**, Normalized values for nasal scraping and blood ILC2s at the 3 times points. *Solid line* is for patients with AERD, and *dotted lines* are for control subjects. **D**, Eosinophils as percentage of enriched granulocytes. Difference between baseline and reaction samples was significant ($P = .044$, paired *t* test). **E**, Change in levels of the urine PGD $_2$ metabolite 11β -PGF $_2\alpha$. Difference between baseline and reaction samples was significant ($P = .008$). **F**, Change in urine LTE $_4$ levels. Difference between baseline and reaction sample was significant ($P = .017$). * $P < .05$ and ** $P < .005$.

numbers in patients with AERD during NSAID reactions. Our novel findings demonstrate that ILC2s are recruited to the nasal mucosa during COX-1 inhibitor-induced reactions in patients with AERD and that the increase in nasal ILC2 numbers from baseline correlates with reaction symptom severity. Concomitantly, ILC2 numbers were reduced in peripheral blood along with eosinophil numbers during NSAID reactions and correlated inversely with duration of the NSAID reaction. Furthermore, levels of lipid mediators that could recruit and activate ILC2s in tissues, including LTE $_4$ and PGD $_2$ metabolite, were increased in urine during reactions.

ILC2s are activated by PGD $_2$ and capable of producing large amounts of type 2 cytokines, leading to tissue eosinophilia.^{10,13,20} Importantly, AERD is also associated with these characteristics, and we postulated that ILC2s might be active in patients with the disease. In this study we found that after ketorolac challenge-induced reactions in patients with AERD, blood ILC2 numbers decreased with a concurrent increase in nasal scraping ILC2 numbers. These changes were contemporaneous with an increase in levels of the stable PGD $_2$ metabolite 11β -PGF $_2\alpha$ and the terminal CysLT LTE $_4$. PGD $_2$ and LTE $_4$ levels are increased at

baseline in patients with AERD and increase after COX-1 inhibitor ingestion,^{5,25} and PGD $_2$ has been shown to promote chemotaxis in ILC2s.^{10,20} Notably, CysLTs have been shown to be chemotactic for T $_H$ 2 cells²⁶ and eosinophils²⁷ and could be contributing also to the ILC2 responses we observed. Numbers of blood ILC2s during reactions negatively correlated with the time it took for the reactions to subside, which could be due to ongoing ILC2 recruitment during more severe reactions, but this hypothesis will require further study.

Changes in nasal mucosal ILC2 numbers also correlated positively with severity of symptoms during reactions. Buchheit et al⁷ reported that increased nasal symptoms in patients with AERD correlated with peak levels of a PGD $_2$ metabolite, which could promote ILC2 recruitment and activation. These data also indicate that subjective patient symptoms, particularly nasocular symptoms, can be correlated with objective laboratory measurements of reaction intensity. Although nasal mucosal ILC2 numbers correlated with reaction severity in our studies, we were unable to show a correlation between change in ILC2 numbers and change in FEV $_1$. There might be several reasons for this finding. First, all of our patients were taking a leukotriene

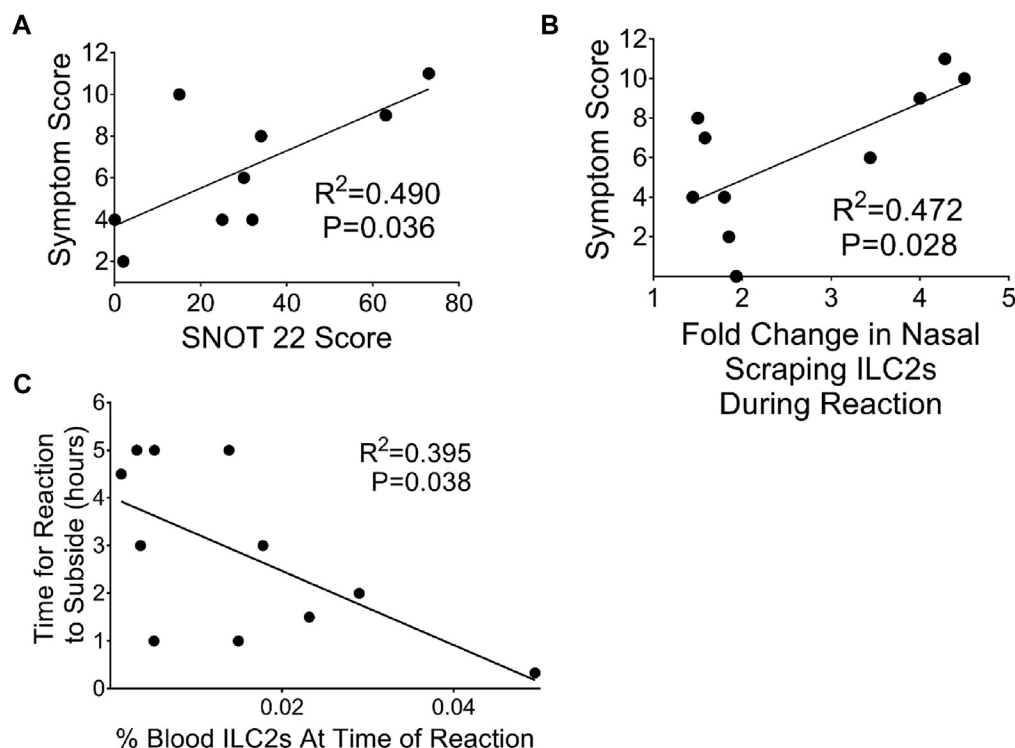


FIG 3. Change in ILC2 numbers as related to reaction characteristics. **A**, Significant correlation was seen between maximum symptom score during reaction and baseline SNOT-22 score (Pearson correlation coefficient, 2-tailed $R^2 = 0.490$, $P = .036$). **B**, Significant correlation was seen between maximum symptom score and fold increase in nasal scraping ILC2 numbers during the reaction (Pearson correlation coefficient, $R^2 = 0.472$, $P = .028$). **C**, Correlation between ILC2 numbers in the blood and time for reaction to subside (Pearson correlation coefficient, 2-tailed $R^2 = 0.395$, $P = .038$).

modifier for at least 1 week, which has been shown to reduce the drop in FEV₁ after aspirin challenge, leading to more upper airway-only reactions.²⁸ Second, all patients were first challenged/desensitized to nasal ketorolac, which also limits lower airway reactions.²² Finally, nasal recruitment of ILC2s might not be completely representative of recruitment and activation of lung ILC2s during AERD reactions.

At the end of the clinical desensitization, we did not observe a consistent pattern of changes in blood ILC2 or eosinophil numbers because values remained low in some patients, whereas others increased back to predesensitization levels. The difference in aspirin dose and protocol duration could contribute to some of this variability, although subgroup analysis with small numbers of patients continued to show a lack of consistent responses in postdesensitization ILC2 and eosinophil numbers. These individual differences did not correlate with systemic steroid use, which could have an effect on ILC2 numbers.¹⁹ Those patients who had symptoms on the last day had a trend toward lower ILC2 numbers in their blood, although this fell short of significance. The differences in postdesensitization levels might be due to ongoing mast cell activation, as noted by continued symptoms, and recruitment of ILC2s into the tissue. Indeed, one mechanism by which aspirin desensitization might work is by preventing cells from homing into the tissues.²⁹ PGD₂ levels are increased in mast cells from patients with AERD,⁷ and high doses of aspirin are able to block both COX-1 and COX-2, which might allow for prevention of high amounts of PGD₂. Cahill et al²⁹ showed that PGD₂ levels decrease after aspirin

desensitization and blood eosinophil numbers actually increase in the blood over time, presumably because of loss of the PGD₂ gradient. Whether there is also an increase in blood ILC2 numbers is not known.

Previous work has shown that AERD can be mediated by innate type 2 immunity. First, the potency of the COX-1 inhibition determines the severity of the reaction, which indicates that it is not adaptive recognition of a specific drug structure that activates the immune system.³⁰ Second, 2 danger signals released from epithelial cells, IL-33, an alarmin-like cytokine, and TSLP, an IL-7–like cytokine, have been shown to be upregulated in nasal polyps from patients with AERD and important drivers for COX-1 inhibitor-induced reactions.^{7,8,31} In a mouse model, blockage of IL-33 abrogated the change in airway resistance and cytokine release after aspirin-lysine challenge,⁸ which supports *in vitro* studies that showed IL-33 alone can induce human mast cell cytokine production.³² Dysregulation of the leukotriene pathway contributes to the pathogenesis of AERD,⁶ and IL-33 expression was found to be dependent on leukotrienes acting on epithelial cells. High PGD₂ levels are characteristic of AERD, and Buchheit et al⁷ showed that TSLP was able to induce PGD₂ generation *in vitro*. Interestingly, IL-33 and TSLP strongly activate ILC2s to produce cytokines capable of driving eosinophilia independently of adaptive immunity.^{11,12} ILC2s also express CysLT1R and generate cytokines in response to stimulation with CysLTs.⁹ Thus, although we have suggested that PGD₂ might be a key mediator for ILC2 influx into the nose, it is possible that IL-33, TSLP, and/or CysLTs are also contributing.

The role of ILC2s after recruitment into tissue is not clear, although the cytokines produced by ILC2s can promote several features of AERD, including eosinophilia and airway hyper-responsiveness.³³ Intensely eosinophilic mucosal tissues is one of the disease's hallmarks, and ILC2s produce abundant amounts of IL-5, which is the major promoter of eosinophils.³⁴ Furthermore, IL-9 is also produced by ILC2s, which contributes to mast cell accumulation and mucus production in animal asthma models.³⁵ Interestingly, both mast cells and eosinophils are an important source of PGD₂,³⁶ indicating a reciprocal relationship between ILC2s and these cell populations. Thus, ILC2s are a good candidate to support the prominent eosinophilic inflammation and mast cell activation seen in patients with AERD, especially given the lack of requirement for specific antigen.

We were able to replicate earlier data that showed a decrease in blood eosinophil numbers after aspirin challenge.^{6,7} Eosinophils have been studied considerably in patients with AERD. Eosinophils, which express CRTH2, respond to PGD₂ by undergoing chemotaxis that can be blocked with a CRTH2 antagonist.³⁷ Eosinophil numbers decrease in the blood of patients with AERD after aspirin challenge,^{6,7} presumably because they are migrating to the tissue in response to the high levels of PGD₂ released during these reactions. Indeed, eosinophil numbers have been shown to increase in nasal lavage fluid after nasal lysine-aspirin challenge in patients with AERD,³⁸ although in this previous study blood eosinophil numbers were not measured to show whether a corresponding decrease occurs. ILC2s also express CRTH2 and undergo chemotaxis in response to PGD₂ *in vitro*,¹⁰ and we hypothesized that they can migrate in response to the high levels of PGD₂ made during COX-1 inhibitor–provoked reactions.

We did not find a direct correlation between increases in ILC2 numbers in tissue and decreases in peripheral blood ILC2 or eosinophil numbers. This could be due to several factors. First, animal studies have shown that ILC2s are recruited from the bone marrow, and the kinetics of replenishment in human subjects is currently unknown.¹³ Furthermore, there might be many more ILC2s in the blood versus the nasal mucosa, and a small change in circulating ILC2 numbers could lead to a much larger change in the nose. Thus a direct inverse correlation might not have been detected because of disproportionate changes in both compartments (nose vs blood), replenishment from bone marrow, and recruitment to other tissues (lung, gastrointestinal tract, and skin) during reactions.

Although our findings are novel in connecting ILC2s to AERD, there are several limitations of our investigations. First, we did not perform functional studies of nasal ILC2s because of very limited cell numbers. Second, CRTH2 was used to identify nasal scraping ILC2s, which can be downregulated in the presence of PGD₂.^{7,10} However, CRTH2 has been shown to be the most specific marker for human ILC2s,³⁹ and we have not reliably found other surface markers, including IL-33 receptor, to be useful. Finally, all of the patients were taking leukotriene modifiers drugs, which have been shown to decrease the airway response seen after aspirin challenge.²⁸ Naso-ocular symptoms are not typically blocked by these drugs, but it is possible that these agents blunted ILC2 recruitment.

In summary, the patients with AERD we studied had increased ILC2 numbers in nasal scrapings and decreased numbers in blood during COX-1 inhibitor challenges, which also corresponded to increases in urinary 11 β -PGF₂ α levels and severity of symptoms.

Thus, ILC2s are recruited to the nasal mucosa in patients with AERD and could contribute to pathogenesis through production of T_H2 cytokines.

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Key messages

- ILC2 numbers increased in nasal mucosa and decreased in blood during COX-1 inhibitor reactions in patients with AERD.
- Eosinophil numbers also decreased in blood, and urinary PGD₂ metabolite and LTE₄ levels increased during AERD reactions.
- Nasal mucosa ILC2 numbers correlated positively with symptom scores.

REFERENCES

1. Rajan JP, Wineinger NE, Stevenson DD, White AA. Prevalence of aspirin-exacerbated respiratory disease among asthmatic patients: a meta-analysis of the literature. *J Allergy Clin Immunol* 2015;135:676-81.e1.
2. Szczeklik A, Nizankowska E, Duplaga M. Natural history of aspirin-induced asthma. AIANE Investigators. European Network on Aspirin-Induced Asthma. *Eur Respir J* 2000;16:432-6.
3. Mjosberg JM, Trifari S, Crellin NK, Peters CP, van Drunen CM, Piet B, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol* 2011;12:1055-62.
4. Stevens WW, Schleimer RP, Kern RC. Chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol Pract* 2016;4:565-72.
5. Bochenek G, Nagraba K, Nizankowska E, Szczeklik A. A controlled study of 9 α -ph₁₁beta-PGF₂ (a prostaglandin D₂ metabolite) in plasma and urine of patients with bronchial asthma and healthy controls after aspirin challenge. *J Allergy Clin Immunol* 2003;111:743-9.
6. Sladek K, Szczeklik A. Cysteinyl leukotrienes overproduction and mast cell activation in aspirin-provoked bronchospasm in asthma. *Eur Respir J* 1993;6:391-9.
7. Buchheit KM, Cahill KN, Katz HR, Murphy KC, Feng C, Lee-Sarwar K, et al. Thymic stromal lymphopoietin controls prostaglandin D₂ generation in patients with aspirin-exacerbated respiratory disease. *J Allergy Clin Immunol* 2016;137:1566-76.e5.
8. Liu T, Kanaoka Y, Barrett NA, Feng C, Garofalo D, Lai J, et al. Aspirin-exacerbated respiratory disease involves a cysteinyl leukotriene-driven IL-33-mediated mast cell activation pathway. *J Immunol* 2015;195:3537-45.
9. Doherty TA, Khorram N, Lund S, Mehta AK, Croft M, Broide DH. Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates TH2 cytokine production. *J Allergy Clin Immunol* 2013;132:205-13.
10. Xue L, Salimi M, Panse I, Mjosberg JM, McKenzie AN, Spits H, et al. Prostaglandin D₂ activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on TH2 cells. *J Allergy Clin Immunol* 2014;133:1184-94.
11. Bartemes KR, Iijima K, Kobayashi T, Kephart GM, McKenzie AN, Kita H. IL-33-responsive lineage- CD25+ CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. *J Immunol* 2012;188:1503-13.
12. Kim HY, Chang YJ, Subramanian S, Lee HH, Albacker LA, Matangkasombut P, et al. Innate lymphoid cells responding to IL-33 mediate airway hyperreactivity independently of adaptive immunity. *J Allergy Clin Immunol* 2012;129:216-27.e6.
13. Doherty TA. At the bench: understanding group 2 innate lymphoid cells in disease. *J Leukoc Biol* 2015;97:455-67.
14. Kim BS, Siracusa MC, Saenz SA, Noti M, Monticelli LA, Sonnenberg GF, et al. TSLP elicits IL-33-independent innate lymphoid cell responses to promote skin inflammation. *Sci Transl Med* 2013;5:170ra16.
15. Bartemes KR, Kephart GM, Fox SJ, Kita H. Enhanced innate type 2 immune response in peripheral blood from patients with asthma. *J Allergy Clin Immunol* 2014;134:671-8.e4.
16. Nagakumar P, Denney L, Fleming L, Bush A, Lloyd CM, Saglani S. Type 2 innate lymphoid cells in induced sputum from children with severe asthma. *J Allergy Clin Immunol* 2016;137:624-6.e6.

17. Doherty TA, Baum R, Newbury RO, Yang T, Dohil R, Aquino M, et al. Group 2 innate lymphocytes (ILC2) are enriched in active eosinophilic esophagitis. *J Allergy Clin Immunol* 2015;136:792-4.e3.
18. Lao-Araya M, Steveling E, Scadding GW, Durham SR, Shamji MH. Seasonal increases in peripheral innate lymphoid type 2 cells are inhibited by subcutaneous grass pollen immunotherapy. *J Allergy Clin Immunol* 2014;134:1193-5.e4.
19. Walford HH, Lund SJ, Baum RE, White AA, Bergeron CM, Husseman J, et al. Increased ILC2s in the eosinophilic nasal polyp endotype are associated with corticosteroid responsiveness. *Clin Immunol* 2014;155:126-35.
20. Chang JE, Doherty TA, Baum R, Broide D. Prostaglandin D2 regulates human type 2 innate lymphoid cell chemotaxis. *J Allergy Clin Immunol* 2014;133:899-901.e3.
21. Beasley RC, Featherstone RL, Church MK, Rafferty P, Varley JG, Harris A, et al. Effect of a thromboxane receptor antagonist on PGD₂- and allergen-induced bronchoconstriction. *J Appl Physiol* 1985;1989(66):1685-93.
22. Lee RU, White AA, Ding D, Dursun AB, Woessner KM, Simon RA, et al. Use of intranasal ketorolac and modified oral aspirin challenge for desensitization of aspirin-exacerbated respiratory disease. *Ann Allergy Asthma Immunol* 2010;105:130-5.
23. Miller RC, Brindle E, Holman DJ, Shofer J, Klein NA, Soules MR, et al. Comparison of specific gravity and creatinine for normalizing urinary reproductive hormone concentrations. *Clin Chem* 2004;50:924-32.
24. Bergmann KC, Zuberbier T, Church MK. Omalizumab in the treatment of aspirin-exacerbated respiratory disease. *J Allergy Clin Immunol Pract* 2015;3:459-60.
25. Higashi N, Taniguchi M, Mita H, Yamaguchi H, Ono E, Akiyama K. Aspirin-intolerant asthma (AIA) assessment using the urinary biomarkers, leukotriene E₄ (LTE₄) and prostaglandin D₂ (PGD₂) metabolites. *Allergol Int* 2012;61:393-403.
26. Parmentier CN, Fuerst E, McDonald J, Bowen H, Lee TH, Pease JE, et al. Human T(H)2 cells respond to cysteinyl leukotrienes through selective expression of cysteinyl leukotriene receptor 1. *J Allergy Clin Immunol* 2012;129:1136-42.
27. Gauvreau GM, Parameswaran KN, Watson RM, O'Byrne PM. Inhaled leukotriene E(4), but not leukotriene D(4), increased airway inflammatory cells in subjects with atopic asthma. *Am J Respir Crit Care Med* 2001;164:1495-500.
28. Stevenson DD, Simon RA, Mathison DA, Christiansen SC. Montelukast is only partially effective in inhibiting aspirin responses in aspirin-sensitive asthmatics. *Ann Allergy Asthma Immunol* 2000;85:477-82.
29. Cahill KN, Bensko JC, Boyce JA, Laidlaw TM. Prostaglandin D(2): a dominant mediator of aspirin-exacerbated respiratory disease. *J Allergy Clin Immunol* 2015;135:245-52.
30. Szczeklik A, Gryglewski RJ, Czerniawska-Mysik G, Zmuda A. Aspirin-induced asthma. Hypersensitivity to fenoprofen and ibuprofen in relation to their inhibitory action on prostaglandin generation by different microsomal enzymic preparations. *J Allergy Clin Immunol* 1976;58:10-8.
31. Nagarkar DR, Poposki JA, Tan BK, Comeau MR, Peters AT, Hulse KE, et al. Thymic stromal lymphopoietin activity is increased in nasal polyps of patients with chronic rhinosinusitis. *J Allergy Clin Immunol* 2013;132:593-600.e12.
32. Allakhverdi Z, Smith DE, Comeau MR, Delespesse G. Cutting edge: the ST2 ligand IL-33 potently activates and drives maturation of human mast cells. *J Immunol* 2007;179:2051-4.
33. Chang YJ, Kim HY, Albacker LA, Baumgarth N, McKenzie AN, Smith DE, et al. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat Immunol* 2011;12:631-8.
34. Holgate ST. Innate and adaptive immune responses in asthma. *Nat Med* 2012;18:673-83.
35. Soroosh P, Doherty TA. Th9 and allergic disease. *Immunology* 2009;127:450-8.
36. Feng X, Ramsden MK, Negri J, Baker MG, Payne SC, Borish L, et al. Eosinophil production of prostaglandin D2 in patients with aspirin-exacerbated respiratory disease. *J Allergy Clin Immunol* 2016;138:1089-97.e3.
37. Hirai H, Tanaka K, Takano S, Ichimasa M, Nakamura M, Nagata K. Cutting edge: agonistic effect of indomethacin on a prostaglandin D2 receptor, CRTH2. *J Immunol* 2002;168:981-5.
38. Kupczyk M, Kurmanowska Z, Kuprys-Lipinska I, Bochenska-Marciniak M, Kuna P. Mediators of inflammation in nasal lavage from aspirin intolerant patients after aspirin challenge. *Respir Med* 2010;104:1404-9.
39. Bal SM, Bernink JH, Nagasawa M, Groot J, Shikhagaie MM, Golebski K, et al. IL-1beta, IL-4 and IL-12 control the fate of group 2 innate lymphoid cells in human airway inflammation in the lungs. *Nat Immunol* 2016;17:636-45.

Scale

0= no symptom

1= mild, barely noticeable symptoms

2= moderate, somewhat troublesome symptoms

3= symptoms are much worse than you generally experience or are making you very uncomfortable

Symptoms Assessed

Nasal congestion

Runny nose

Sneezing

Itchy nose

Itchy/watery eyes

Tight throat

Wheezing

Chest tightness

FIG E1. Modified AERD symptom score.

Scripps Clinic*Day 1*

Ketorolac Challenge

- 1.26 mg ketorolac, 1 spray in 1 nostril. Wait 30 minutes.
- 2.53 mg ketorolac, 1 spray in each nostril. Wait 30 minutes.
- 5.05 mg ketorolac, 2 sprays in each nostril. Wait 30 minutes.
- 7.58 mg ketorolac, 3 sprays in each nostril. Wait 60 minutes.

Aspirin Challenge/Desensitization

- Aspirin 81 mg. Wait 90 minutes.
- Aspirin 81 mg. Wait 90 minutes. If stable, discharge for day.

Day 2

- Aspirin 325 mg. Wait 3 hours. End of desensitization.

UCSD*Day 1*

Ketorolac Challenge

- 1.26 mg ketorolac, 1 spray in 1 nostril. Wait 30 minutes.
- 2.53 mg ketorolac, 1 spray in each nostril. Wait 30 minutes.
- 5.05 mg ketorolac, 2 sprays in each nostril. Wait 30 minutes.
- 7.58 mg ketorolac, 3 sprays in each nostril. Wait 60 minutes.

Aspirin Challenge/Desensitization

- Aspirin 40.5 mg. Wait 90 minutes.
- Aspirin 40.5 mg. Wait 90 minutes. If stable, discharge for day.

Day 2

- Aspirin 81 mg. Wait 3 hours.
- Aspirin 162 mg. Wait 3 hours
- Aspirin 325 mg. Wait 3 hours. If stable, discharge for day

Day 3

- Aspirin 650 mg. Wait 3 hours. End of desensitization.

FIG E2. NSAID challenge and desensitization protocols. *UCSD*, University of California, San Diego.

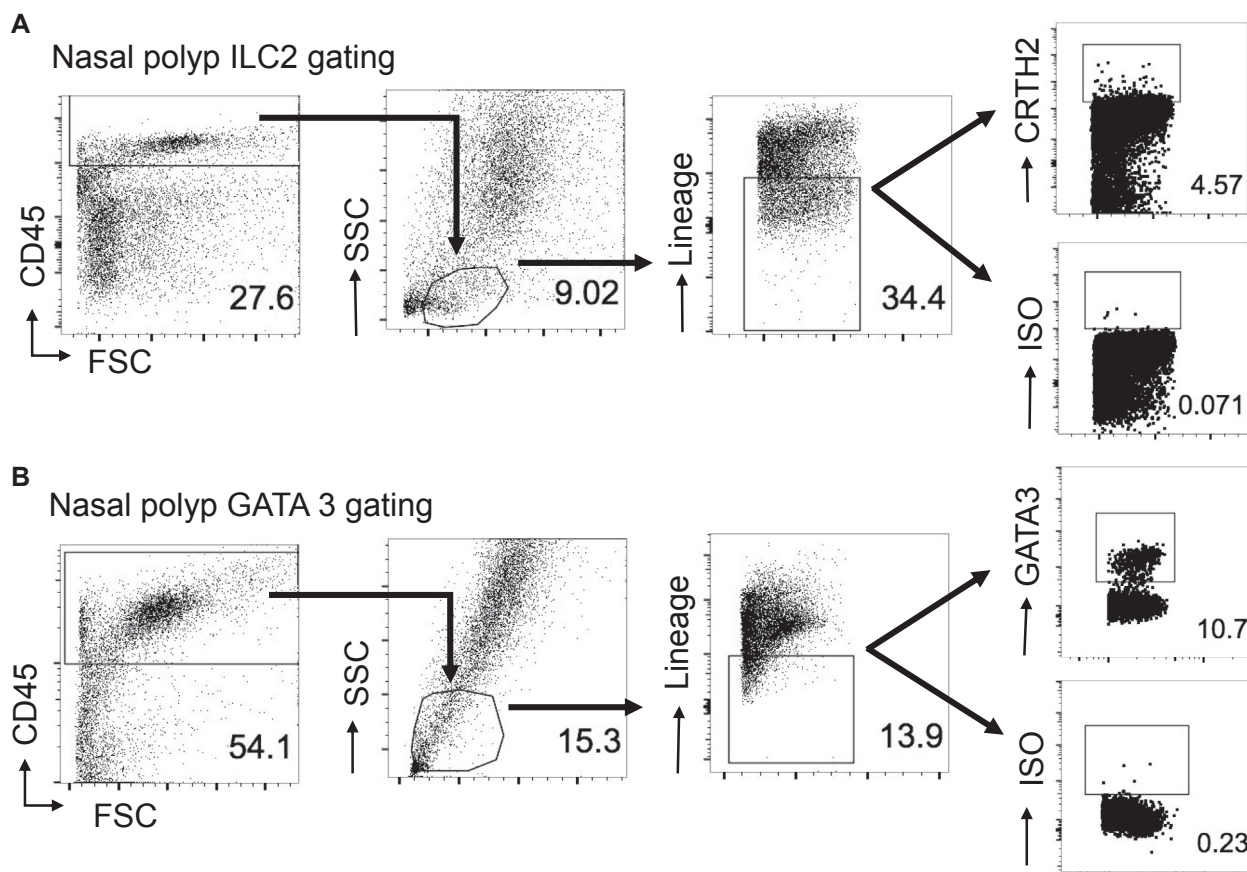


FIG E3. Gating strategy for nasal scrapings and blood ILC2s derived from nasal polyps. Representative gating from a surgically removed nasal polyp from a patient who subsequently underwent aspirin desensitization. Nasal polyp ILC2s were identified as CD45⁺ lymphocytes that are Lin⁻ and express either CRTH2 (**A**) or GATA-3 (**B**). FSC, Forward scatter; SSC, side scatter.