

Thymic stromal lymphopoietin controls prostaglandin D₂ generation in patients with aspirin-exacerbated respiratory disease



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Background: Prostaglandin (PG) D₂ is the dominant COX product of mast cells and is an effector of aspirin-induced respiratory reactions in patients with aspirin-exacerbated respiratory disease (AERD).

Objective: We evaluated the role of the innate cytokine thymic stromal lymphopoietin (TSLP) acting on mast cells to generate PGD₂ and facilitate tissue eosinophilia and nasal polyposis in patients with AERD.

Methods: Urinary eicosanoid levels were measured in aspirin-tolerant control subjects and patients with AERD. Nasal polyp specimens from patients with AERD and chronic rhinosinusitis were analyzed by using quantitative PCR, Western blotting, and immunohistochemistry. Human cord blood- and peripheral blood-derived mast cells were stimulated with TSLP *in vitro* to assess PGD₂ generation.

Results: Urinary levels of a stable PGD₂ metabolite (uPGD-M) were 2-fold higher in patients with AERD relative to those in control subjects and increased further during aspirin-induced reactions. Peak uPGD-M levels during aspirin reactions correlated with reductions in blood eosinophil counts and lung function and

increases in nasal congestion. Mast cells sorted from nasal polyps expressed PGD₂ synthase (hematopoietic PGD₂ synthase) mRNA at higher levels than did eosinophils from the same tissue. Whole nasal polyp *TSLP* mRNA expression correlated strongly with mRNA encoding hematopoietic PGD₂ synthase ($r = .75$), the mast cell-specific marker carboxypeptidase A3 ($r = .74$), and uPGD-M ($r = 0.74$). Levels of the cleaved active form of TSLP were increased in nasal polyps from patients with AERD relative to those in aspirin-tolerant control subjects. Recombinant TSLP induced PGD₂ generation by cultured human mast cells.

Conclusions: Our study demonstrates that mast cell-derived PGD₂ is a major effector of type 2 immune responses driven by TSLP and suggests that dysregulation of this innate system contributes significantly to the pathophysiology of AERD. (J Allergy Clin Immunol 2016;137:1566-76.)

Key words: Aspirin-exacerbated respiratory disease, Samter triad, nasal polyps, thymic stromal lymphopoietin, prostaglandin D₂, cysteinyl leukotrienes, innate immunity, mast cells, eosinophils

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Supported by National Institutes of Health grants T32 AI007306-29, AI007306-27, U19 AI095219-01, 5U19AI070412-06, and K23HL111113-02 and by generous contributions from the Vinik Family and the Kaye Family.

Disclosure of potential conflict of interest: K. M. Buchheit, H. R. Katz, K. C. Murphy, and C. Feng have received grants from the National Institutes of Health (NIH). K. N. Cahill has received a grant and provision of writing assistance, medicine, equipment, or administrative support from the NIH; has received money for her institution from the Vinik and Kaye families; and is employed by Knopp Biosciences. N. Bhattacharyya has consultant arrangements with Sanofi, IntersectENT, and Entellus. E. Israel has consultant arrangements with AstraZeneca, Novartis, Philips Respironics, Regeneron Pharmaceuticals; is on the speakers' bureau for Merck; and provided expert testimony for Campbell, Campbell, Edwards & Conroy, Crammer, Bishop, and O'Brien, Ficksman & Conley, Fox Rothschild, and Ryan Deluca; has received travel/meeting expenses from Research in Real Life (RiRL) and TEVA Specialty Pharmaceuticals; has received royalties from UpToDate; and has received grants from Genentech, Boehringer Ingelheim, GlaxoSmithKline, Merck, Sunovion, and TEVA. J. A. Boyce has received grants from the NIH/National Institute of Allergy and Infectious Diseases; is a board member for the American Board of Allergy and Immunology; and has consultant arrangements with Amnylam Pharmaceuticals. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication May 19, 2015; revised September 22, 2015; accepted for publication October 5, 2015.

Available online December 12, 2015.

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0091-6749/\$36.00

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<http://dx.doi.org/10.1016/j.jaci.2015.10.020>

Eosinophil-rich tissue pathology is an important feature of immune defense against helminths¹ and is also a typical histologic finding in human diseases, such as asthma,² chronic rhinosinusitis (CRS),³ and certain gastrointestinal disorders.⁴ Although such pathology can reflect the effector arm of adaptive immune responses involving T_H2 cells and allergen-specific IgE, a complementary pathway mediated by the innate immune system can drive similar pathology, either alone or as an amplifier of adaptive type 2 responses. This innate type 2 immune pathway is initiated by cytokines, such as thymic stromal lymphopoietin (TSLP),⁵ IL-33,⁶ and IL-25,⁷ which derive largely from epithelial and other barrier cells that are disturbed by microbes or toxins. TSLP is an IL-7-like cytokine thought to be important in a number of human diseases, including asthma,⁸ atopic dermatitis,⁹ and nasal polyposis,¹⁰⁻¹² and polymorphic variants of *TSLP* and TSLP receptor (*TSLPR*) are risk alleles for asthma and other diseases.¹³ TSLP induces type 2 cytokine generation by mast cells,¹⁴ type 2 innate lymphoid cells (ILC2s),^{15,16} and CD34⁺ hematopoietic progenitor cells¹⁷ and can activate eosinophils¹⁸ and basophils.¹⁹ An mAb against TSLP showed promise in a proof-of-concept study in atopic asthmatic patients.⁵ To date, however, there has been no direct demonstration of a mechanism by which this system contributes to tissue inflammation and pathology in human subjects.

Prostaglandin (PG) D₂, the dominant COX pathway product of mast cells, was originally recognized as a bronchoconstrictor²⁰ and vasodilator²¹ when directly administered to human subjects. Subsequently, PGD₂ was identified as the preferred ligand for chemoattractant receptor homologue expressed by T_H2 cells

Abbreviations used

AERD:	Aspirin-exacerbated respiratory disease
ATA:	Aspirin-tolerant asthma
CPA3:	Carboxypeptidase A3
CRS:	Chronic rhinosinusitis
CRTH2:	Chemoattractant receptor homologue expressed by T _H 2 cells
EpCAM:	Epithelial cell adhesion molecule
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
hPGDS:	Hematopoietic PGD ₂ synthase
ILC2:	Type 2 innate lymphoid cell
5-LO:	5-Lipoxygenase
LT:	Leukotriene
NSAID:	Nonsteroidal anti-inflammatory drug
PG:	Prostaglandin
PGD-M:	9 α ,11 β -Dihydroxy-15-oxo-2,3,18,19-tetranorprost-5-ene-1,20-dioic acid
SCF:	Stem cell factor
TSLP:	Thymic stromal lymphopoietin
TX:	Thromboxane
uPGD-M:	Urinary prostaglandin D metabolite

(CRTH2), a G protein–coupled receptor expressed by human type 2 cells, eosinophils, and basophils.^{22,23} PGD₂ acts at CRTH2 to induce chemotaxis of these cell types *in vitro*²³ and *in vivo* in mouse models of allergen-induced pulmonary and cutaneous inflammation.^{24,25} Recently, human ILC2s were found to express high CRTH2 levels,²⁶ and PGD₂ was found to potently induce cytokine generation by ILC2s.²⁷ These studies suggest an important role for PGD₂ and potentially for mast cells in the innate type 2 pathway that drives tissue eosinophilia. Whether PGD₂ and mast cells are integrated with TSLP in innate type 2 immunity is unknown.

Nasal polyps are outgrowths of inflamed sinonasal mucosa that occur in patients with CRS and often require surgical excision. They are densely infiltrated by eosinophils, contain activated mast cells and relatively large numbers of ILC2s,^{26,28,29} and have increased TSLP expression and activity compared with healthy nasal tissue.^{10–12} They frequently arise in subjects with no evidence of IgE-dependent allergic sensitization.³⁰ As such, nasal polyps reflect part of the spectrum of tissue pathology induced by the innate type 2 pathway. Nasal polyps are especially aggressive and recurrent in patients with aspirin-exacerbated respiratory disease (AERD), a distinctive and severe adult-onset respiratory syndrome. AERD is associated with eosinophilic asthma³¹ and ongoing activation of mast cells. It is defined by pathognomonic, non–IgE-mediated respiratory reactions on ingestion of aspirin and other drugs that inhibit COX-1. Mast cell activation is a typical feature of these clinical reactions, with systemic release of multiple mediators, including tryptase and PGD₂.³²

We recently demonstrated that patients with AERD who generated the highest baseline levels of PGD₂, as determined by measurement of a stable metabolite of PGD₂ (9 α ,11 β -dihydroxy-15-oxo-2,3,18,19-tetranorprost-5-ene-1,20-dioic acid [PGD-M]) in urine, experienced the most severe clinical reactions.³³ In the current study we sought to identify factors that could influence the synthesis of PGD₂ in patients with AERD. We tested the hypothesis that TSLP might contribute to PGD₂ generation by mast cells and that PGD₂ might, in turn, be an effector of the capacity for TSLP to drive type 2 pathology.

METHODS

Patient characterization

Patients between the ages of 18 and 70 years were recruited at the Brigham and Women's Hospital (Boston, Massachusetts) Allergy and Immunology clinic and Asthma Research Center between April 2011 and June 2015 and classified according to their clinical characteristics. Nonasthmatic control subjects had no history of asthma or intolerance to aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs). Control subjects with aspirin-tolerant asthma (ATA) had physician-diagnosed persistent asthma and had taken aspirin or an NSAID within the previous 6 months without adverse reactions. Patients were suspected of having AERD if they had asthma, nasal polyposis, and a history of respiratory reaction on ingestion of a COX inhibitor. In all subjects with that compatible clinical history, the diagnosis of AERD was then confirmed with a graded oral challenge to aspirin that resulted in characteristic sinonasal symptoms and/or a decrease in FEV₁ of at least 15%. Patients were excluded from the study if they smoked cigarettes or were pregnant. All patients with AERD were treated with the cysteinyl leukotriene (cysLT) 1 receptor blocker montelukast during aspirin challenge, and none were receiving the 5-lipoxygenase (5-LO) inhibitor zileuton before the challenge.

For the nasal polyp and nasal tissue studies, patients were recruited and tissue was collected at the time of elective endoscopic sinus surgery from patients with AERD or from aspirin-tolerant control subjects with CRS with and without nasal polyps. Details of the subjects' characteristics, including age, sex, and asthma severity, are included in [Table 1](#).

The institutional human subjects institutional review board approved the study, and all subjects provided written informed consent.

Aspirin challenge protocol

Aspirin challenges were performed while patients were not receiving the 5-LO inhibitor zileuton so that cysLT production could be monitored. Patients took their regularly prescribed inhaled corticosteroids with or without long-acting β -agonists the morning of challenge, as applicable. All subjects received montelukast (10 mg) the evening before aspirin challenge to attenuate the severity of respiratory symptoms during the reaction. All subjects underwent the challenge protocol in one of our outpatient clinics or in the Asthma Research Center at the Brigham and Women's Hospital. Oral aspirin challenges started with 40 mg of aspirin, followed by dose increases (81, 162, and 325 mg) every 90 minutes. Patients were observed for respiratory symptoms, ocular injection, flushing, rash, and abdominal pain. The aspirin dose that caused upper and/or lower respiratory symptoms was recorded as the provocative dose. FEV₁ for each patient was recorded at baseline, before each dose, and at the time of reaction. Total Nasal Symptom Score questionnaires were recorded by patients at baseline, and again every 30 minutes for 3 hours after the onset of reactions. Demographic and clinical data were extracted from the medical record at the time of challenge.

Urinary eicosanoid measurements

For patients with AERD who underwent aspirin challenge, urine was collected at baseline (before aspirin administration), at the onset of symptoms of an aspirin-induced reaction, and again 90 and 180 minutes after the onset of the reaction. Baseline urine samples were collected from subjects with ATA who had been off NSAIDs for at least 1 week. Urine samples were stored at –80°C and analyzed by using gas chromatography–mass spectrometry at Vanderbilt University. As described previously, concentrations of the major urinary PGD₂ metabolite PGD-M were measured and reported as nanograms per milligram of creatinine.³⁴

Peripheral blood leukocyte counts

Blood was collected for complete blood counts (LabCorp, Burlington, NC) from patients with AERD at baseline (before aspirin administration) and again 60 minutes after onset of their aspirin-induced reactions.

TABLE I. Patients' characteristics

	Patients with AERD	Patients with ATA	Healthy control subjects (for urine studies in Fig 1, A)	Subjects with CRS
No.	52	7	9	20
Sex (male/female)	21/31	1/6	2/7	11/9
Median age (range [y])	47 (28-70)	27 (20-56)*	35 (25-56)†	44 (20-67)
Asthma (%)	100	100	0	15
Baseline FEV ₁ (% predicted), mean ± SD	89 ± 15	84 ± 9	96 ± 8	Unavailable
Baseline ACQ7 score, mean ± SD	0.60 ± 0.66	0.52 ± 0.21	NA	Unavailable
Receiving daily inhaled corticosteroids (no.)	50/52	6/7	0/9	2/3

ACQ7, Seven-item Asthma Control Questionnaire.

**P* = .023 for ages of patients with ATA compared with ages of patients with AERD.

†*P* = .036 for ages of healthy control subjects compared with ages of patients with AERD.

CRTH2 surface expression measurement

Whole peripheral blood was collected in heparinized tubes, kept at room temperature, and assayed within 1 hour of collection. Forty microliters of unstimulated blood was incubated for 10 minutes with mAbs specific for CD45 (BD Biosciences, San Jose, Calif), CCR3 (BioLegend, San Diego, Calif), and CRTH2/CD294 (BD Biosciences) or appropriate isotype controls and fixed in 1% paraformaldehyde. At least 1000 CCR3⁺ cells were recorded for each sample on a BD FACSCanto Flow Cytometer and analyzed with FlowJo Version v.X.0.7 (TreeStar, Ashland, Ore). Eosinophils were identified as CCR3⁺CD45⁺ cells within the granulocyte scatter gate and were assayed for the presence of CRTH2.

Polyp procurement and tissue specimen preparation

Nasal tissue was excised at the time of surgery; one tissue segment was immediately preserved in RNAlater (Qiagen, Valencia, Calif) for RNA extraction, and the remaining tissue was placed in RPMI with 1 U/mL penicillin-streptomycin for transport to the laboratory. Within 2 hours of surgery, the tissue was removed from RPMI and divided into segments. One segment was transferred into CellLytic 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 was fixed in 4% paraformaldehyde, embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, Calif), and kept at -80°C until sectioning. Sections of 10-μm thickness were freshly cut, thaw mounted onto slides, fixed in 4% paraformaldehyde, and stored at -80°C until stained.

For some patients, a tissue segment was also placed in media containing 10% FBS and chopped with a straight razor blade and then digested with 400 U/mL Type IV collagenase (Worthington Biochemical Corporation, Lakewood, NJ) and 200 μg/mL DNase (Sigma-Aldrich). The resulting suspension was passed through a 70-μm filter to retrieve a single-cell suspension for flow cytometric sorting. These cells were stained with mAbs against CD45, CD117, CD90, epithelial cell adhesion molecule (EpCAM), platelet-endothelial cell adhesion molecule (CD31), CCR3 (BD Biosciences), and FcεRI (BioLegend) and sorted into purified cell populations with a BD FACSAria Fusion Cell Sorter to collect tissue mast cells (CD45⁺CD117^{hi}FcεRI⁺), eosinophils (CD45⁺CCR3⁺ within the granulocyte forward and side scatter gate), fibroblasts (CD45⁻EpCAM⁻CD31⁻CD90⁺), epithelial cells (CD45⁻EpCAM⁺CD31⁻CD90⁻), and endothelial cells (CD45⁻EpCAM⁻CD31⁺CD90⁻) separately. Purified mast cell populations were confirmed by means of toluidine blue staining, and purified eosinophil populations were confirmed with staining comparable with the Wright-Giemsa method (Fisher Scientific, Waltham, Mass). Cells were sorted into RNeasy Protect (Qiagen) and stored at -20°C until RNA was extracted. Generally, between 3 and 50 × 10³ mast cells were obtained per specimen.

Quantitative PCR

RNA was extracted from the whole nasal tissue specimens with Tri Reagent (Qiagen) and converted to cDNA by using the RT² First Strand Kit (Qiagen).

Expression of *TSLP*, *COX-2* (*PTGS2*), carboxypeptidase A3 (*CPA3*), hematopoietic PGD₂ synthase (*HPGDS*), 5-LO (*ALOX5*), and leukotriene (LT) C₄ (*LTC₄S*) synthase transcripts was examined by using RT² SYBR Green qPCR Master Mix (Qiagen).

RNA was extracted from sorted cells with the RNeasy Micro Kit (Qiagen) and converted to cDNA as above. Expression of *HPGDS*, *COX-1* (*PTGS1*), *COX-2* (*PTGS2*), and TSLP receptor (*TSLPR*) transcripts were examined in mast cells and eosinophils, and *TSLP* expression was measured in epithelial cells, endothelial cells, and fibroblasts, as described above. The identities of the sorted cells were further verified by monitoring mRNA-encoding eosinophil peroxidase in eosinophils, EpCAM in epithelial cells, CD31 in endothelial cells, and collagen in fibroblasts. Expression levels of transcripts were normalized to expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*); all primers from Qiagen.

Western blot analysis

Protein-extracted supernatants were used to generate gels and then transferred onto Immobilon-P Membranes (Bio-Rad Laboratories, Hercules, Calif) and blocked with 5% milk in tris(hydroxymethyl)aminomethane-buffered saline to measure TSLP protein in the nasal polyp tissue. Blots were incubated with primary rabbit polyclonal anti-TSLP (catalog no. ABT330; Millipore, Danvers, Mass) or anti-GAPDH (Cell Signaling Technology, Danvers, Mass) antibodies, washed, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich) and visualized by means of enhanced chemiluminescence (GE HealthCare, Pittsburgh, Pa). The molecular weight of TSLP was determined by loading one lane of each gel with 10 μL of SDS-PAGE Molecular Weight Standards, Broad Range (Bio-Rad Laboratories) and comparing the distance migrated by the protein with the distance migrated by the dye. TSLP protein was quantified by means of densitometry and expressed relative to GAPDH. A blot of 4 nasal polyp samples was probed with a new additional polyclonal anti-TSLP antibody (NB110-55234; Novus Biologicals, Littleton, Colo), which was raised against a known 19-amino-acid TSLP peptide, to confirm that the staining of TSLP was specific. That blot was then stripped of bound antibody and incubated for 1 hour with 1 μL/mL of the neutralizing/blocking peptide NB110-55234PEP (Novus Biologicals), which was used as an immunogen for NB110-55234. The blocked blot was then reprobbed with the NB110-55234 antibody to show the disappearance of now blocked protein. The same blot was reincubated with the original anti-TSLP antibody ABT330 to verify that the original antibody used in our studies also recognized the same 10- and 15-kDa TSLP bands.

Immunohistochemistry

Frozen sections were prepared from nasal polyps, incubated with the mouse anti-human tryptase mAb AA1 (Dako, Carpinteria, Calif) or isotype control, and developed with the EnVision + System-HRP for mouse primary antibodies (Dako). Sections were counterstained with hematoxylin. For quantification of mast cells, numbers of tryptase-positive cells in photomicrographs encompassing approximately 1 mm² of subepithelial tissue (quantified with Image J software) were counted and expressed per square millimeter.

Mast cell culture and stimulation

Human cord blood mast cells were derived from CD34⁺ progenitor cells and cultured for 6 to 8 weeks in RPMI supplemented with stem cell factor (SCF), IL-6, and IL-10, as previously described.³⁵ Mast cell purity was confirmed with toluidine blue staining. Cells (10⁵) were incubated for 6 and 24 hours in the presence of TSLP (10 ng/mL; PeproTech, Rocky Hill, NJ) with SCF at 100 ng/mL. Human peripheral blood mast cells were derived from CD34⁺ progenitor cells and cultured for 5 weeks, as previously described,³⁶ except for use of StemSpan Serum-Free Media (Stem Cell Technologies, Vancouver, British Columbia, Canada) instead of charcoal-filtered FCS and with the addition of the retinoic acid receptor antagonist LE540 at 1 μmol/L (Wako Chemicals, Richmond, Va) and human LDL at 10 μg/mL (Stem Cell Technologies), as suggested by Karl Nocka (Pfizer Pharmaceuticals, New York, NY). Mast cell purity was confirmed with toluidine blue staining. Cells (10⁵) were incubated for 6 hours in the presence of 10 ng/mL SCF and 10 ng/mL TSLP, 10 ng/mL IL-33 (PeproTech, Rocky Hill, NJ), or both TSLP and IL-33 at 10 ng/mL.

The cell supernatants were collected, and a PGD₂ ELISA (Cayman, Ann Arbor, Mich) was performed according to the manufacturer's instructions, with the assay performed in duplicate. Cysteinyl LT and thromboxane (TX) B₂ ELISAs (Cayman) were also performed on the cord blood mast cell supernatants, according to the manufacturer's instructions, with the assay performed in duplicate.

Statistical analysis

Data are presented as means plus SEMs, unless otherwise stated. Differences in values in normally distributed data were analyzed with the paired or unpaired *t* test, as appropriate, with significance defined as a *P* value of less than .05, and all tests were 2-tailed. Nonnormally distributed data were analyzed with the Mann-Whitney test, with significance defined as a *P* value of less than .05. Linear dependence was measured with the Pearson correlation coefficient.

RESULTS

Study populations and baseline demographics

The patients with AERD and those with CRS were close in age, with the patients with ATA and healthy control subjects being significantly younger. The baseline FEV₁ and Asthma Control Questionnaire scores were similar between the patients with AERD and those with ATA. All patients with AERD and ATA had physician-diagnosed asthma, and 15% of patients with CRS had a known diagnosis of asthma (Table I). Because the patients with CRS were recruited at the time of surgery, not all clinical data were available, and some asthma diagnoses might not have been captured. Baseline urinary prostaglandin D metabolite (uPGD-M) levels did not correlate significantly with subjects' age (*r* = 0.27) or baseline FEV₁ (*r* = -0.28).

PGD₂ production directly mediates effector responses in patients with AERD

Aspirin challenges in patients with AERD result in respiratory tract mast cell activation through IgE-independent mechanisms,³⁰ presumably reflecting depletion of homeostatic PGE₂. To identify the contributions of PGD₂ to the end-organ effects of this activation, we monitored uPGD-M levels and their relationship to clinical and physiologic responses to aspirin challenge. Urine obtained from patients with AERD before aspirin challenge contained approximately 2-fold more uPGD-M than did that obtained from patients with ATA and healthy control subjects (Fig 1, A). All patients with AERD reacted to 162 mg of aspirin or less. There was no significant correlation between the

provocative aspirin dose and peak uPGD-M levels, and there was no relationship between asthma severity (determined by FEV₁ and Asthma Control Questionnaire scores) and uPGD-M levels at baseline (data not shown). Cytofluorographic analysis revealed that eosinophils in the blood of patients with AERD expressed substantially lower levels of surface CRTH2 than did eosinophils in the blood of control subjects with ATA (Fig 1, B). Eosinophil CRTH2 expression inversely correlated with uPGD-M levels at baseline in patients with ATA and AERD (*r* = -0.79; Fig 1, C). After aspirin ingestion, all patients with AERD had symptoms of sinonasal congestion, sneezing, and ocular pruritus, and many also had chest tightness, wheezing, and a decrease in FEV₁. During aspirin-induced reactions, uPGD-M levels increased in a time-dependent manner in all patients with AERD (Fig 1, D). Peak levels of uPGD-M correlated strongly with reductions in blood eosinophil counts (*r* = -0.95; Fig 1, E), as well as with decreases in FEV₁ (*r* = -0.59; Fig 1, F) and increases in nasal symptom scores (*r* = 0.64; Fig 1, G).

Mast cells are the primary site of hPGDS expression in nasal polyps

To determine the likely source of PGD₂ in respiratory tissue, we performed cytofluorographic, immunohistochemical, and transcript analyses of nasal polyps that were surgically excised from patients with AERD and control subjects with CRS.

By using flow cytometry, mast cells, defined as CD45⁺CD117^{hi}FcεRI⁺ cells within the granulocyte scatter gate, and eosinophils, defined as CD45⁺CCR3⁺ cells (Fig 2, A and B), were sorted into purified cell populations. Mast cell purity was confirmed with toluidine blue staining (Fig 2, C), and eosinophil purity was confirmed with staining comparable with the Wright-Giemsa method (Fig 2, D). Levels of *HPGDS* mRNA expressed by the polyp mast cells were 10- to 3000-fold greater than those expressed by eosinophils obtained from the same samples (*P* < .0001; Fig 2, E), with similar trends observed in samples from both patients with AERD and subjects with CRS. COX-2 (*PTGS2*) mRNA expression by mast cells and eosinophils varied between subjects, but the 2 cell types expressed similar amounts of COX-1 (*PTGS1*) mRNA (see Fig E1 in this article's Online Repository at www.jacionline.org),³⁷ and mast cells expressed significantly higher levels of COX-2 than COX-1.

Immunostaining for tryptase revealed no difference in numbers of mast cells in patients with AERD versus subjects with CRS (Fig 3, A). Flow cytometry of dispersed nasal polyps revealed that mast cells accounted for 0.53% to 6.3% of all CD45⁺ cells in nasal polyps from patients with AERD and 0.19% to 4.7% of all CD45⁺ cells in nasal tissue of control subjects with CRS and also revealed no difference between patients with AERD and subjects with CRS (Fig 3, B).

TSLP expression in nasal polyps correlates with hPGDS and mast cell marker levels

Whole nasal polyps were used to generate RNA and protein for quantitative PCR and Western blotting. *TSLP* mRNA was detected in all nasal polyp samples. Steady-state levels of mRNA encoding *TSLP* were similar between patients with AERD and subjects with CRS (Fig 4, A). There was significantly more proteolytically processed (approximately 10 kDa) TSLP protein in samples from patients with AERD than in those from control subjects

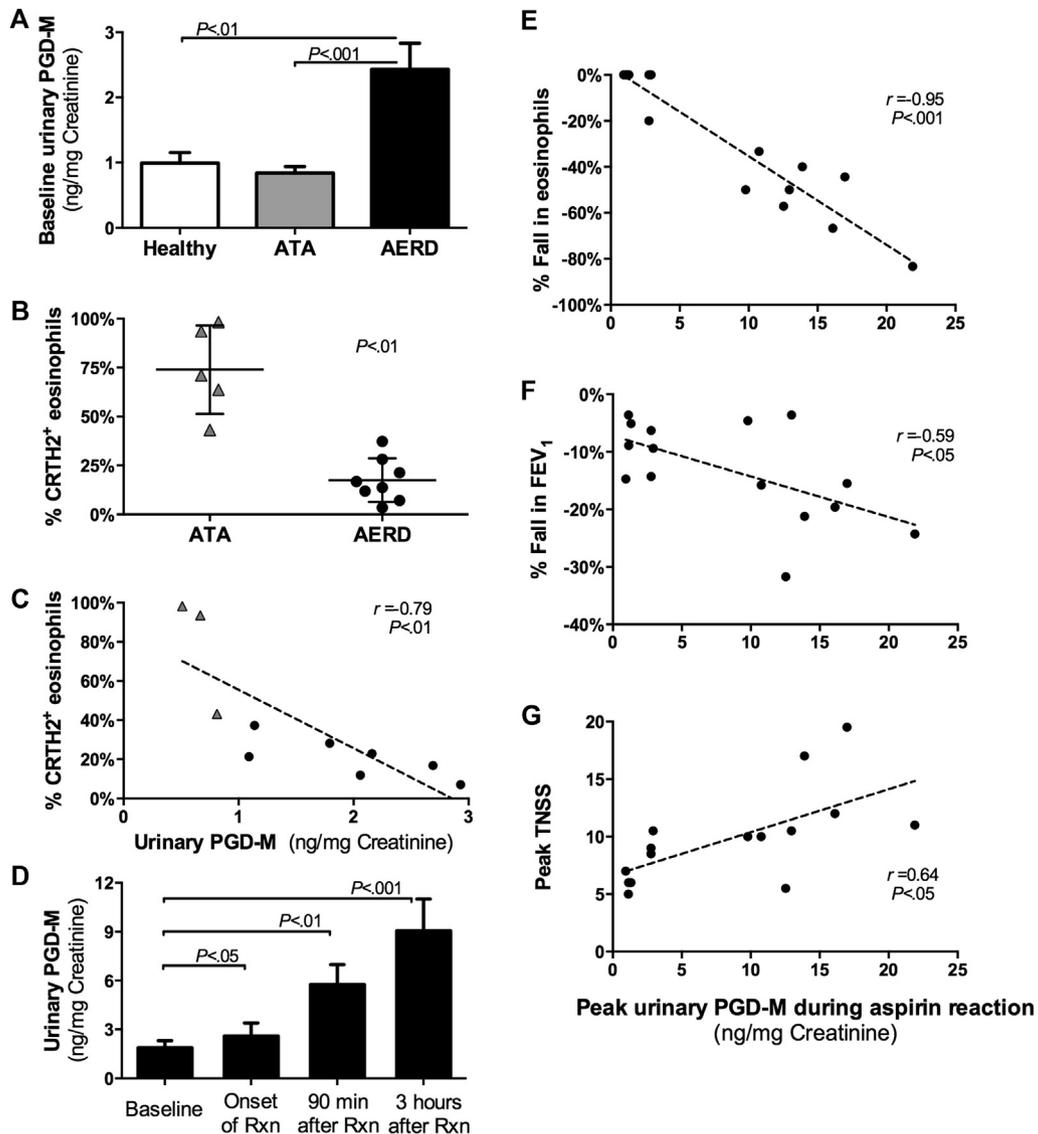


FIG 1. PGD₂ production mediates effector responses in patients with AERD. **A**, Baseline uPGD-M levels from healthy control subjects ($n = 9$), control subjects with ATA ($n = 7$), and patients with AERD ($n = 29$). **B**, Surface CRTH2 expression by blood eosinophils from control subjects with ATA and patients with AERD. **C**, Correlation of surface CRTH2 expression by blood eosinophils with uPGD-M levels. **D**, Time-dependent changes in uPGD-M levels during reactions to oral aspirin challenges. *Rxn*, Reaction. **E-G**, Correlations of peak uPGD-M levels with changes in peripheral blood eosinophil counts (Fig 1, *E*), FEV₁ (Fig 1, *F*), and Total Nasal Symptom Scores (Fig 1, *G*). Fig 1, *A* and *D*, Data are shown as means + SEMs. Fig 1, *B*, Data are shown as individual points with group means. Fig 1, *C* and *E-G*, Data are shown as linear dependence measured with the Pearson correlation coefficient.

(Fig 4, *B* and *C*). The identity of TSLP was confirmed with 2 separate TSLP antibodies and by blocking with a neutralizing immunogenic peptide (see Fig E2 in this article's Online Repository at www.jacionline.org). The presence of the higher-molecular-weight 15-kDa TSLP band was variable in nasal polyp lysate samples (see Fig E3 in this article's Online Repository at www.jacionline.org). Cell sorting of dispersed nasal polyp cells revealed TSLP mRNA derived from EpCAM⁺ epithelial cells, CD90⁺ fibroblasts, and CD31⁺ endothelial cells (see Fig E4 in this article's Online Repository at www.jacionline.org).

To determine the relationship between TSLP, mast cell phenotype, and PGD₂ generation, we compared levels of TSLP mRNA with mast cell-specific transcripts and PGD₂-generating

enzymes in nasal polyps of patients with AERD. TSLP mRNA levels correlated strongly with both CPA3 ($r = 0.74$, $P < .0001$; Fig 5, *A*) and HPGDS ($r = 0.75$, $P < .0001$; Fig 5, *B*) transcripts but not with COX-2, 5-LO, or LTC₄ synthase (data not shown). Nasal polyp TSLP mRNA correlated significantly with baseline urinary levels of PGD-M ($r = 0.74$, $P < .01$; Fig 5, *C*) but not with those of LTE₄ ($r = 0.05$, data not shown). Levels of uPGD-M, but not LTE₄, also tended to correlate with nasal polyp expression of HPGDS ($r = 0.68$, $P < .05$; Fig 5, *D*) and CPA3 ($r = 0.51$, $P = .08$; data not shown). Urinary levels of a stable TXA₂ metabolite (TX-M) also correlated with TSLP mRNA expression ($r = 0.64$, data not shown). We did not have sufficient numbers of matched urine and polyp samples to determine

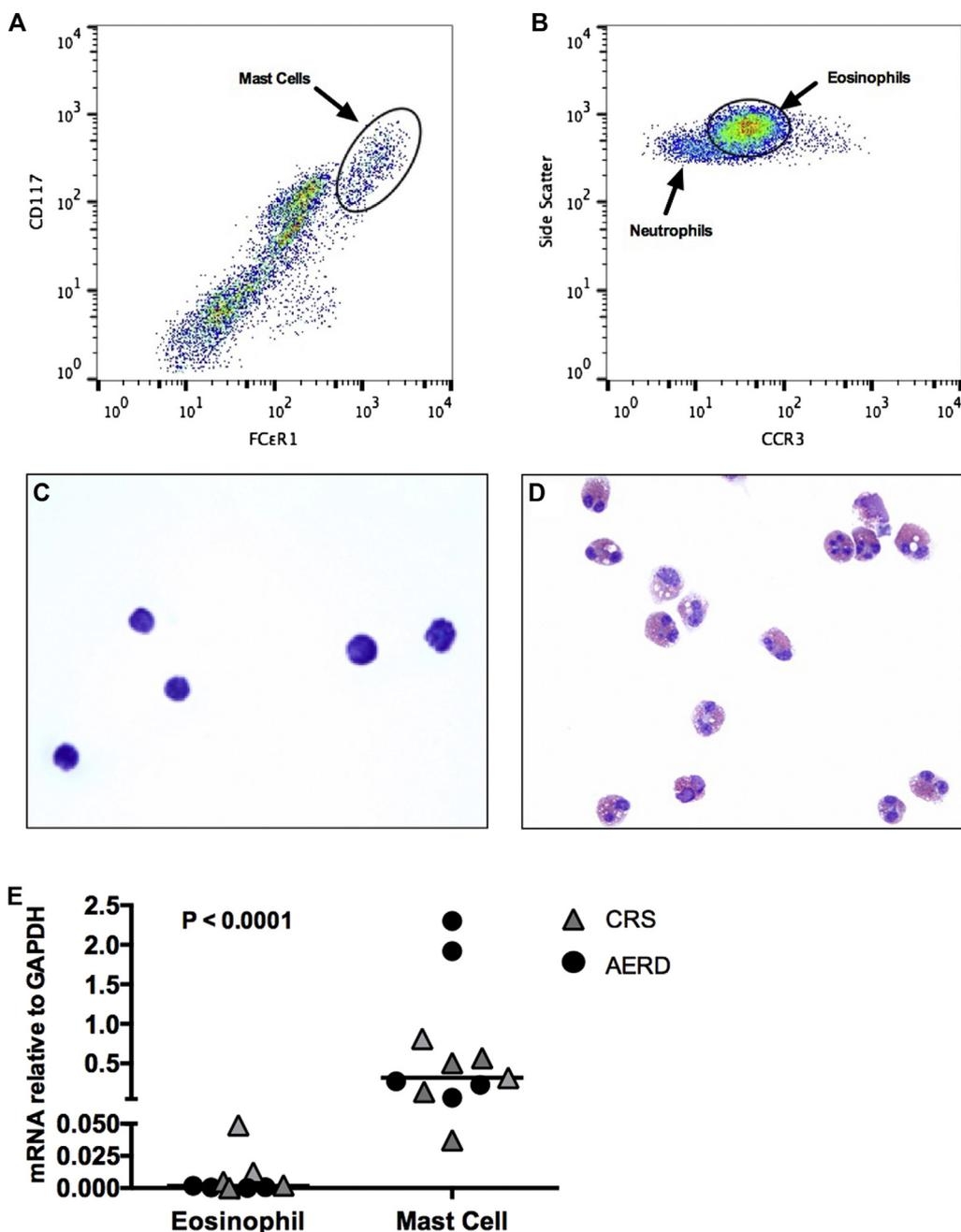


FIG 2. Expression of PGD₂ synthetic enzymes by nasal polyp effector cells. **A** and **B**, Cytofluorographic detection of mast cells (Fig 2, **A**) and eosinophils (Fig 2, **B**) in enzymatically dispersed nasal polyps. **C**, Toluidine blue staining of sorted nasal polyp mast cells. **D**, Staining of sorted nasal polyp eosinophils. **E**, *HPGDS* mRNA expression from sorted nasal polyp eosinophils and mast cells. **E**, Data are shown as individual points with group medians, as measured by using the Mann-Whitney test.

whether these quantitative relationships were also evident in subjects with CRS.

TSLP stimulates PGD₂ production by mast cells *in vitro*

We stimulated human cord blood–derived mast cells with recombinant TSLP to determine whether this cytokine could directly elicit PGD₂ production. Unstimulated mast cells generated small quantities of PGD₂. TSLP increased the production of PGD₂ at 6 hours in each of 3 mast cell cultures,

although there was significant variability in production of PGD₂ between donors, and therefore no statistical difference was reached (Fig 6, **A**). At 24 hours, levels of PGD₂ production by stimulated mast cells had decreased to approximately 30% of the levels measured at 6 hours but were still greater than unstimulated baseline values (data not shown). TSLP stimulation also elicited TXA₂ release, as indicated by detection of the stable metabolite TXB₂, but did not induce cysLT formation (see Fig E5 in this article’s Online Repository at www.jacionline.org).

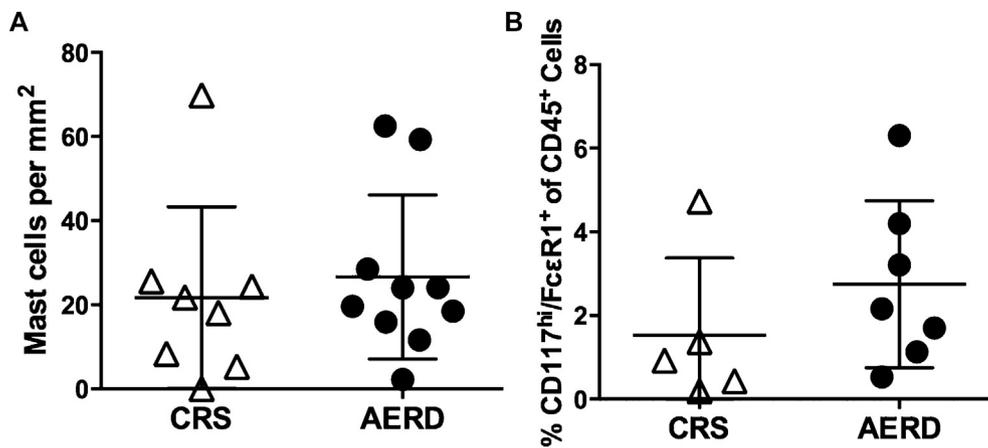


FIG 3. Comparison of nasal tissue mast cell counts in patients with AERD and aspirin-tolerant control subjects with CRS. **A**, Number of mast cells per square millimeter, as determined by means of immunohistochemistry in control subjects with CRS and patients with AERD. **B**, Percentage of CD45⁺ cells that are CD117^{hi}/FcεRI⁺ in patients with CRS and those with AERD. Data are shown as individual points with group means.

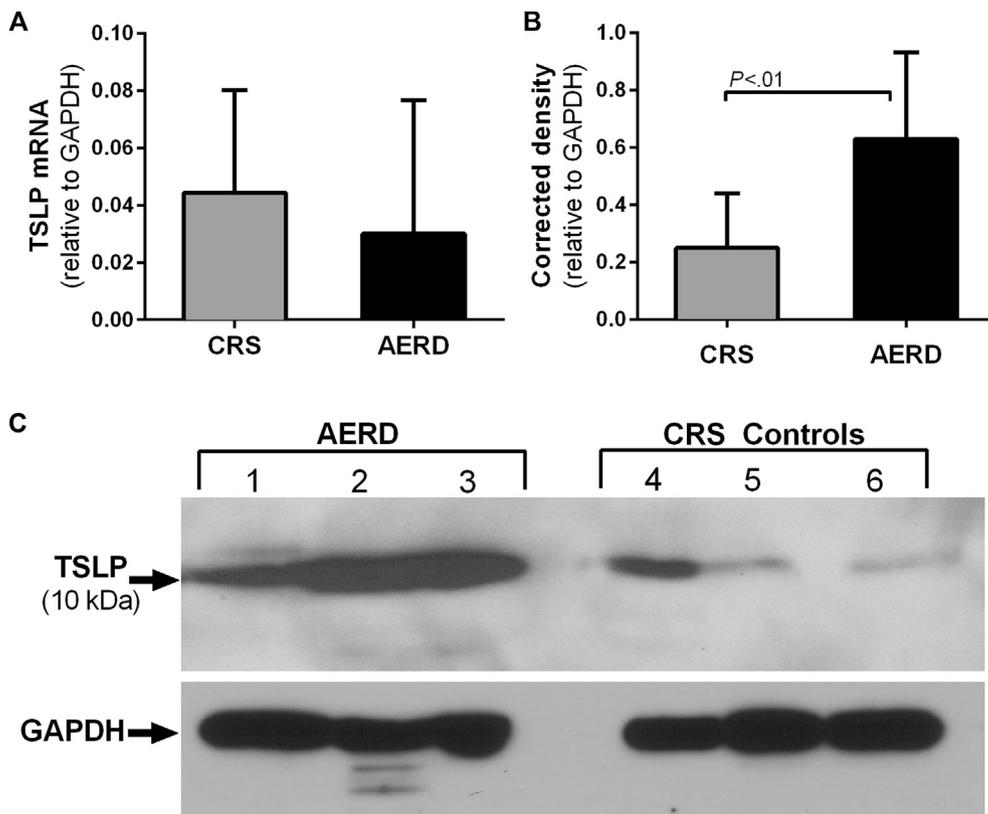


FIG 4. Expression of TSLP in nasal polyps from patients with AERD and control subjects with CRS. **A**, Whole nasal polyp *TSLP* mRNA in subjects with CRS (n = 7) and patients with AERD (n = 18). **B**, Densitometric analysis of the cleaved active form of TSLP from patients with CRS and AERD (n = 11, both groups). **C**, Total protein levels of TSLP in patients with AERD and subjects with CRS measured by using Western blotting of 3 representative samples per group. Fig 4, A and B, Data are shown as means + SEMs. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.

To test the ability of TSLP to induce PGD₂ generation in an alternative human mast cell system, we stimulated human peripheral blood–derived mast cells with recombinant TSLP with and without IL-33. At 6 hours, TSLP alone did not increase

peripheral blood–derived mast cell generation of PGD₂ to greater than baseline levels (data not shown). However, TSLP and IL-33 together led to greater PGD₂ generation over that of IL-33 alone, with a 23%, 74%, and 87% increase in PGD₂ production in each

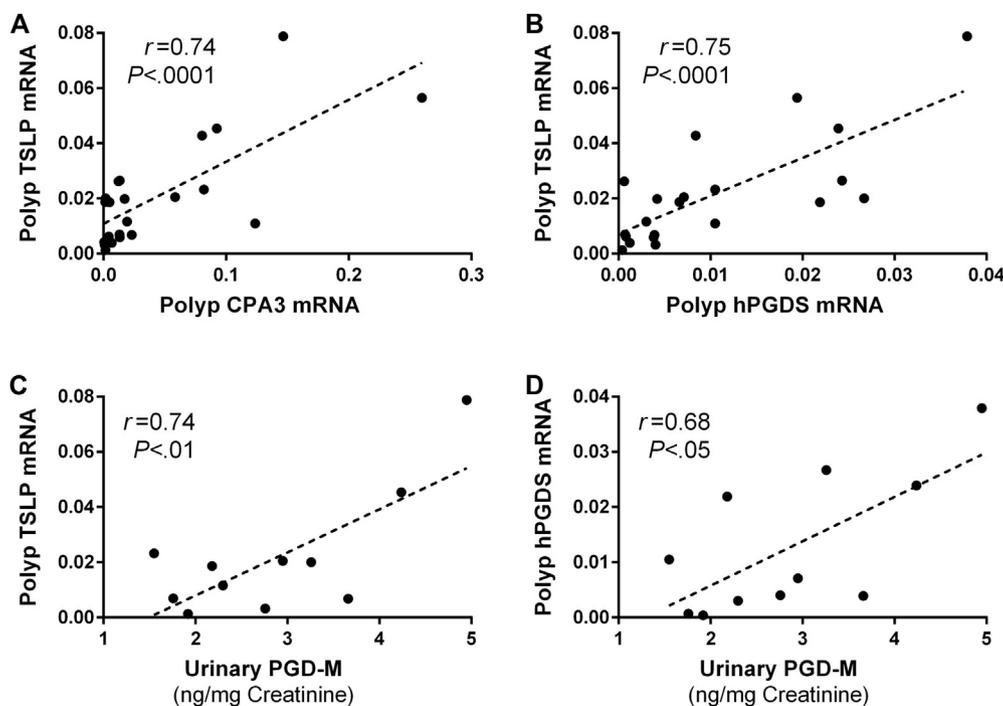


FIG 5. Relationships between nasal polyp *TSLP* mRNA expression, mast cell markers, and systemic PGD₂ production in patients with AERD. **A**, Correlation between *TSLP* and *CPA3* mRNA expression. **B**, Correlation between *TSLP* and *HPGDS* mRNA expression. **C**, Correlation between *TSLP* expression and baseline uPGD-M levels. **D**, Correlation between *HPGDS* mRNA expression and baseline uPGD-M levels. Data are shown as linear dependence measured with the Pearson correlation coefficient.

of the individual donors on addition of TSLP. This indicates a synergistic effect of IL-33 and TSLP (Fig 6, B).

DISCUSSION

PGD₂ is considered an effector of IgE-dependent type 1 hypersensitivity reactions,³⁸ but its role in innate immunity is largely unexplored. Innate type 2 immune responses initiated by epithelially derived cytokines involve both myeloid (eosinophils and basophils)²³ and lymphoid (ILC2s)²⁶ effector cells that express CRTH2, suggesting that PGD₂ plays a role in this system. AERD in particular and nasal polyposis in general frequently occur in nonatopic subjects,³⁰ yet consistently involve chronic eosinophilic sinonasal pathology, suggesting that innate type 2 immunity contributes to the disease. TSLP is an important component of the innate type 2 immune system that is expressed in nasal polyps and that acts on mast cells in combination with IL-1β and IL-33 to induce type 2 cytokine production.^{14,39} Given our recent study demonstrating that the severity of clinical reactions to aspirin challenge in patients with AERD relates to the level of PGD₂ production,³³ we conducted this study to determine the relationship between PGD₂ production, mast cell activity, and the potential involvement of TSLP using well-phenotyped patients with and without AERD to reflect a spectrum of PGD₂ production.

Aspirin challenges in patients with AERD elicit bronchoconstriction and sinonasal congestion,⁴⁰ presumably reflecting non-IgE-dependent mast cell activation that is “unbraked” by COX-1 inhibition.^{41,42} As blood eosinophil counts decrease during aspirin-induced reactions,⁴³ eosinophil counts simultaneously increase in nasal lavage fluid,⁴⁴ suggesting that these effector cells

are acutely recruited to the site of mast cell activation. This recruitment into respiratory tissues occurs without changes in the levels of eosinophil-active chemokines. Because eosinophils are CRTH2⁺, we sought to determine whether PGD₂ released during reactions to aspirin might be responsible for their recruitment, along with additional physiologic manifestations of the reaction. During the course of aspirin challenge in a cohort of patients with AERD, we monitored uPGD-M levels along with a range of clinical, biochemical, and cellular parameters. All subjects were treated with a cysLT₁ receptor antagonist to eliminate the potential confounding influences of cysLTs on lower airway responses to aspirin. The high baseline levels of uPGD-M in samples from patients with AERD (Fig 1, A) were paralleled by significantly lower levels of membrane expression of CRTH2 by their blood eosinophils (Fig 1, B) when compared with those in control subjects with ATA, suggesting *in vivo* exposure to high levels of PGD₂ leads to CRTH2 receptor downregulation.^{27,45} Baseline uPGD-M levels did not correlate with asthma severity. uPGD-M levels increased during reactions, and peak uPGD-M levels did not correlate with the provocative dose of aspirin, which was 162 mg or less in all patients with AERD. Aspirin selectively inhibits COX-1 at this dosing range. Notably, AERD involves impaired COX-2 expression by nasal polyp cells, such as fibroblasts, which generate PGE₂. As a result, the ratio of PGD₂ to PGE₂ is substantially higher in polyps from patients with AERD than in control subjects with CRS.⁴¹ It seems likely that depletion of residual COX-1-derived PGE₂ permits mast cell activation in patients with AERD,⁴⁶ resulting in PGD₂ generation by polyp mast cells, which strongly express COX-2 (see Fig E1). This suggests a mechanism that preserves the capacity of mast cells to generate PGD₂ during aspirin challenge.

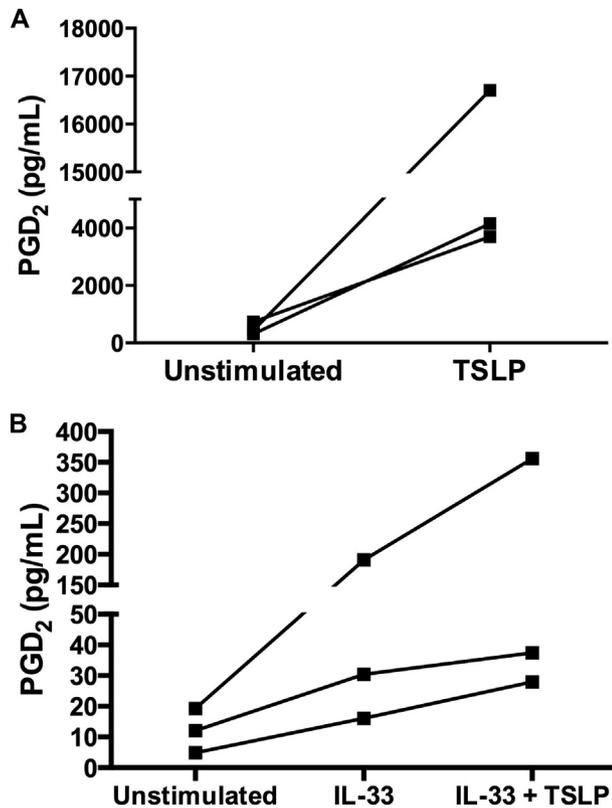


FIG 6. *In vitro* stimulation of mast cells with TSLP. **A**, TSLP (10 ng/mL) increases human cord blood-derived mast cell generation of PGD₂ *in vitro* at 6 hours. **B**, TSLP (10 ng/mL) with IL-33 (10 ng/mL) synergistically increases human peripheral blood-derived mast cell generation of PGD₂ *in vitro* at 6 hours compared with IL-33 alone. Data are displayed for individual experiments with cells from 3 different donors; differences are not statistically significant.

Impaired homeostatic PGE₂ generation in the respiratory tissue at baseline could also contribute to the high baseline levels of uPGD-M observed in patients with AERD. The correlation between peak uPGD-M levels and the decrease in blood eosinophil counts (Fig 1, E) strongly suggests that PGD₂, acting at CRTH2, is a dominant chemotactic stimulus that accounts for effector cell recruitment in this context. Moreover, the relationship between peak uPGD-M levels during reactions and the decrease in FEV₁ (Fig 1, F) and the increase in nasal symptoms (Fig 1, G) could well reflect actions of PGD₂ at T prostanoid receptors and D prostanoid 1 receptors, which respectively account for the bronchoconstrictive and vasodilatory effects of PGD₂.^{47,48} Thus PGD₂ plays a major end-organ effector role in this innate type 2 immune response involving mast cell activation.

We next sought to identify the cellular source or sources of PGD₂ in the respiratory tissue and the mechanism or mechanisms responsible for its production. Both mast cells⁴⁹ and eosinophils⁵⁰ express *HPGDS*, and both are found in nasal polyps. Quantitative immunohistochemical studies suggest that the number of hPGDS⁺ cells in nasal polyps correlates with disease severity.⁵¹ hPGDS can convert the precursor PGH₂ from either COX-1, an exquisitely aspirin-sensitive enzyme, or COX-2, which is less aspirin sensitive. Induction of COX-2 expression by mast cells *in vitro* results in robust and sustained generation of PGD₂.³⁷ We directly monitored the expression of *HPGDS* and the COX

enzymes (*PTGS1* and *PTGS2*) in cytofluorographically purified nasal polyp mast cells and comparing these values with those in eosinophils from the same samples (Fig 2, C and D). To our knowledge, this is the first such analysis to be reported. The markedly stronger expression of *HPGDS* by polyp mast cells compared with eosinophils (Fig 2, E), along with their robust expression of COX-2 (*PTGS2*) (see Fig E1), supports the thesis that mast cells are the primary source of PGD₂ in respiratory tissue. As noted above, COX-2 expression might preserve the ability of mast cells to generate PGD₂ when their activation is induced by depletion of COX-1-derived PGE₂ in patients with AERD. In turn, the aspirin-resistant generation of PGD₂ by mast cells facilitates the baseline pathology, as well as the physiologic responses to aspirin challenge.

Among many cellular targets, TSLP facilitates mast cell development *in vivo*⁵² and mast cell activation *in vitro*.¹⁴ Because TSLP is strongly expressed by nasal polyps,^{10,11} we focused on its potential role as a driver of PGD₂ generation both *in vivo* and *in vitro*. We found TSLP mRNA and protein in all whole nasal tissue samples and verified that its transcript was expressed by epithelial cells, fibroblasts, and endothelial cells, regardless of disease phenotype (see Fig E4). TSLP undergoes processing by leukocyte-derived proteases, yielding bioactive fragments¹² that are consistent with the 10-kDa size of the molecular species identified by means of Western blotting in our studies (Fig 4, C, and see Figs E2 and E3). Remarkably, the levels of *TSLP* mRNA expression by nasal polyps from patients with AERD correlated with polyp *CPA3* and *HPGDS* transcript expression (Fig 5, A and B), as well as with baseline uPGD-M (Fig 5, C) and TX-M (data not shown) levels but not with urinary LTE₄ levels. The correlation between *TSLP* mRNA, mast cell markers, and PGD₂ levels *in vivo* suggests a spectrum of AERD pathophysiology that could reflect some combination of inductive effects of TSLP on mast cell markers or the promotion of mast cell proliferation, survival, or both by TSLP, as suggested by mouse studies.⁵² Additionally, tissue mast cells promote TSLP expression in a model of allergic rhinitis,⁵³ suggesting that the relationship between mast cells and TSLP in the tissue might be bilateral. The low COX-2 expression at the whole polyp level (primarily caused by impaired expression by PGE₂-generating structural cells) likely accounts for the lack of a relationship between COX-2 and TSLP. Moreover, TSLP induced PGD₂ production by human cord blood-derived cultured mast cells (Fig 6, A) and by human peripheral blood-derived cultured mast cells when combined with IL-33 (Fig 6, B) *ex vivo* but did not induce cysLT production (see Fig E5). Thus TSLP can directly and selectively induce PGD₂ generation by mast cells in nasal polyps, whereas additional factors might be responsible for inducing cysLT production. Although we have no evidence that TSLP is responsible for the dramatic and acute release of PGD₂ during aspirin-induced reactions, we suspect that TSLP is one of the main factors responsible for priming the tissue mast cells for chronic overproduction of PGD₂.

Our findings strongly link the actions of TSLP to the activation of mast cells and the generation of PGD₂ *in vivo*. PGD₂ is a logical candidate effector in patients with AERD because of its persistent high-level production, its capacity to recruit eosinophils and basophils, and its ability to directly induce cytokine production by ILC2s,²⁷ as well as conventional type 2 cells.⁵⁴ We propose that mast cell-derived PGD₂, driven at least in part by TSLP, amplifies and perpetuates the innate type 2–like axis of

inflammation caused by phenotypically altered structural cells and their cytokines. Both anti-TSLP and CRTH2 antagonists are in advanced stages of clinical development for the treatment of asthma. Our findings suggest that both of these modalities might be efficacious in patients with AERD.

Key messages

- TSLP contributes to the generation of PGD₂ by mast cells.
- PGD₂ production correlates with several pathogenic features of AERD.
- Anti-TSLP and CRTH2 antagonists are potential treatment modalities to be considered in patients with AERD.

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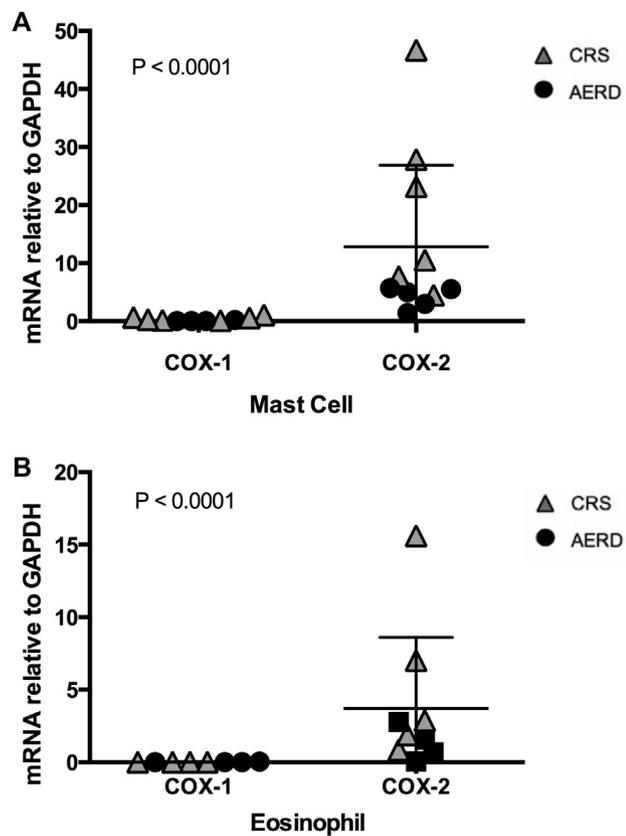


FIG E1. COX-1 and COX-2 mRNA expression in mast cells and eosinophils. COX-1 and COX-2 mRNA expression in sorted nasal polyp mast cells (A) and eosinophils (B) in patients with AERD and subjects with CRS. Data are shown as individual points with group medians, as measured by using the Mann-Whitney test.

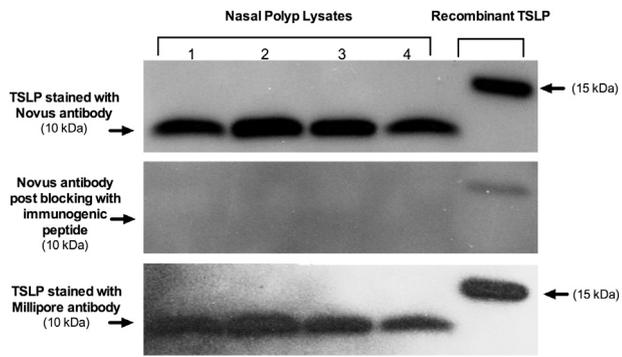


FIG E2. Confirmation of TSLP protein in nasal polyp samples measured by using Western blotting. *Top panel*, 10 kDa of TSLP protein measured in nasal polyp lysates and 15 kDa of TSLP protein measured from recombinant TSLP (Novus antibody). *Middle panel*, Same Western blot after blocking with immunogenic peptide (Novus) plus Novus antibody. *Bottom panel*, Same Western blot using Millipore antibody to identify TSLP.

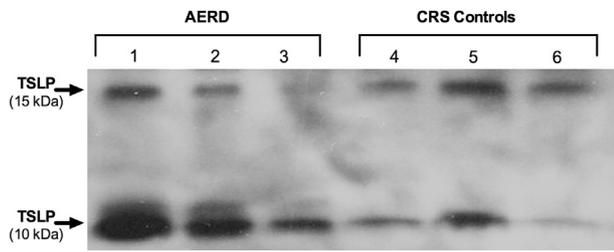


FIG E3. Multiple TSLP protein bands (15 and 10 kDa) expressed in nasal polyp lysates from patients with AERD and subjects with CRS.

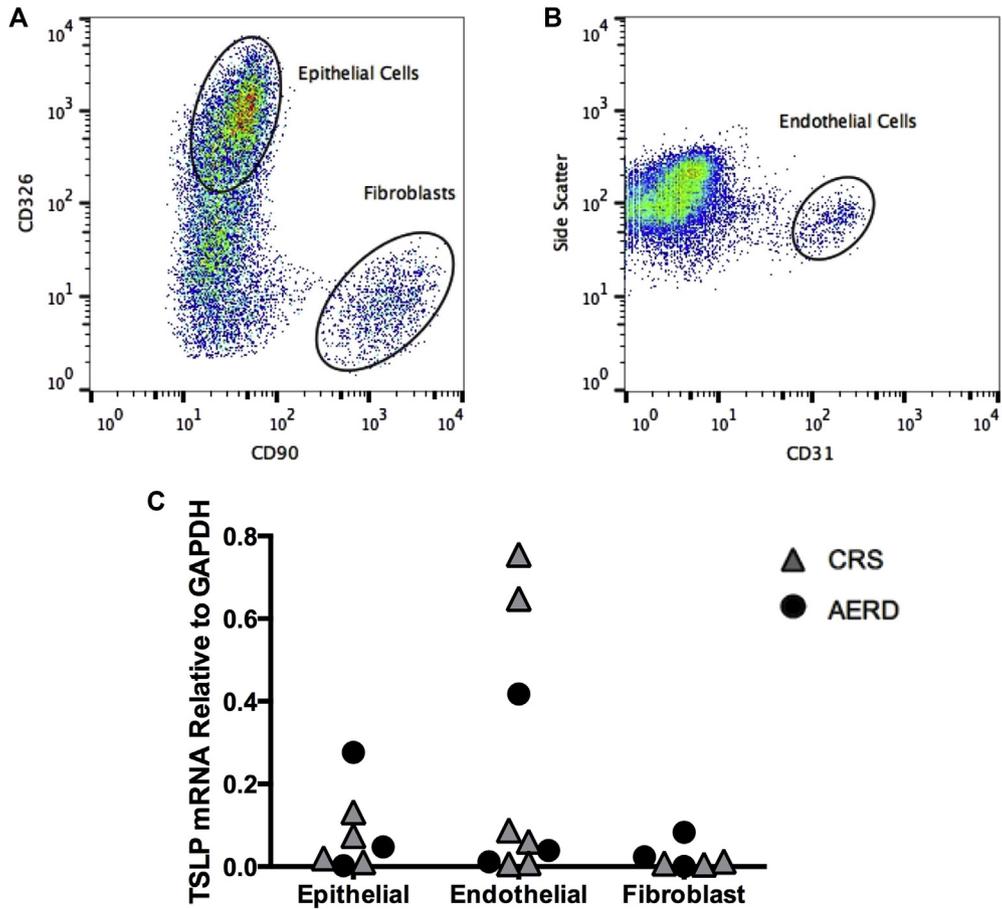


FIG E4. *TSLP* mRNA expression in sorted nasal polyp structural cells. **A** and **B**, Cytofluorographic detection of epithelial cells and fibroblasts (Fig E4, **A**) and endothelial cells (Fig E4, **B**). **C**, *TSLP* mRNA expression from sorted nasal polyp epithelial cells, endothelial cells, and fibroblasts. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.

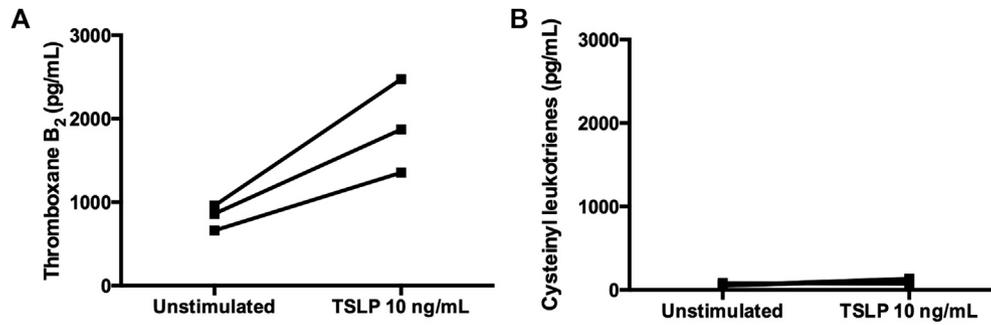


FIG E5. Evaluation of arachidonic acid pathway products after *in vitro* stimulation of human cord blood-derived mast cells with TSLP. **A**, TSLP (10 ng/mL) increases mast cell generation of TXB₂ *in vitro* at 6 hours. **B**, TSLP (10 ng/mL) stimulation does not increase mast cell generation of cysLTs at 6 hours. Data are displayed for individual experiments with cells from 3 different donors.

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