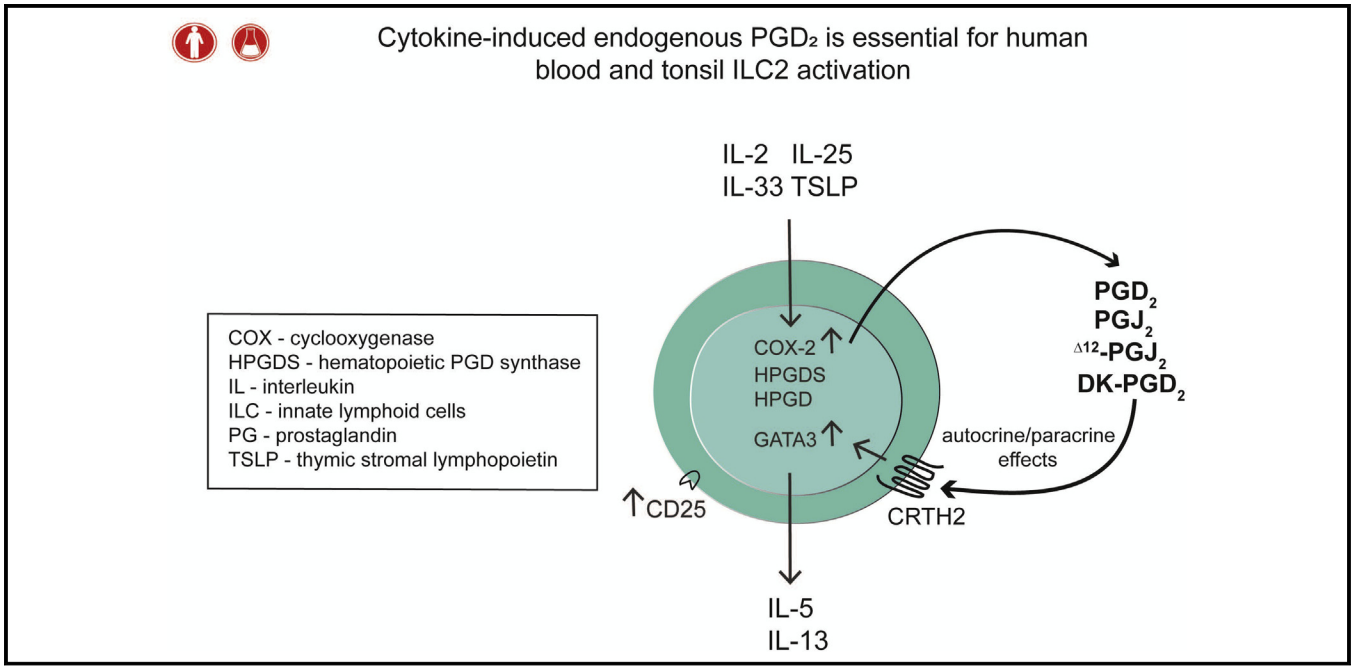
Cytokine-induced endogenous production of prostaglandin D_2 is essential for human group 2 innate lymphoid cell activation

Jovana Maric, PhD,[a,b] Avinash Ravindran, MSc,[c] Luca Mazzurana, MSc,[b] Aline Van Acker, PhD,[b] Anna Rao, PhD,[b] Efthymia Kokkinou, MSc,[b] Maria Ekoff, PhD,[c] Dominique Thomas, PhD,[d] Alexander Fauland, PhD,[e] Gunnar Nilsson, PhD,[c,f] Craig E. Wheelock, PhD,[e] Sven-Erik Dahlén, MD, PhD,[g] Nerea Ferreirós, PhD,[d] Gerd Geisslinger, MD, PhD,[d,h] Danielle Friberg, MD, PhD,[i,j] Akos Heinemann, MD,[a] Viktoria Konya, PhD,[a,b]* and Jenny Mjösberg, PhD[b,k]*

Graz, Austria; Stockholm, Uppsala, and Linköping, Sweden; and Frankfurt, Germany

GRAPHICAL ABSTRACT



Background: Group 2 innate lymphoid cells (ILC2s) play a key role in the initiation and maintenance of type 2 immune responses. The prostaglandin (PG) D_2–chemoattractant receptor–homologous molecule expressed on T_H2 cells (CRTH2) receptor axis potently induces cytokine production and ILC2 migration.

From [a]the Otto Loewi Research Center, Pharmacology Section, Medical University of Graz, and BioTechMed, Graz; [b]the Center for Infectious Medicine, Department of Medicine Huddinge, [c]the Division of Physiological Chemistry II, Department of Medical Biochemistry and Biophysics, [g]Experimental Asthma and Allergy Research, Institute of Environmental Medicine, and [i]the Department of Clinical Science, Intervention and Technology, CLINTEC, Karolinska Institutet, Stockholm; [c]the Immunology and Allergy Unit, Department of Medicine, Solna, Karolinska Institutet, and Clinical Immunology and transfusion medicine, Karolinska University Hospital, Stockholm; [d]the Institute of Clinical Pharmacology, Goethe-University Frankfurt, Pharmazentrum Frankfurt/ZAFES, Frankfurt; [f]the Department of Medical Sciences, Uppsala University; [h]the Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Project group Translational Medicine & Pharmacology TMP, Frankfurt; [j]the Department of Surgical Science, Uppsala University; and [k]the Department of Clinical and Experimental Medicine, Linköping University.

*These authors contributed equally to this work.

This project received funding from the Swedish Research Council (521-2013-2791), the Swedish Cancer Society (130396), the Foundation for Strategic Research (ICA12-0023), and the Swedish Society for Medical Research (to J. Mjösberg); the Austrian Science Fund FWF (grant P25531-B23; to V.K.); PhD Program DK-MOLIN (FWF-W1241; to J. Maric); Deutsche Forschungsgemeinschaft DFG (SFB 1039, Z01; to G.G.); the Swedish Research Council, and the Swedish Heart-Lung Foundation (to G.N.). A.F. was funded by the Karin and Sten Mörstedt Initiative on Anaphylaxis. C.E.W. was supported by the Swedish Heart-Lung Foundation (HLF 20150640 and HLF 20140469). S.-E.D. acknowledges the ChAMP (Centre for Allergy Research Highlights Asthma Markers of Phenotype) consortium, which is funded by the Swedish Foundation for Strategic Research, the Karolinska Institutet, AstraZeneca & the Science for Life Laboratory Joint Research Collaboration, and the Vårdal Foundation.

Disclosure of potential conflict of interest: A. Heinemann has received consultancy fees from AstraZeneca and Bayer. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication September 20, 2017; revised August 8, 2018; accepted for publication October 11, 2018.

Corresponding author: Jenny Mjösberg, PhD, Center for Infectious Medicine, F59, Karolinska Institutet, S-14186 Stockholm, Sweden. E-mail: jenny.mjosberg@ki.se. Or: Viktoria Konya, PhD, Otto Loewi Research Center, Pharmacology Section, Medical University of Graz, Graz, Austria. E-mail: viktoria.konya@medunigraz.at.

0091-6749/$36.00

https://doi.org/10.1016/j.jaci.2018.10.069

Objective: We set out to examine PG production in human ILC2s and the implications of such endogenous production on ILC2 function.

Methods: The effects of the COX-1/2 inhibitor flurbiprofen, the hematopoietic prostaglandin D_2 synthase (HPGDS) inhibitor KMN698, and the CRTH2 antagonist CAY10471 on human ILC2s were determined by assessing receptor and transcription factor expression, cytokine production, and gene expression with flow cytometry, ELISA, and quantitative RT-PCR, respectively. Concentrations of lipid mediators were measured by using liquid chromatography–tandem mass spectrometry and ELISA.

Results: We show that ILC2s constitutively express HPGDS and upregulate COX-2 upon IL-2, IL-25, and IL-33 plus thymic stromal lymphopoietin stimulation. Consequently, PGD_2 and its metabolites can be detected in ILC2 supernatants. We reveal that endogenously produced PGD_2 is essential in cytokine-induced ILC2 activation because blocking of the COX-1/2 or HPGDS enzymes or the CRTH2 receptor abolishes ILC2 responses.

Conclusion: PGD_2 produced by ILC2s is, in a paracrine/autocrine manner, essential in cytokine-induced ILC2 activation. Hence we provide the detailed mechanism behind how CRTH2 antagonists represent promising therapeutic tools for allergic diseases by controlling ILC2 function. (J Allergy Clin Immunol 2019;■■■:■■■-■■■.)

Key words: *Group 2 innate lymphoid cells, prostaglandin D_2, chemoattractant receptor–homologous molecule expressed on T_H2 cells, allergy*

Abbreviations used

CBMC:	Cord blood–derived mast cell
CRTH2:	Chemoattractant receptor–homologous molecule expressed on T_H2 cells
DK-PGD_2:	13, 14-Dihydro-15-keto prostaglandin D_2
DP1:	Prostaglandin D_2 receptor 1
15d-PGJ_2:	15-Deoxy-delta-12, 14-PGJ_2
HPGD:	15-Hydroxyprostaglandin dehydrogenase
HPGDS:	Hematopoietic prostaglandin D_2 synthase
ILC2:	Group 2 innate lymphoid cell
IMDM:	Iscove modified Dulbecco medium
LC-MS/MS:	Liquid chromatography–tandem mass spectrometry
LPGDS:	Lipocalin prostaglandin D_2 synthase
LT:	Leukotriene
NHS:	Normal human serum
PE:	Phycoerythrin
PGD_2:	Prostaglandin
PPAR-γ:	Peroxisome proliferator–activated receptor γ
RT-qPCR:	Quantitative RT-PCR
TSLP:	Thymic stromal lymphopoietin
TXB_2:	Thromboxane B_2

Group 2 innate lymphoid cells (ILC2s) play important roles in the initiation and amplification of type 2 immune responses.[1] Under inflammatory conditions, ILC2s are stimulated by the innate cytokines IL-33, IL-25, and thymic stromal lymphopoietin (TSLP), which are products of activated epithelial cells and macrophages.[2] Optimal activation of human ILC2s requires a combination of these 3 cytokines, as well as IL-2.[3,4]

Furthermore, ILC2s can be activated by proinflammatory lipid mediators.[5] Leukotriene (LT) D_4, LTE_4,[6,7] and prostaglandin (PG) D_2 are potent stimulators of mouse and human ILC2s.[8-10] PGD_2 has been shown to induce IL-5 and IL-13 production, as well as migration of ILC2s.[8,10,11] Human ILC2s were initially identified based on the expression of one of the receptors for PGD_2.[12] The PGD_2 receptor chemoattractant receptor–homologous molecule expressed on T_H2 cells (CRTH2) now serves as an important cell-surface marker of human ILC2s. CRTH2 is a Gi protein–coupled receptor, and in type 2 immune cells, such as T_H2 cells, eosinophils, basophils, and ILC2s, CRTH2 activation increases free intracellular calcium levels.[13] Activation of these cells is crucial in the development of allergic inflammatory processes.

PGD_2, belonging to the eicosanoid family, is derived from arachidonic acid, which is liberated from membrane phospholipids by phospholipase A_2. Activation of COX-1, COX-2, or both leads to a 2-step metabolism of arachidonic acid into PGG_2 and subsequently to PGH_2, which is the substrate for enzymes specialized in generating different PGs. There are 2 types of PGD_2 synthases: hematopoietic prostaglandin D_2 synthase (HPGDS), which is expressed on immune cells,[14-16] and lipocalin prostaglandin D_2 synthase (LPGDS), which is found mainly in nervous system, heart, and adipose tissue.[17,18] Mast cells are the major source of PGD_2,[19] whereas dendritic cells, macrophages,[20] eosinophils,[21] and T_H2 cells release smaller amounts of PGD_2.[22] In the metabolism of PGD_2, 15-hydroxyprostaglandin dehydrogenase (HPGD) converts PGD_2 into 13, 14-dihydro-15-keto prostaglandin D_2 (DK-PGD_2),[23,24] a natural selective agonist of CRTH2 receptor.[25] Furthermore, PGD_2 can be nonenzymatically converted into PGJ_2 and its derivatives, metabolites with biological activity.[26]

Because CRTH2 is a key receptor in allergic inflammatory processes and mediates the PGD_2-induced activation of ILC2s and other type 2 immune cells, the PGD_2-CRTH2 axis is an emerging therapeutic target to control allergic diseases.[22,27,28] The role of exogenous PGD_2 in ILC2 function has been clearly established; however, it has not been addressed whether endogenously produced PGD_2 plays a role in ILC2 activation.

Our study shows that human ILC2s express HPGDS and that IL-2, IL-25, and IL-33 plus TSLP activation of human ILC2s leads to upregulation of the rate-limiting COX-2 enzyme. This renders ILC2s capable of producing PGD_2 and its metabolites. Endogenously produced PGD_2 is essential in cytokine-induced activation of human ILC2s because a COX-1/2 blocker potently prevents IL-5 and IL-13 production from the cells. Similarly, selective antagonism of CRTH2 on cytokine stimulation inhibits ILC2 activation. Thus we demonstrate that human ILC2s critically depend on endogenously produced PGD_2 for activation by cytokines. Our results have implications for the treatment of allergic inflammation.

METHODS

A detailed description of the methods and materials used in this study is provided in the Methods section in this article's Online Repository at www.jacionline.org.

Isolation and flow cytometric sorting of tonsillar and blood ILC2s

Human ILC2s were obtained from tonsils and buffy coats. Cells were isolated by means of flow cytometric sorting with antibodies against specific cell-surface markers, as previously described.[29] Tonsils were obtained from both children and adults. Because we did not observe any differences in the results depending on age or sex, we present data pooled from children and adults of both sexes. A detailed description can be found in the Methods section in this article's Online Repository.

Culture, expansion, and treatments of ILC2s

Freshly sorted tonsillar ILC2s were expanded for 2 weeks in culture with a mixture of feeder cells, IL-2 (100 U/mL), PHA, and IL-4, as previously described.[12] After 2 weeks of expansion, the majority of the feeder cells were engulfed by the ILC2s, rendering ILC2s greater than 85% pure, and the remaining feeder cells were not viable, as determined by means of dead cell marker discrimination in flow cytometry (data not shown). ILC2s were preincubated for 40 minutes with flurbiprofen (1 μmol/L) or a specific HPGDS inhibitor developed by Sanofi-Aventis (Paris, France; 2-phenyl-pyrimidine-5-carboxylic acid [2,3-dihidro-indol-1-yl]-amide [United States patent 8,202,863], which for this research study was synthesized by Cayman Chemical [Ann Arbor, Mich], given the internal code KMN698, and used at a concentration of 100 nmol/L) or for 20 minutes with the CRTH2 antagonist CAY10471 (1 μmol/L) before IL-2 (10 U/mL) with or without IL-33, TSLP, and IL-25 (50 ng/mL each). Expanded ILC2s were incubated for 6 and 24 hours. Freshly sorted blood and tonsillar ILC2s were cultured for 5 days.

Flow cytometric staining and analysis of intracellular cytokines

Cell-surface expression of CD25 and CRTH2 was detected by using specific mAbs. Intracellular IL-5 and IL-13 protein, as well as the transcription factor GATA-3, were detected after fixation and permeabilization of the cells with appropriate antibodies (see the Methods section in this article's Online Repository). After staining, cells were immediately analyzed on an LSR Fortessa flow cytometer (BD Biosciences, San Jose, Calif).

Mast cell culture and stimulation

Human cord blood–derived mast cells (CBMCs) were isolated and then maintained, as previously described.[30,31] CBMCs were preincubated with 10 ng/mL human IL-4 for 4 days and received 1 μg/mL IgE overnight before activation. CBMCs were activated in Iscove modified Dulbecco medium (IMDM)/1% normal human serum (NHS) by using either 2 μg/mL anti-human IgE for 6 hours or 2 μmol/L of the calcium ionophore A23187 for 2 hours. Supernatants were collected and stored at −80°C.

ELISA

Concentrations of IL-5 and IL-13 in cell supernatants were analyzed by using an IL-5 ELISA Duo-Set kit (R&D Systems, Minneapolis, Minn) or an IL-13 ELISA kit. Concentrations of PGD_2 in ILC2 supernatants in some experiments were determined by using the PGD_2–MOX (methoxime) ELISA Kit (Cayman Chemical).

Liquid chromatography–tandem mass spectrometry

Lipid mediators in ILC2 supernatants were detected after indicated treatments for 24 hours by using liquid chromatography–tandem mass spectrometry (LC-MS/MS). LC-MS/MS was carried out after the supernatants were spiked with the respective stable isotopes as internal standards and extracted by using ethyl acetate. A detailed description can be found in the Methods section in this article's Online Repository.

Quantitative RT-PCR

Total RNA from expanded tonsillar ILC2 cells was isolated by using the RNeasy Micro Kit (Qiagen, Hilden, Germany). cDNA was synthesized by using the iScript cDNA Synthesis Kit, and quantitative PCR reactions were performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, Calif). Primers for human transcripts for molecules important for PGD_2 metabolism and action, *COX1*, *COX2*, *HPGDS*, *LPGDS*, *HPGD*, prostaglandin D_2 receptor 1 *(DP1)*, and *CRTH2* and the housekeeping gene *RPS18*, were from Bio-Rad Laboratories. Quantitative RT-PCR (RT-qPCR) was performed in the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories).

Statistical analyses

Differences between 2 groups were analyzed by using the matched-pairs *t* test, and 3 or more groups were compared by using 1-way ANOVA for repeated measurements and the Dunnett multiple comparisons test. Analyses were performed by using GraphPad Prism 6 software (GraphPad Software, La Jolla, Calif).

RESULTS

Production of IL-5 and IL-13 by ILC2s is dependent on endogenous PGs

Recently, we showed that human ILC2s express HPGDS[32] and therefore hypothesized that ILC2s are able to produce PGs, which might be important for ILC2 activation. Because ILC2s are typically activated by cytokines, we sought to investigate the role of endogenous PGs in the context of cytokine-induced ILC2 activation. We incubated human tonsillar ILC2s with cytokines known to activate ILC2s: IL-33, TSLP, and IL-25 (each 50 ng/mL) plus IL-2 (10 U/mL). To investigate the importance of endogenous PGs in ILC2 activation, we pretreated ILC2s with flurbiprofen. Inhibition of the COX-1/2 enzymes almost completely prevented IL-5 and IL-13 secretion from cytokine-stimulated ILC2s (Fig 1, *A* and *B*). Suppression of type 2 cytokine production on a per-cell basis was confirmed by analyzing intracellular cytokine production, demonstrating that flurbiprofen inhibited the synthesis of IL-5 and IL-13 in ILC2s (Fig 1, *C-E*) without hampering cell viability (data not shown). Because IL-2 is an important costimulator of ILC2s,[4] we determined how CD25 (IL-2 receptor α) expression is affected by ILC2-derived PGs. Although stimulatory cytokines upregulated CD25 expression on ILC2s (Fig 1, *F-H*), coincubation of ILC2s with flurbiprofen largely abolished the cytokine-triggered CD25 upregulation (Fig 1, *F-H*). Because the transcription factor GATA-3 is important for maintenance and activation of ILC2s, we examined whether its expression is affected by inhibition of PG production. Indeed, inhibition of COX-1/2 by flurbiprofen reduced cytokine-induced GATA-3 upregulation (Fig 1, *I-K*).

These data suggest that PGs produced by ILC2s on cytokine stimulation are necessary for optimal ILC2 activation.

The PGD_2 pathway is turned on in stimulated human ILC2s

Based on the dramatically reduced ILC2 function on inhibition of PG production, we aimed to investigate which PGs can be released by activated ILC2s. It has already been shown that exogenous PGD_2 acts as a strong stimulator of ILC2s through engaging the CRTH2 receptor.[8] However, it has not yet been determined whether ILC2s themselves can produce PGD_2.

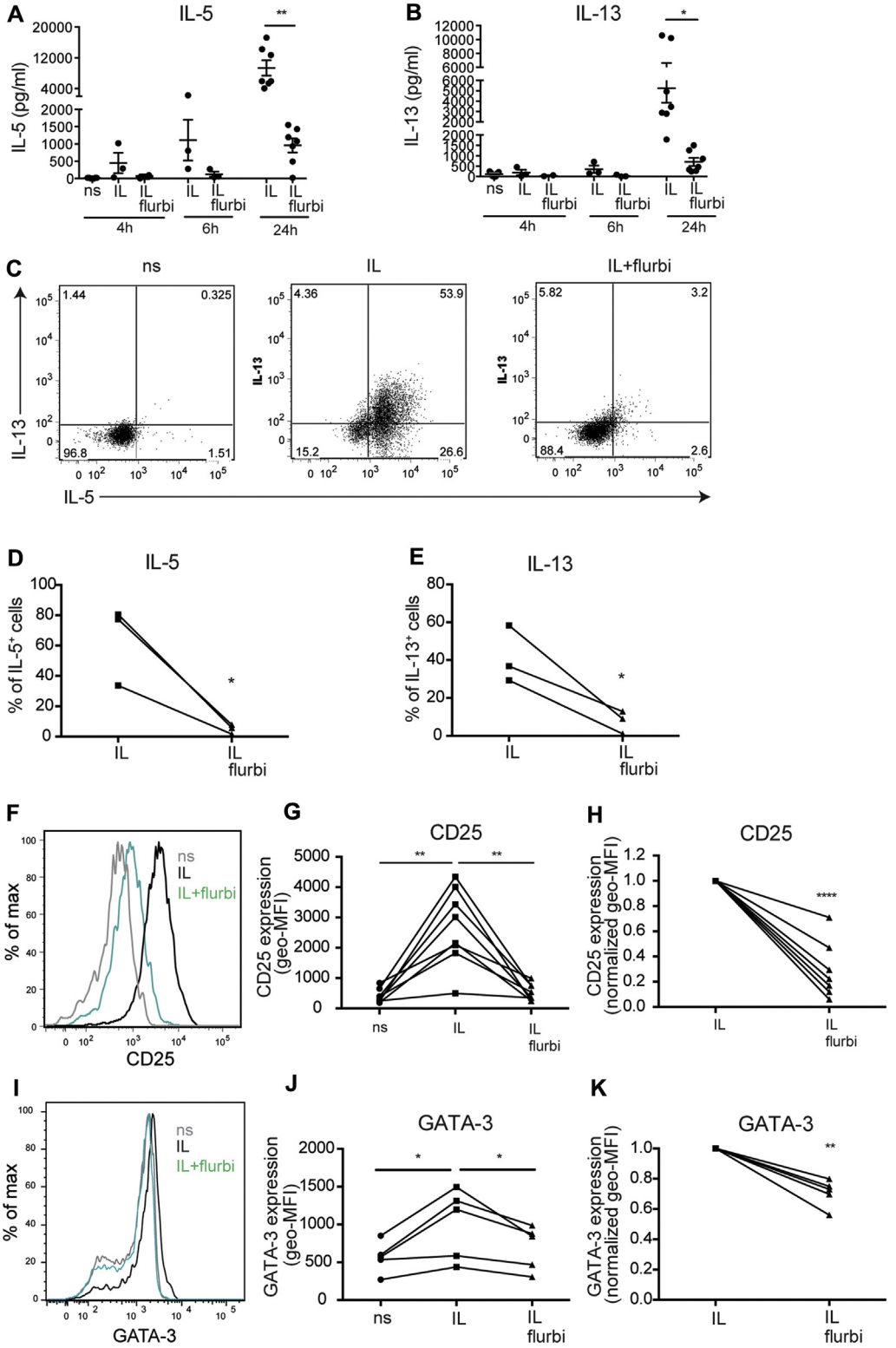


FIG 1. Production of IL-5 and IL-13 by ILC2s is dependent on endogenously produced PGs. **A** and **B,** Sorted and expanded ILC2s in the presence of IL-2 (10 U/mL) were stimulated with IL-33, IL-25, and TSLP (50 ng/mL each) or left nonstimulated for 4, 6, and 24 hours. Flurbiprofen (1 μmol/L) was used at 40 minutes before treatment. Concentrations of IL-5 (Fig 1, *A*) and IL-13 (Fig 1, *B*) in ILC2 supernatants were detected by using ELISA; graphs show individual concentrations and means ± SEMs. **P* < .05 and ***P* < .01, 3 donors for the 4- and 6-hour time points and 7 donors for 24 hours. **C,** Intracellular expression of IL-5 and IL-13 in ILC2s was assessed after 24 hours by using flow cytometry. **D** and **E,** Graphs show individual percentages of IL-5–producing (Fig 1, *D*) and IL-13–producing (Fig 1, *E*) ILC2s. **P* < .05, 3 donors. **F** and **I,** Expression of CD25 (Fig 1, *F*)

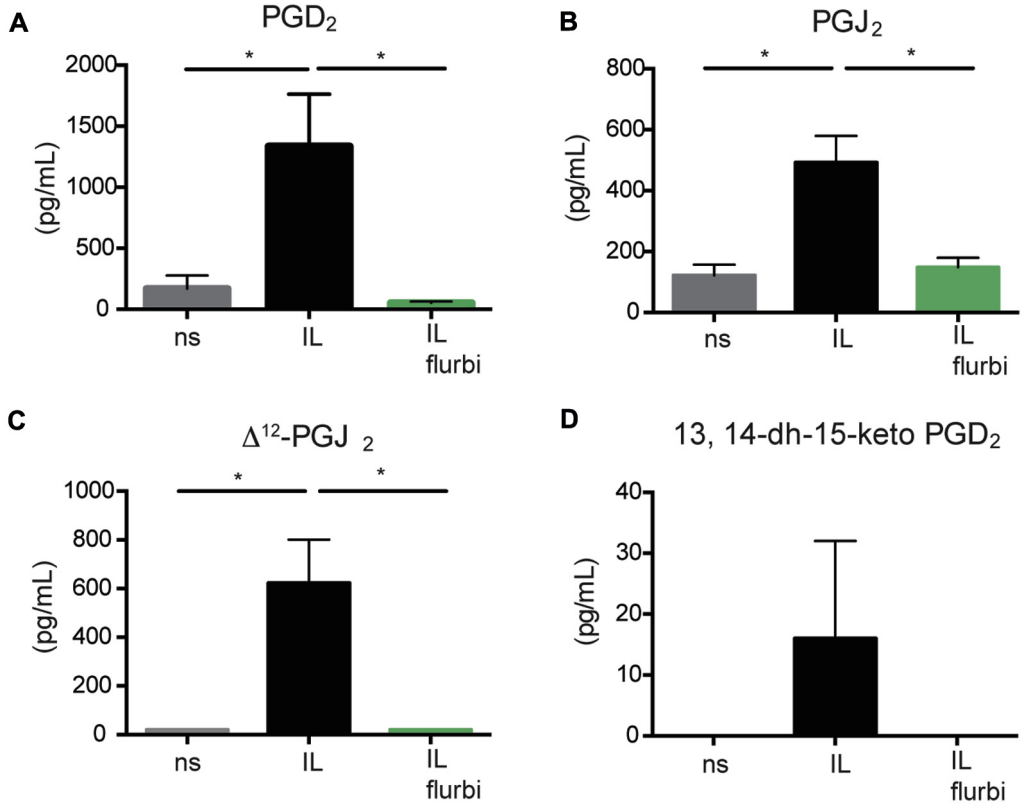


FIG 2. Human tonsillar ILC2s produce PGD_2 and its metabolites on cytokine stimulation. Sorted and expanded tonsillar ILC2s were stimulated as indicated for 24 hours with or without flurbiprofen (1 μmol/L, 40 minutes before treatment). Mediators of the PGD_2 pathway were detected in ILC2 supernatants by using LC-MS/MS. Graphs show mean + SEM concentrations of PGD_2 (*$P < .05$, 5 donors for the nonstimulated *[ns]* and IL-33, IL-25, and TSLP *[IL]* groups and 4 donors for the IL plus flurbiprofen group; **A**), PGJ_2 (*$P < .05$, 7 donors; **B**), Δ^{12}-PGJ_2 (*$P < .05$, 7 donors; **C**), and DK-PGD_2 **(D)** from 4 donors. Samples were compared by using ANOVA matched-pair measurements with the Dunnett posttest, in which other groups were compared with the IL group, except for PGD_2 data, for which ANOVA nonpaired measurement was performed. *flurbi*, Flurbiprofen; *IL*, IL-33, IL-25, and TSLP; *ns*, nonstimulated.

Therefore we collected supernatants of *in vitro*–expanded human tonsillar ILC2s and performed a highly sensitive mass spectrometric analysis for detection of lipid mediator release. Nonstimulated ILC2s did not constitutively release any lipids, whereas upon cytokine stimulation, we were able to detect PGD_2 and its metabolites PGJ_2, Δ^{12}-PGJ_2, and, in lower concentrations, DK-PGD_2 (Fig 2). Coincubation with flurbiprofen completely inhibited the release of these lipid mediators. Additionally, we detected thromboxane B_2 (TXB_2) in ILC2 supernatants, which was also completely inhibited by flurbiprofen (see Fig E1 in this article's Online Repository at www.jacionline.org). Upon cytokine stimulation, we additionally detected the PGD_2 metabolite 15-deoxy-delta-12, 14-PGJ_2 (15d-PGJ_2; data not shown). Other prostanoids, such as PGE_2, PGI_2, and metabolites, were not detected in the supernatants of stimulated ILC2s (data not shown).

To address the relative contribution of ILC2s to the PGD_2 pool, we analyzed lipid mediators produced by mast cells, which are known to be the richest source of PGD_2.[33-36] We detected high amounts of PGD_2 on IgE receptor activation of human CBMCs (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). Compared with CBMCs, ILC2s clearly produced less PGD_2, suggesting most likely an autocrine but potentially also paracrine role of PGD_2 in ILC2 activation rather than contributing to the systemic levels of PGD_2.

Furthermore, in addition to detecting PGD_2 and its metabolites in ILC2 supernatants, we analyzed gene expression of the enzymes involved in PGD_2 metabolism: COX-1, COX-2, HPGDS, and HPGD (Fig 3). Although nonstimulated ILC2s constitutively expressed *HPGDS* mRNA, they did not express *COX1* or *COX2* mRNA, providing an explanation for the lack of PG production

and GATA-3 (Fig 1, *I*) was analyzed by using flow cytometry; representative histograms are shown. **G**, **H**, **J**, and **K**, Graphs show individual geometric mean fluorescence intensity of CD25 expression (**$P < .01$, 8 donors; Fig 1, *G*) and GATA-3 expression (*$P < .05$, 5 independent experiments, 4 donors; Fig 1, *J*), and geometric mean fluorescence intensity normalized to stimulated cells of CD25 (****$P < .0001$, 8 donors; Fig 1, *H*), and GATA-3 expression (**$P < .01$, 5 independent experiments, 4 donors; Fig 1, *K*) after 24 hours of culture. *flurbi*, Flurbiprofen; *IL*, IL-33, IL-25, and TSLP; *ns*, nonstimulated.

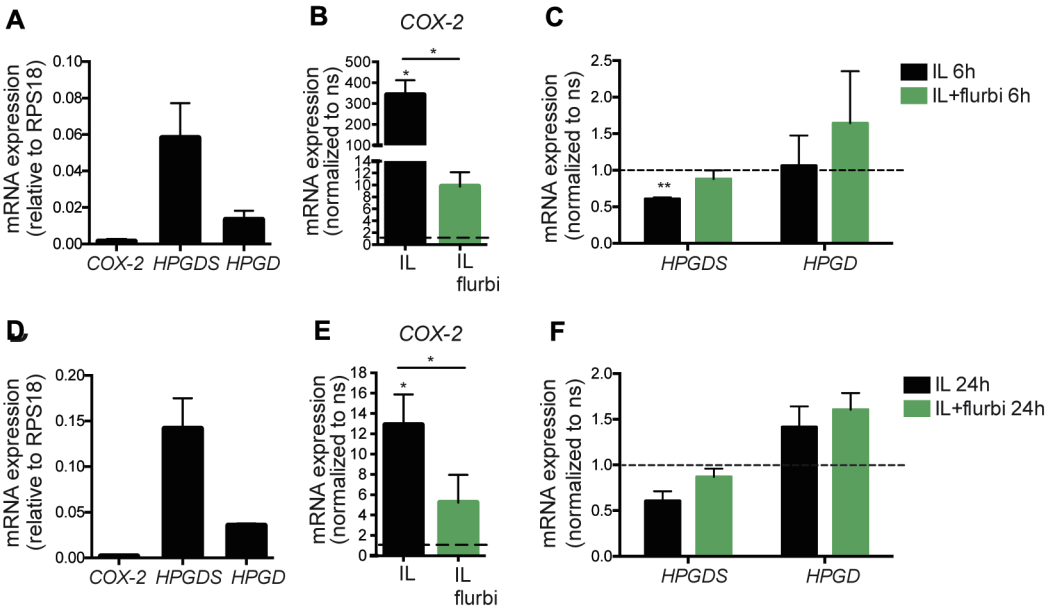


FIG 3. ILC2s express enzymes necessary for PGD_2 production and metabolism. Sorted and expanded tonsillar ILC2s were stimulated as indicated for 6 **(A-C)** and 24 **(D-F)** hours, with or without flurbiprofen (1 μmol/L, 40 minutes before treatment). Basal mRNA expression of *COX2, HPGDS,* and *HPGD* in nonstimulated ILC2s after 6 (Fig 3, *A*) and 24 (Fig 3, *D*) hours of culture were analyzed by using quantitative PCR. Data are shown as means + SEMs (data are from 4 donors). Changes of gene expression normalized to nonstimulated cells after 6 hours of stimulation (**P* < .05 between IL-33, IL-25, and TSLP *[IL]* treatment and the nonstimulated group and between IL plus flurbiprofen and IL for *COX2* and ***P* < .01 between the IL treatment and nonstimulated groups for *HPGDS*, 4 donors for COX-2 data and 3 donors for HPGDS and HPGD; Fig 3, *B* and *C*) and after 24 hours (**P* < .05 between the IL treatment and nonstimulated groups and between the IL plus flurbiprofen and IL groups, 4 donors; Fig 3, *E* and *F*) are shown as means + SEMs. *flurbi*, Flurbiprofen; *IL*, IL-33, IL-25, and TSLP; *ns*, nonstimulated.

from resting ILC2s. However, after cytokine stimulation, ILC2s upregulated *COX2* expression approximately 300-fold (Fig 3, *A*, *B*, *D*, and *E*). *COX1* expression was not induced on cytokine stimulation (data not shown). Interestingly, the COX-1/2 inhibitor flurbiprofen partially prevented upregulation of *COX2* mRNA, indicating a possible regulatory role of COX-2 activation in transcription of COX-2 in ILC2s (Fig 3, *B* and *E*). Although ILC2s expressed *HPGDS* transcripts under basal conditions (Fig 3, *A* and *D*), they lacked *LPGDS* expression (data not shown). These findings are in line with our previous observation that freshly isolated human ILC2s express *HPGDS* transcripts, as detected by using single-cell RNA sequencing.[32] Additionally, we revealed here that nonstimulated ILC2s express *HPGD* (Fig 3, *A* and *D*), the enzyme responsible for conversion of PGD_2 to DK-PGD_2.[23] Interestingly, DK-PGD_2 is a natural selective agonist of the CRTH2 receptor,[25] and we were able to detect small amounts of this PGD_2 metabolite in ILC2 supernatants (Fig 2, *D*). Cytokine stimulation caused downregulation of *HPGDS* expression after 6 hours (Fig 3, *C*) and slightly but not significantly reduced *HPGDS* expression after 24 hours (Fig 3, *F*). Coincubation with flurbiprofen did not influence *HPGDS* expression, and ILC2 stimulation did not affect the expression of *HPGD* (Fig 3, *C* and *F*).

Overall, we demonstrated that ILC2s express the enzymes required for PGD_2 synthesis, *HPGDS* and *COX2*, with the latter being highly upregulated on cytokine stimulation. As a consequence, cytokine activation of ILC2s leads to endogenous PG synthesis, as demonstrated by our detection of PGD_2 and its metabolites in the supernatant of stimulated ILC2s. Additionally, we showed that ILC2s express *HPGD* and that its product, the selective CRTH2 agonist DK-PGD_2, can be detected in ILC2 supernatants.

Activation of ILC2s on cytokine stimulation depends on endogenous PGD_2

We found that PGD_2 is the major PG released by ILC2s. To confirm that the observed inhibitory effects of flurbiprofen are due to abolished PGD_2 production and thereby reduced paracrine/autocrine action of PGD_2, we incubated ILC2s with the selective HPGDS inhibitor KMN698 and the CRTH2 antagonist CAY10471 before cytokine treatment. The HPGDS inhibitor blocked release of PGD_2 from stimulated ILC2s, showing its specific effects (Fig 4, *A*). Inhibition of PGD_2 production reduced IL-5 and IL-13 release from cytokine-stimulated ILC2s, confirming a crucial role of PGD_2 production in ILC2 stimulation (Fig 4, *B* and *C*). Furthermore, we observed that the HPGDS inhibitor reduced cytokine-induced upregulation of CD25 (Fig 4, *D-F*) and GATA-3 expression (Fig 4, *G-I*).

Our data also revealed that the selective CRTH2 antagonist almost completely abolished IL-5 and IL-13 secretion from cytokine-stimulated ILC2s (Fig 5, *A* and *B*). Furthermore, blocking of the CRTH2 receptor prevented the increase in intracellular IL-5 and IL-13 production in activated ILC2s (Fig 5, *C-E*). CRTH2 antagonism also prevented CD25 (Fig 5, *F-H*) and GATA-3 upregulation (Fig 5, *I-K*) of stimulated ILC2s. These data clearly show that endogenous PGD_2 produced by ILC2s on

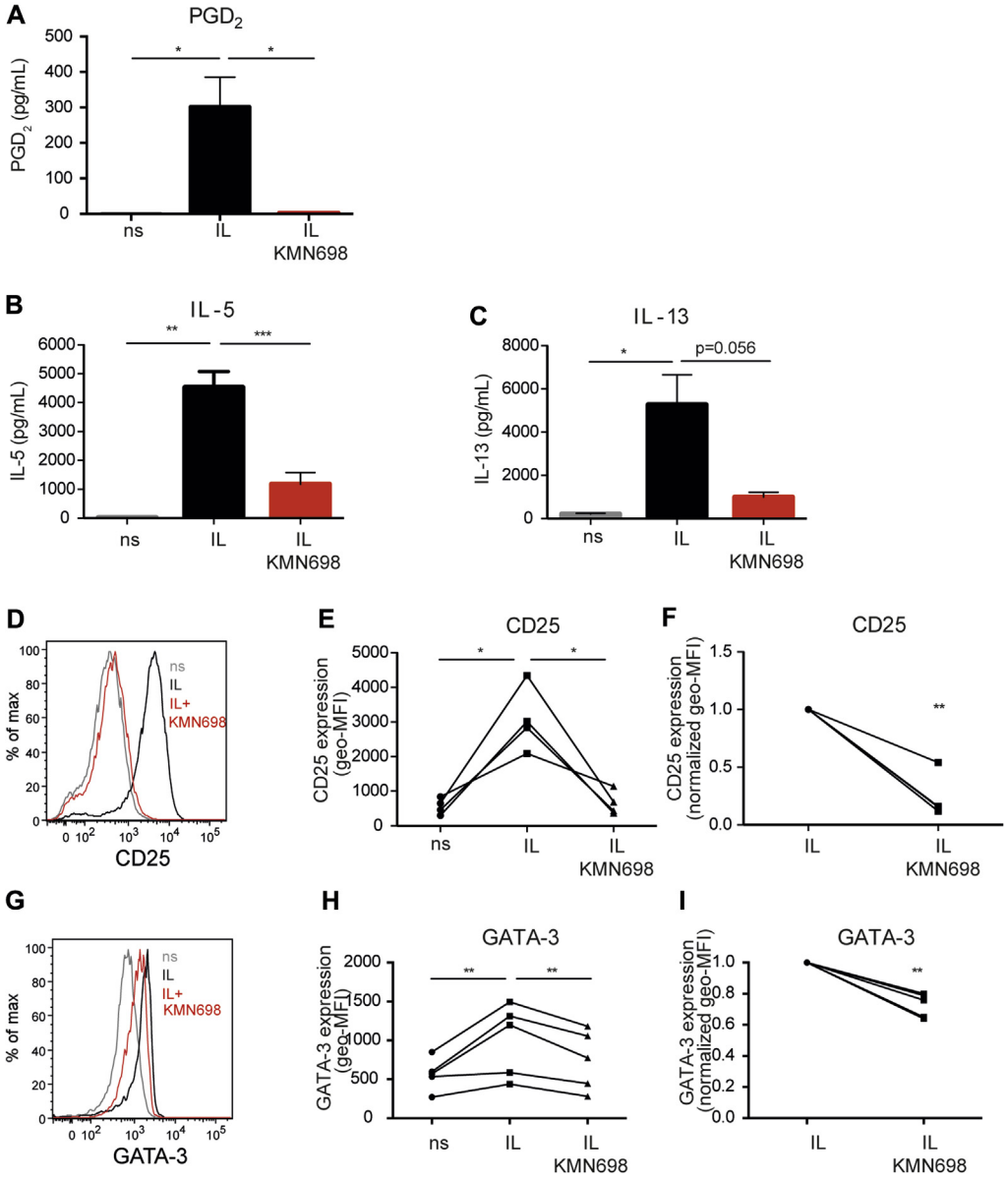


FIG 4. Endogenously produced PGD_2 is essential for ILC2 stimulation. Sorted and expanded tonsillar ILC2s were stimulated as indicated with or without the HPGDS inhibitor KMN698 (100 nmol/L, 40 minutes before treatment) for 24 hours. **A,** Concentration of released PGD_2 was measured by using ELISA; graph shows means + SEMs (*P < .05, data are from 2 donors, 4 independent experiments). **B** and **C,** Concentrations of released IL-5 (Fig 4, *B*) and IL-13 (Fig 4, *C*) were measured by using ELISA; graphs show means + SEMs (*P < .05, **P < .01, and ***P < .001, 4 donors, 5 independent experiments). **D** and **G,** Expression of CD25 (Fig 4, *D*) and GATA-3 (Fig 4, *G*) was analyzed by using flow cytometry; representative histograms are shown. **E F, H,** and **I,** Graphs show individual geometric mean fluorescence intensity of CD25 (*P < .05, 3 donors, 4 independent experiments; Fig 4, *E*) and GATA-3 expression (**P < .01, 4 donors, 5 independent experiments; Fig 4, *H*) and geometric mean fluorescence intensity normalized to stimulated cells of CD25 (**P < .01, 3 donors, 4 independent experiments; Fig 4, *F*) and GATA-3 expression (**P < .01, 4 donors, 5 independent experiments; Fig 4, *I*). *IL*, IL-33, IL-25, and TSLP; *ns*, nonstimulated.

cytokine stimulation is essential for activation of ILC2s acting through the CRTH2 receptor.

It has been demonstrated that exogenous PGD_2 directly stimulates ILC2s through binding to the CRTH2 receptor and that such engagement leads to downregulation of CRTH2 expression at both the mRNA and protein levels.[8] Thus we set out to investigate the role of endogenous PGs in regulating expression of CRTH2 in ILC2s.

Although we detected high levels of *CRTH2* transcripts in nonstimulated ILC2s, only low expression of the *DP1* receptor could be found (see Fig E3, *A* and *B*, in this article's Online Repository at www.jacionline.org). Cytokine stimulation significantly

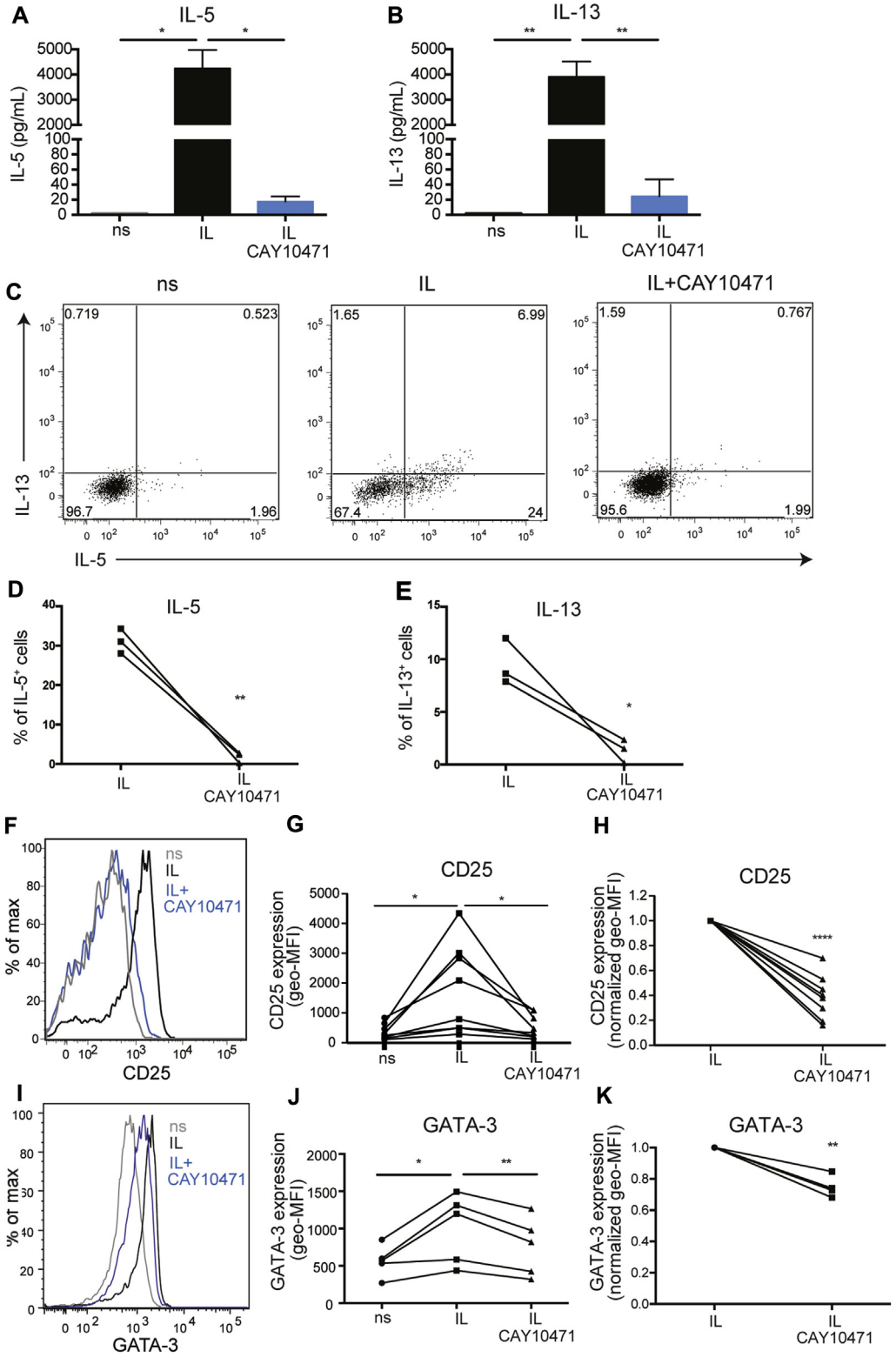


FIG 5. Endogenously produced PGD_2 acts through the CRTH2 receptor on ILC2s. **A** and **B,** Sorted and expanded ILC2s were treated as indicated in the presence or absence of the CRTH2 selective antagonist CAY10471 (1 μmol/L, 20 minutes before treatment) for 24 hours. **A** and **B,** Concentrations of IL-5 (Fig 5, *A*) and IL-13 (Fig 5, *B*) in ILC2 supernatants were detected by using ELISA; graphs show means + SEMs (*$P < .05$ and **$P < .01$, 4 donors). **C-E,** Intracellular expression of IL-5 and IL-13 in ILC2s was assessed after 24 hours by using flow cytometry; graphs show individual percentages of IL-5–producing (Fig 5, *D*) and IL-13–producing (Fig 5, *E*) ILC2s (*$P < .05$ and **$P < .01$, 3 donors). **F** and **I,** Expression of CD25 (Fig 5, *F*) and GATA-3 (Fig 5, *I*) was analyzed by using flow cytometry; representative histograms are shown. **G, H, J,**

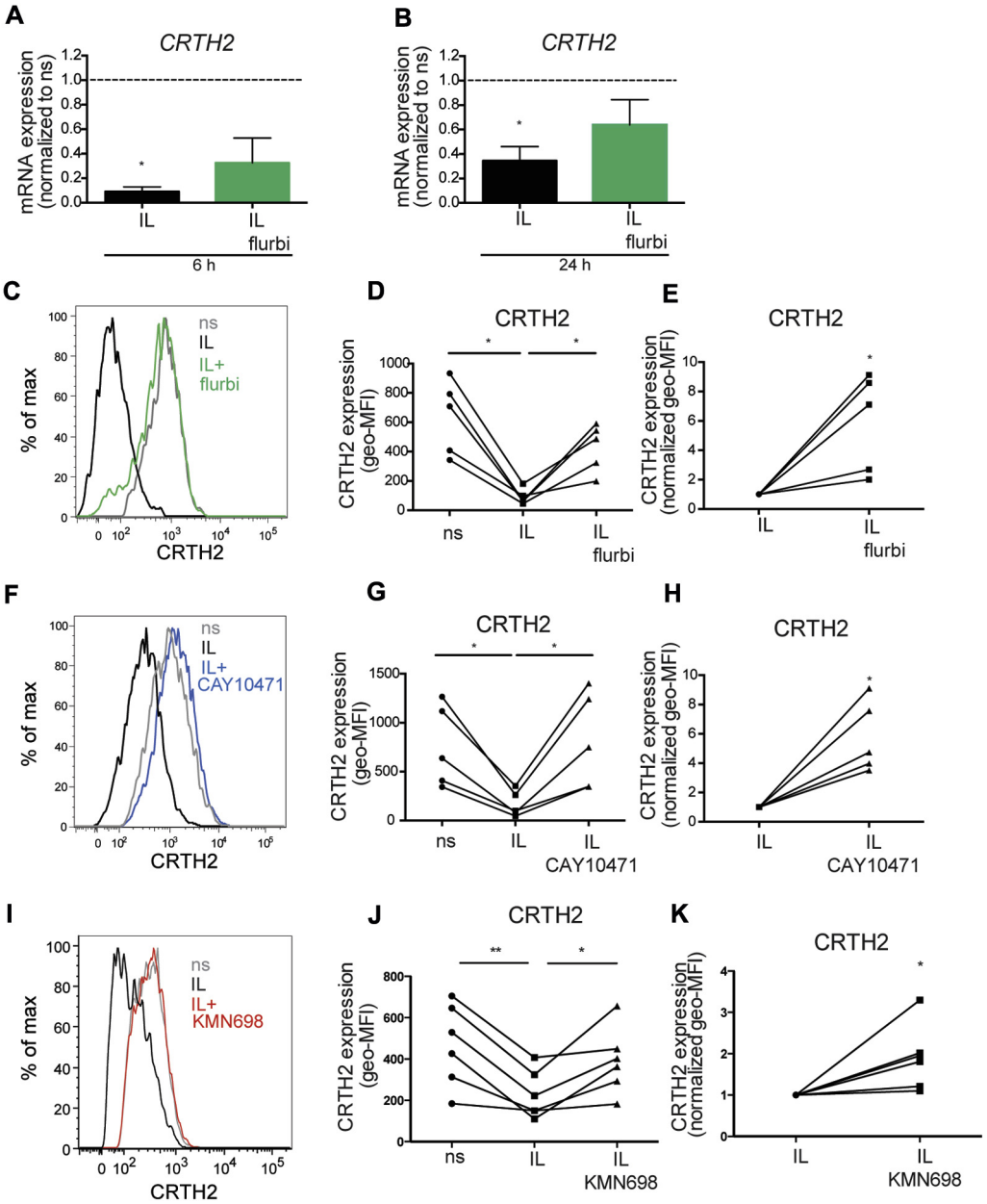


FIG 6. Cytokine-induced downregulation of CRTH2 is prevented by inhibition of endogenous PG production or antagonism of CRTH2 receptor. Tonsillar ILC2s were stimulated as indicated for 6 or 24 hours, with pretreatment of flurbiprofen (1 μmol/L, 40 min; **A-E**), CAY10471 (1 μmol/L, 20 minutes; **F-H**), or the HPGDS inhibitor KMN698 (100 nmol/L, 40 minutes; **I-K**). Changes in *CRTH2* mRNA expression normalized to nonstimulated cells after 6 hours of stimulation (**P* < .05, 3 donors; Fig 6, *A*) and after 24 hours (**P* < .05, 4 donors; Fig 6, *B*) are shown as means + SEMs. Fig 6, *C*, *F*, and *I*, CRTH2 protein expression was analyzed by using flow cytometry. Graphs show individual geometric mean fluorescence intensity (Fig 6, *D*, *G*, and *J*) and individual geometric mean fluorescence intensity normalized to stimulated cells of CRTH2 expression after 24 hours of culture (Fig 6, *E*, *H*, and *K*; **P* < .05 and ***P* < .01, for flurbiprofen and CAY10471 data are from 5 donors and for KMN698 from 4 donors, 6 independent experiments). *flurbi*, Flurbiprofen; *IL*, IL-33, IL-25, and TSLP; *MFI*, mean fluorescence intensity; *ns*, nonstimulated.

and **K,** Graphs show individual geometric mean fluorescence intensity of CD25 (**P* < .05, 7 donors, 8 independent experiments; Fig 5, *G*) and GATA-3 expression (**P* < .05 and ***P* < .01, 5 independent experiments, 4 donors; Fig 5, *J*) and geometric mean fluorescence intensity normalized to stimulated cells of CD25 (*****P* < .0001, 7 donors, 8 independent experiments; Fig 5, *H*) and GATA-3 expression (***P* < .01, 4 donors, 5 independent experiments; Fig 5, *K*) after 24 hours of culture. *IL*, IL-33, IL-25, and TSLP; *MFI*, mean fluorescence intensity; *ns*, nonstimulated.

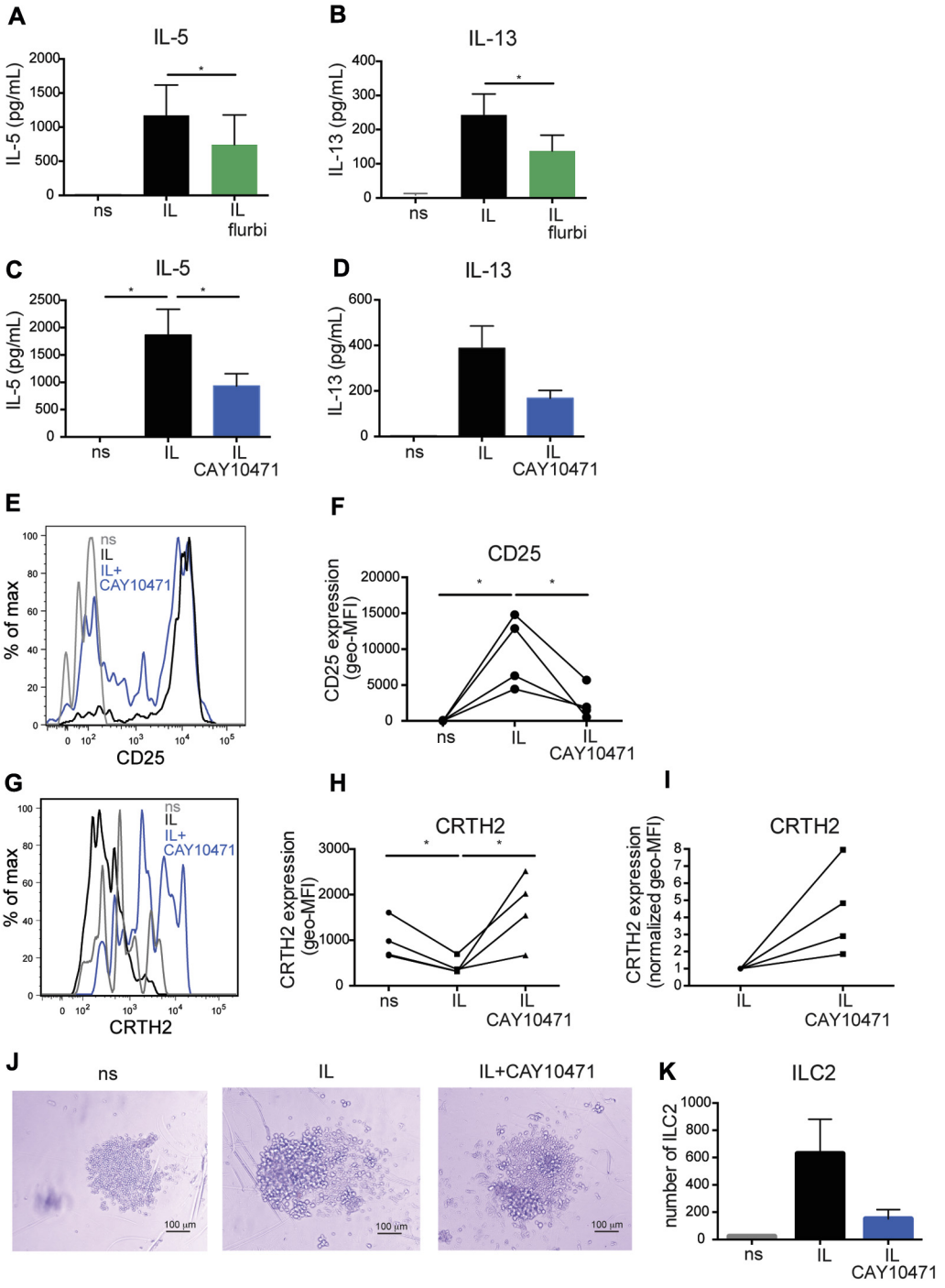


FIG 7. Endogenously produced PGD_2 contributes to activation of freshly sorted tonsillar and blood ILC2s on cytokine stimulation. Freshly sorted human tonsillar (**A** and **B**) and blood **(C-K)** ILC2s were incubated as indicated for 5 days in the presence or absence of flurbiprofen (1 μmol/L; Fig 7, *A* and *B*) or CAY10471 (1 μmol/L; Fig 7, *C-K*). Concentrations of released IL-5 from tonsillar ILC2s (Fig 7, *A*) and blood ILC2s (Fig 7, *C*) and IL-13 from tonsillar ILC2s (Fig 7, *B*) and blood ILC2s (Fig 7, *D*) were measured by means of ELISA; graphs show means + SEMs (**P* < .05, 4 donors for blood ILC2s and 3 donors for tonsillar ILC2s). Fig 7, *E* and *G*, Expression of CD25 and CRTH2 in freshly sorted blood ILC2s was analyzed by using flow cytometry; representative histograms are shown. Graphs show individual geometric mean fluorescence intensity of CD25 expression in blood ILC2s after 5 days of culture (**P* < .05, 4 donors; Fig 7, *F*) and of CRTH2 expression (**P* < .05, 4 donors; Fig 7, *H*) and individual geometric mean fluorescence intensity of CRTH2 expression normalized to stimulated cells (4 donors; Fig 7, *I*). Fig 7, *J*, Images of freshly sorted blood ILC2s after 5 days of treatments (×10 magnification, *scale bar* = 100 μm). Fig 7, *K*, Numbers of freshly sorted blood ILC2s acquired by using flow cytometry from 4 donors. *flurbi*, Flurbiprofen; *IL*, IL-33, IL-25, and TSLP; *MFI*, mean fluorescence intensity; *ns*, nonstimulated.

reduced *CRTH2* mRNA expression in ILC2s, which was partially but not significantly reversed on inhibition of COX-1/2 by using flurbiprofen (Fig 6, *A* and *B*). *DP1* expression was not modulated by cytokine stimulation after 6 hours but was downregulated after 24 hours of stimulation (see Fig E3, *C* and *D*). Moreover, we showed that stimulatory cytokines also reduce CRTH2 protein expression on the cell surface (Fig 6). Of note, pretreatment of ILC2s with either flurbiprofen (Fig 6, *C-E*), CRTH2 antagonist (Fig 6, *F-H*), or a HPGDS inhibitor (Fig 6, *I-K*) reversed cell-surface downregulation of CRTH2 to the expression level of nonstimulated ILC2s. These findings strongly suggest that endogenously released PGD_2 is responsible for cytokine-induced reduction of CRTH2 expression.

Endogenously produced PGD_2 is crucial for cytokine-induced activation of freshly isolated blood and tonsillar ILC2s

Next, we aimed to confirm our findings based on *in vitro*–expanded tonsillar ILC2s by using freshly isolated and sort-purified ILC2s. Additionally, we extended our studies to include not only tissue (tonsillar) ILC2s but also blood ILC2s, which display a more naive phenotype.[37] ILC2s freshly isolated from tonsils (Fig 7, *A* and *B*) and blood (Fig 7, *C-K*) elaborated IL-5 and IL-13 after 5 days of *in vitro* culture with IL-2, IL-25, and IL-33 plus TSLP. As observed in *in vitro*–expanded ILC2s, cotreatment with flurbiprofen (Fig 7, *A* and *B*) or the CRTH2 antagonist (Fig 7, *C* and *D*) significantly reduced IL-5 and IL-13 secretion.

We also determined changes in CD25 expression in freshly isolated blood ILC2s. Cytokine stimulation markedly upregulated CD25 expression in ILC2s, which was prevented by means of preincubation with the CRTH2 antagonist (Fig 7, *E* and *F*). Similar to our observation with *in vitro*–expanded ILC2s, cytokine-induced downregulation of CRTH2 in freshly isolated ILC2s was reversed by coincubation with the CRTH2 antagonist (Fig 7, *G-I*). Furthermore, we captured microscopic images of freshly isolated ILC2s that were cultured for 5 days. These images revealed that specific inhibition of the CRTH2 receptor allowed less prominent cell growth compared with the treatment with only stimulatory cytokines (Fig 7, *J*). The microscopic analysis was confirmed by quantification of the number of viable ILC2s by using flow cytometry (Fig 7, *K*), which showed that blocking of CRTH2 prevented cytokine-induced increase of ILC2 numbers.

Altogether, we show here that endogenously produced PGD_2 is essential in complete activation of freshly isolated human blood and tissue ILC2s.

DISCUSSION

ILC2s require several signals for optimal activation. Synergy between the signal transducer and activator of transcription activators TSLP and IL-2, as well as the nuclear factor κB stimulators IL-33 and IL-25, potently activates human ILC2s.[3,38] Interestingly, ILC2s can also be activated by the lipid mediators PGD_2, LTD_4, and LTE_4.[6-8] The PGD_2-CRTH2 axis is described to be important in identification and function of ILC2s.[7,8,39] Because ILC2s are crucial in the initial steps and maintenance of type 2 immune responses during allergic inflammation,[1,40] better understanding of regulation of these cells is required for development of novel therapeutic approaches. Although recent studies highlight the role of lipid mediators in ILC2 function,[5] it is still unknown whether ILC2s are able to produce PG levels on their own. We demonstrate here that upon cytokine stimulation, ILC2s, which constitutively express *HPGDS*, upregulate *COX2* and start producing PGD_2, which in turn leads to engagement of the CRTH2 receptor. We prove that this endogenous PGD_2-CRTH2 pathway is essential for cytokine-induced ILC2 activation.

In our study we detected PGD_2 and its metabolites PGJ_2, Δ^{12}-PGJ_2, and 15d-PGJ_2, as well as TXB_2, in supernatants of stimulated ILC2s. Importantly, we observed that ILC2s exclusively produce these lipid mediators. The COX-1/2 inhibitor flurbiprofen inhibited prostanoid production of ILC2s. To our surprise, flurbiprofen almost completely abolished IL-5 and IL-13 production of ILC2s on cytokine stimulation, suggesting an interesting mechanism of ILC2 activation by endogenously produced PGs.

We were able to confirm *HPGDS* mRNA expression in *in vitro*–expanded tonsillar ILC2s, as previously shown in freshly isolated ILC2s.[32] HPGDS is also expressed by other immune cells, such as mast cells, T_H2 and dendritic cells, macrophages, and eosinophils, which are all able to produce PGD_2.[14,16,22] Even though *HPGDS* is constitutively expressed by ILC2s, nonstimulated ILC2s do not produce PGD_2, which is most probably due to the lack of *COX1* and *COX2* expression. In contrast, mast cells express COX-1 and release PGD_2 already under basal conditions, whereas COX-2 can be induced for instance on immunologic activation with IgE crosslinking.[15] Here we show that stimulation of ILC2s with the cytokines IL-33, TSLP, and IL-25 plus IL-2 induces approximately 300-fold upregulation of *COX2* mRNA expression. COX-2 is the rate-limiting enzyme in PGD_2 production because it provides the substrate PGH_2 for the already highly expressed HPGDS in ILC2s. We used a cytokine mixture for activating ILC2s because this approach ensures full stimulation of human ILC2s.[3,12]

We have not investigated the effect of single cytokines on COX-2 induction. However, IL-33, a member of the IL-1 family, has previously been shown to induce PGD_2 synthesis in mast cells,[41] and a recent study demonstrates that TSLP synergistically with IL-33 contributes to PGD_2 production by human mast cells.[36] Similar to ILC2s, T_H2 cells can also upregulate COX-2 on different stimuli and hence are capable of PGD_2 production.[16,42] Interestingly, the COX inhibitor flurbiprofen also attenuated upregulation of *COX2* mRNA in stimulated ILC2s. It is likely that endogenous PGD_2, acting through a positive feedback loop, further contributes to *COX2* upregulation and, consequently, inhibition of its production reduces *COX2* expression in ILC2s. Positive feedback regulation of PGs on *COX2* expression has been shown.[43]

Importantly, we demonstrate that endogenous PGD_2 is responsible for ILC2 activation on cytokine stimulation by using an HPGDS inhibitor and a selective antagonist of CRTH2. It was previously shown that exogenous PGD_2 treatment of ILC2s led to downregulation of CRTH2 expression both at the mRNA and protein levels.[8] Furthermore, PGD_2 was reported to induce internalization and desensitization of CRTH2 in HEK293 cells and in human eosinophils.[25,44] We observed a reduction in CRTH2 mRNA and protein expression on cytokine stimulation of ILC2s. Most likely, restored cell-surface expression of CRTH2 after flurbiprofen, CRTH2 antagonist, and HPGDS inhibitor treatment is due to hampering of endogenous PGD_2 production and

CRTH2 binding. Thus in addition to the positive regulatory role of ILC2-derived PGs in COX-2 upregulation, there is an additional self-limiting mechanism in the PGD_2-caused reduction of CRTH2 expression in activated ILC2s. Furthermore, because reduced *CRTH2* mRNA expression on cytokine stimulation was not completely reversed by flurbiprofen, it is likely that ILC2-stimulatory cytokines can also directly affect *CRTH2* gene expression in a PGD_2-independent manner.

We show that ILC2s also express *HPGD*, and we detected the product of the enzyme DK-PGD_2 in ILC2 supernatants. DK-PGD_2 is a naturally occurring selective CRTH2 agonist that can activate human T_H2 cells and eosinophils.[13,25] Additionally, other PGD_2 metabolites present in ILC2 supernatants, such as PGJ_2, Δ^{12}-PGJ_2, and 15d-PGJ_2, can also mediate their effects through the CRTH2 receptor.[26,27] These metabolites, along with PGD_2, could be additionally responsible for CRTH2 receptor–dependent ILC2 activation. 15d-PGJ_2, in addition to exerting its effects through the CRTH2 receptor, also binds to the peroxisome proliferator-activated receptor γ (PPAR-γ). Although PPAR-γ activation can lead to anti-inflammatory effects,[45] other studies showed that it triggered human eosinophil chemotaxis[46] and the type 2 response in mouse T_H2 cells and ILC2s.[47] Hence the 15d-PGJ_2–PPAR-γ axis might also be partly involved in ILC2 activation. We show that ILC2s also express low levels of *DP1* receptor. However, because of the powerful inhibitory effect of the CRTH2 antagonist, it is unlikely that the DP1 receptor is involved in ILC2 activation, which is in agreement with earlier studies.[11]

In addition to metabolites of the PGD_2 pathway, we also detected low levels of TXB_2; although there is no evidence yet for thromboxane receptor expression on ILC2s, thromboxane metabolites still could play some role in ILC2 activation because they have been shown to act as CRTH2 agonists.[48]

Importantly, we confirmed the role of endogenous PGD_2 in freshly isolated ILC2s by culturing these cells with cytokines and COX-1/2 inhibitor or CRTH2 antagonists for 5 days. We chose this rather long incubation time because we have observed that freshly isolated, compared with *in vitro*–expanded, ILC2s require that for activation. In comparison with *in vitro*–expanded ILC2s, the effect of COX-1/2 inhibition and CRTH2 antagonism on freshly isolated cells was more modest, indicating that *in vitro* expansion of ILC2s leads to a greater dependence on PGD_2 rather than the cytokines themselves. An alternative explanation could be that during this long incubation time, the reversible inhibitory effects of the CRTH2 antagonist and flurbiprofen were less pronounced. Nevertheless, we confirmed that also freshly isolated ILC2s use endogenous PGD_2 as a mechanism for cytokine-induced activation.

As an intermediate step in ILC2 activation, we investigated IL-2 receptor α (CD25) receptor expression because IL-2 is an important costimulator of ILC2s.[4,7] Suppression of cytokine-induced CD25 upregulation by the CRTH2 antagonist and flurbiprofen indicates that regulation of CD25 expression is one of the downstream mechanisms of the PGD_2-CRTH2 pathway. Furthermore, we showed that endogenous PGD_2 is of importance for GATA-3 upregulation.

In our *in vitro* system, we analyzed purified tonsillar and blood ILC2s. It is possible that *in vivo* PGD_2 released from other cellular sources, such as mast cells, can have additional effects on ILC2s. However, in early innate immune responses, the paracrine/autocrine effects of PGD_2 produced by stimulated ILC2s might be crucial.

In conclusion, we revealed that cytokine-stimulated ILC2 activation largely depends on endogenous PGD_2 production. We showed that human ILC2s express *HPGDS*; upregulate *COX2* on stimulation with IL-33, TSLP, and IL-25 plus IL-2; and consequently produce PGD_2. We could also demonstrate that endogenously produced PGD_2 and its metabolites, acting through the CRTH2 receptor, are essential for CD25 upregulation and subsequent production of IL-5 and IL-13 in cytokine-stimulated ILC2s. Our data reveal a novel mechanism by which ILC2s critically depend on endogenously produced PGD_2 induced by stimulating cytokines for optimal activation. Hence our data highlight the importance of therapeutic CRTH2 antagonists in control of allergic inflammation.

We thank the Department of Medicine, Huddinge Flow Cytometry Facility, Karolinska Institutet, for allowing us to use the cell sorters.

Key messages

- **ILC2s express HPGDS, upregulate COX-2, and consequently produce PGD_2 upon cytokine stimulation**
- **Endogenously produced PGD_2, acting through the CRTH2 receptor, is responsible for cytokine-induced activation of ILC2s.**
- **CRTH2 antagonists prevent cytokine-induced ILC2 activation and might serve as promising therapeutic tools.**

REFERENCES

1. Mjösberg J, Spits H. Human innate lymphoid cells. J Allergy Clin Immunol 2016; 138:1265-76.
2. Hazenberg MD, Spits H. Human innate lymphoid cells. Blood 2014;124:700-9.
3. Mjösberg J, Bernink J, Golebski K, Karrich JJ, Peters CP, Blom B, et al. The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. Immunity 2012;37:649-59.
4. 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.
5. Konya V, Mjösberg J. Lipid mediators as regulators of human ILC2 function in allergic diseases. Immunol Lett 2016;179:36-42.
6. 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.
7. Salimi M, Stoger L, Liu W, Go S, Pavord I, Klenerman P, et al. Cysteinyl leukotriene E4 activates human group 2 innate lymphoid cells and enhances the effect of prostaglandin D2 and epithelial cytokines. J Allergy Clin Immunol 2017;140: 1090-100.e11.
8. Xue L, Salimi M, Panse I, Mjösberg 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.
9. 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.
10. Wojno ED, Monticelli LA, Tran SV, Alenghat T, Osborne LC, Thome JJ, et al. The prostaglandin D(2) receptor CRTH2 regulates accumulation of group 2 innate lymphoid cells in the inflamed lung. Mucosal Immunol 2015;8:1313-23.
11. Barnig C, Cernadas M, Dutile S, Liu X, Perrella MA, Kazani S, et al. Lipoxin A4 regulates natural killer cell and type 2 innate lymphoid cell activation in asthma. Sci Transl Med 2013;5:174ra26.
12. Mjösberg 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.
13. Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, et al. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. J Exp Med 2001;193: 255-61.

14. Shimura C, Satoh T, Igawa K, Aritake K, Urade Y, Nakamura M, et al. Dendritic cells express hematopoietic prostaglandin D synthase and function as a source of prostaglandin D2 in the skin. Am J Pathol 2010;176:227-37.
15. Dichlberger A, Schlager S, Maaninka K, Schneider WJ, Kovanen PT. Adipose triglyceride lipase regulates eicosanoid production in activated human mast cells. J Lipid Res 2014;55:2471-8.
16. Mitson-Salazar A, Yin Y, Wansley DL, Young M, Bolan H, Arceo S, et al. Hematopoietic prostaglandin D synthase defines a proeosinophilic pathogenic effector human T(H)2 cell subpopulation with enhanced function. J Allergy Clin Immunol 2016;137:907-18.e9.
17. Zayed N, Li X, Chabane N, Benderdour M, Martel-Pelletier J, Pelletier JP, et al. Increased expression of lipocalin-type prostaglandin D2 synthase in osteoarthritic cartilage. Arthritis Res Ther 2008;10:R146.
18. Seo MJ, Oh DK. Prostaglandin synthases: molecular characterization and involvement in prostaglandin biosynthesis. Prog Lipid Res 2017;66:50-68.
19. Nantel F, Fong C, Lamontagne S, Wright DH, Giaid A, Desrosiers M, et al. Expression of prostaglandin D synthase and the prostaglandin D2 receptors DP and CRTH2 in human nasal mucosa. Prostaglandins Other Lipid Mediat 2004;73:87-101.
20. Jandl K, Stacher E, Balint Z, Sturm EM, Maric J, Peinhaupt M, et al. Activated prostaglandin D2 receptors on macrophages enhance neutrophil recruitment into the lung. J Allergy Clin Immunol 2016;137:833-43.
21. Luna-Gomes T, Magalhaes KG, Mesquita-Santos FP, Bakker-Abreu I, Samico RF, Molinaro R, et al. Eosinophils as a novel cell source of prostaglandin D2: autocrine role in allergic inflammation. J Immunol 2011;187:6518-26.
22. Pettipher R, Hansel TT, Armer R. Antagonism of the prostaglandin D2 receptors DP1 and CRTH2 as an approach to treat allergic diseases. Nat Rev Drug Discov 2007;6:313-25.
23. Liu X, Zhang S, Arora JS, Snyder NW, Shah SJ, Blair IA. 11-Oxoeicosatetraenoic acid is a cyclooxygenase-2/15-hydroxyprostaglandin dehydrogenase-derived antiproliferative eicosanoid. Chem Res Toxicol 2011;24:2227-36.
24. Gheorghe KR, Sadique S, Leclerc P, Idborg H, Wobst I, Catrina AI, et al. Limited effect of anti-rheumatic treatment on 15-prostaglandin dehydrogenase in rheumatoid arthritis synovial tissue. Arthritis Res Ther 2012;14:R121.
25. Sedej M, Schroder R, Bell K, Platzer W, Vukoja A, Kostenis E, et al. D-type prostanoid receptor enhances the signaling of chemoattractant receptor-homologous molecule expressed on T(H)2 cells. J Allergy Clin Immunol 2012;129:492-500, e1-9.
26. Heinemann A, Schuligoi R, Sabroe I, Hartnell A, Peskar BA. Delta 12-prostaglandin J2, a plasma metabolite of prostaglandin D2, causes eosinophil mobilization from the bone marrow and primes eosinophils for chemotaxis. J Immunol 2003;170:4752-8.
27. Schuligoi R, Sturm E, Luschnig P, Konya V, Philipose S, Sedej M, et al. CRTH2 and D-type prostanoid receptor antagonists as novel therapeutic agents for inflammatory diseases. Pharmacology 2010;85:372-82.
28. Peinhaupt M, Sturm EM, Heinemann A. Prostaglandins and their receptors in eosinophil function and as therapeutic targets. Front Med (Lausanne) 2017;4:104.
29. Konya V, Czarnewski P, Forkel M, Rao A, Kokkinou E, Villablanca EJ, et al. Vitamin D downregulates the IL-23 receptor pathway in human mucosal group 3 innate lymphoid cells. J Allergy Clin Immunol 2018;141:279-92.
30. Xiang Z, Moller C, Nilsson G. IgE-receptor activation induces survival and Bfl-1 expression in human mast cells but not basophils. Allergy 2006;61:1040-6.
31. Gela A, Kasetty G, Jovic S, Ekoff M, Nilsson G, Morgelin M, et al. Eotaxin-3 (CCL26) exerts innate host defense activities that are modulated by mast cell proteases. Allergy 2015;70:161-70.
32. Bjorklund AK, Forkel M, Picelli S, Konya V, Theorell J, Friberg D, et al. Corrigendum: the heterogeneity of human CD127(+) innate lymphoid cells revealed by single-cell RNA sequencing. Nat Immunol 2016;17:740.
33. Okayama Y, Hagaman DD, Metcalfe DD. A comparison of mediators released or generated by IFN-gamma-treated human mast cells following aggregation of Fc gamma RI or Fc epsilon RI. J Immunol 2001;166:4705-12.
34. Xue L, Barrow A, Pettipher R. Interaction between prostaglandin D and chemoattractant receptor-homologous molecule expressed on Th2 cells mediates cytokine production by Th2 lymphocytes in response to activated mast cells. Clin Exp Immunol 2009;156:126-33.
35. Frandsen PM, Krohn IJ, Hoffmann HJ, Schiotz PO. The influence of IgE on cultured human mast cells. Allergy Asthma Immunol Res 2013;5:409-14.
36. 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.
37. Forkel M, Berglin L, Kekalainen E, Carlsson A, Svedin E, Michaelsson J, et al. Composition and functionality of the intrahepatic innate lymphoid cell-compartment in human nonfibrotic and fibrotic livers. Eur J Immunol 2017;47:1280-94.
38. Duerr CU, Fritz JH. Regulation of group 2 innate lymphoid cells. Cytokine 2016;87:1-8.
39. Huang T, Hazen M, Shang Y, Zhou M, Wu X, Yan D, et al. Depletion of major pathogenic cells in asthma by targeting CRTh2. JCI Insight 2016;1:e86689.
40. Dhariwal J, Cameron A, Trujillo-Torralbo MB, Del Rosario A, Bakhsoliani E, Paulsen M, et al. Mucosal type 2 innate lymphoid cells are a key component of the allergic response to aeroallergens. Am J Respir Crit Care Med 2017;195:1586-96.
41. Moulin D, Donze O, Talabot-Ayer D, Mezin F, Palmer G, Gabay C. Interleukin (IL)-33 induces the release of pro-inflammatory mediators by mast cells. Cytokine 2007;40:216-25.
42. Tanaka K, Ogawa K, Sugamura K, Nakamura M, Takano S, Nagata K. Cutting edge: differential production of prostaglandin D2 by human helper T cell subsets. J Immunol 2000;164:2277-80.
43. Vichai V, Suyarnsesthakorn C, Pittayakhajonwut D, Sriklung K, Kirtikara K. Positive feedback regulation of COX-2 expression by prostaglandin metabolites. Inflamm Res 2005;54:163-72.
44. Gallant MA, Slipetz D, Hamelin E, Rochdi MD, Talbot S, de Brum-Fernandes AJ, et al. Differential regulation of the signaling and trafficking of the two prostaglandin D2 receptors, prostanoid DP receptor and CRTH2. Eur J Pharmacol 2007;557:115-23.
45. Scher JU, Pillinger MH. 15d-PGJ2: the anti-inflammatory prostaglandin? Clin Immunol 2005;114:100-9.
46. Kobayashi Y, Ueki S, Mahemuti G, Chiba T, Oyamada H, Saito N, et al. Physiological levels of 15-deoxy-Delta12,14-prostaglandin J2 prime eotaxin-induced chemotaxis on human eosinophils through peroxisome proliferator-activated receptor-gamma ligation. J Immunol 2005;175:5744-50.
47. Chen T, Tibbitt CA, Feng X, Stark JM, Rohrbeck L, Rausch L, et al. PPAR-gamma promotes type 2 immune responses in allergy and nematode infection. Sci Immunol 2017;2.
48. Bohm E, Sturm GJ, Weiglhofer I, Sandig H, Shichijo M, McNamee A, et al. 11-Dehydro-thromboxane B2, a stable thromboxane metabolite, is a full agonist of chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2) in human eosinophils and basophils. J Biol Chem 2004;279:7663-70.

METHODS

Isolation and flow cytometric sorting of tonsillar and blood ILC2s

Human tonsils were freshly received from tonsillectomies of patients with obstructive sleep apnea syndrome at the Ear Nose Throat Clinic at the Karolinska University Hospital, Huddinge, Sweden. Collection and use of tonsils were approved by the regional ethical board at the Karolinska Institutet. Buffy coats were provided by the blood bank at the Karolinska University Hospital Huddinge and were collected according to the approval of the regional ethical board at Karolinska Institutet. Isolation of ILCs from tonsils was performed, as previously described.[E1] Briefly, tonsils were dissected into small pieces, ground through a cell strainer, and centrifuged. After Lymphoprep centrifugation (STEMCELL Technologies, Vancouver, British Columbia, Canada), layers of mononuclear cells were collected, and cells were depleted from lineage-positive cells through magnetic separation by using directly conjugated anti-CD3, anti-CD19, and anti-CD14 microbeads (markers for T cells, B cells, and monocytes, respectively) and LD MACS columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Lineage-depleted PBMCs from buffy coats were additionally enriched by using the CD127 MicroBead Kit (Miltenyi Biotec). Lineage-depleted cells were incubated with the following specific mAbs: fluorescein isothiocyanate–conjugated anti-CD14 (TÜK4; Dako, Glostrup, Denmark); anti-CD3 (SK7), anti-FcεRIα (AER-37 CRA-1), anti-CD34 (581), anti-CD123 (6H6), anti-CD1a (HI149), anti–T-cell receptor αβ (IP26), anti–T-cell receptor γδ (B1), and anti-CD94 (DX22; all from BioLegend, San Diego, Calif); anti-BDCA2 (AC144; Miltenyi Biotech) and anti-CD19 (4G7; BD Biosciences). Additionally, we used Brilliant Violet 605–conjugated anti-CD161 (HP-3G10), Brilliant Violet 711–conjugated anti-CD56 (HCD56; both from BioLegend); phycoerythrin (PE)-CF594–conjugated anti-CRTH2 (BM16) and V500-conjugated anti-CD45 (HI30; both from BD Biosciences); and PE-Cy7–conjugated anti-CD127 (R34.34), PE-Cy5.5–conjugated anti-CD117 (104D2D1), and PE-Cy5–conjugated anti-NKp44 (Z231; all from Beckman Coulter, Fullerton, Calif).

In addition, cells were stained with the LIVE/DEAD Fixable Green Dead Cell Stain Kit (Life Technologies, Grand Island, NY). ILC2s were sort-purified based on their specific phenotype as $Lin^{-}CD127^{+}CD161^{+}CRTH2^{+}$ cells by using the FACSAria Fusion cell sorter (BD Bioscience) equipped with the FACSDiva software, version 8.

Culture and expansion of ILC2s

Freshly sorted ILC2s were cultured in IMDM (Gibco, Carlsbad, Calif) supplemented with Yssel medium and 1% NHS (Human AB serum; Invitrogen, Carlsbad, Calif) with penicillin (100 U/mL; HyClone, San Angelo, Tex) and streptomycin (0.1 mg/mL; HyClone). ILC2s were expanded for 2 weeks in culture with a mixture of feeder cells containing PBMCs irradiated at 25 Gy and JY cells irradiated at 50 Gy, with IL-2 (100 U/mL), PHA (1 μg/mL), and addition of IL-4 (25 ng/mL; R&D Systems).

Freshly sorted ILC2s were kept in Yssel medium/1% NHS with IL-2 (10 U/mL) in a 96-well plate (2000 cells per sample) and treated the following day.

Treatments of ILC2s

After expansion for 2 weeks, ILC2s were rested in Yssel medium/1% NHS with IL-2 (2 U/mL) for 3 days before the experiments. Cells were seeded in 96-well U-bottom plates at 5×10^4 cells/well. ILC2s were incubated for 40 minutes with flurbiprofen (1 μmol/L) or 20 minutes with the CRTH2 antagonist CAY10471 (1 μmol/L) before adding IL-2 (10 U/mL) with or without IL-33 (PeproTech, Rocky Hill, NJ), TSLP (PeproTech), and IL-25 (R&D Systems) at 50 ng/mL each.

For LC-MS/MS and quantitative PCR experiments, expanded ILC2s were seeded at a density of 2.5×10^5 cells/well in IMDM/1% NHS without Yssel supplement and incubated for 6 and 24 hours with IL-2 (10 U/mL) and with or without combination of IL-33, TSLP, and IL-25 (50 ng/mL each). Where indicated, flurbiprofen (1 μmol/L) was used 40 minutes before treatment.

Freshly sorted blood and tonsillar ILC2s were kept in the presence of IL-2 (10 U/mL) and nonstimulated or treated with the combination of IL-33, TSLP, and IL-25 (50 ng/mL each) for 5 days. As pretreatments, flurbiprofen (1 μmol/L) and a specific HPGDS inhibitor developed by Sanofi-Aventis (2-phenyl-pyrimidine-5-carboxylic acid [2,3-dihidro-indol-1-yl]-amide [US patent 8,202,863], which for this research study was synthesized by Cayman, given the internal code KMN698, and used at a concentration of 100 nmol/L) were added at 40 minutes, whereas the CRTH2 antagonist CAY10471 (1 μmol/L) was added at 20 minutes before the cytokines. Flurbiprofen and CAY10471 treatments were repeated on days 2 and 4 of incubation. Microscopic images of freshly sorted blood ILC2s were captured after 5 days of culture at ×10 magnification by using a camera and the ToupView (ToupView Photonics, Zhejiang, China) program.

Flow cytometry and analysis of intracellular cytokines

Cell-surface staining of ILC2s was determined by using PE-Cy7–conjugated anti-CD127, PE-Cy5.5–conjugated anti-CD117, Brilliant Violet 605–conjugated anti-CD161, PE-CF594–conjugated anti-CRTH2, and Brilliant Violet 711–conjugated anti-CD56. Dead cells were excluded by using the LIVE/DEAD Fixable Green Dead Cell Stain Kit (Life Technologies). CD25 receptor expression was assessed by using Brilliant Violet 650–conjugated anti-CD25 (BC96; BioLegend).

For intracellular detection of IL-5 and IL-13, ILC2s were stimulated for 24 hours, as already described, with Golgi Plug (containing Brefeldin A) and Golgi Stop (containing monensin) added after 20 hours. Thereafter, ILC2s were fixed (2% paraformaldehyde), permeabilized (FACS Permeabilizing Solution 2; BD Bioscience), and stained with BD Biosciences antibodies for intracellular IL-13 (allophycocyanin-conjugated anti–IL-13; JES10-5A2) and IL-5 (PE-conjugated anti–IL-5; TRFK5). Intranuclear GATA-3 expression was assessed by using allophycocyanin-conjugated antibody (clone TWAJ; eBioscience) after fixation and permeabilization (Foxp3/Transcription factor staining buffer set; eBioscience).

All samples were measured on an LSR Fortessa flow cytometer (BD Biosciences) equipped with FACSDiva software version 8, and data were analyzed by using FlowJo software (TreeStar, Ashland, Ore).

Mast cell culture and stimulation

CBMCs were derived and then maintained, as previously described.[E2,E3] CBMCs were preincubated with 10 ng/mL human IL-4 (PeproTech) for 4 days and received 1 μg/mL IgE (Calbiochem, Merck Millipore, Darmstadt, Germany) overnight before activation. For activation, cells (250,000 cells/well; equivalent to 1.25×10^6 cells/mL) were activated in IMDM supplemented with 1% normal human serum (NHS) and penicillin (100 U/mL) and streptomycin (0.1 mg/mL) by using either 2 μg/mL anti-human IgE (Sigma-Aldrich) for 6 hours or 2 μmol/L calcium ionophore A23187 (Sigma-Aldrich) for 2 hours. Supernatants were collected and stored at −80°C.

ELISA

Concentrations of released IL-5 and IL-13 in ILC2 supernatants were assessed by means of ELISA. We used the human IL-5 ELISA Duo-Set Kit (R&D Systems) and the human IL-13 ELISA kit (Sanquin, Amsterdam The Netherlands).

In some experiments PGD_2 concentrations in ILC2 supernatants were measured by using the ELISA Prostaglandin D_2-MOX ELISA Kit (Cayman Chemicals). PGD_2 was converted to a stable methoxime derivate by mixing cell supernatants or standards with methyl oximating reagents (1:1) and heated to 60°C for 30 minutes. Afterward, PGD_2-methoxime and standards were used for ELISA, and their concentrations were determined spectrophotometrically.

LC-MS/MS analysis of prostanoids and metabolites

Analysis of cell-culture supernatants was performed by using LC-MS/MS. All analytic and internal standards were purchased from Cayman Chemical. Solvents (LC-MS grade) were obtained from Carl Roth (Karlsruhe, Germany).

For extraction of prostanoids and metabolites, 200 μL of cell-culture supernatant were spiked with isotopically labeled internal standards (PGE_2-d4, PGD_2-d4, TXB_2-d4, $PGF_{2\alpha}$-d4, 6-keto $PGF_{1\alpha}$-d4, 20-HETE-d6, and

13,14-dh-15-keto PGE_2), 100 μL of EDTA solution (0.15 mol/L), and 600 μL of ethyl acetate. Samples were vortexed and centrifuged at 20,000*g* for 5 minutes. The organic phase was removed, and the extraction was repeated with 600 μL of ethyl acetate. Organic fractions were combined and evaporated at a temperature of 45°C under a gentle stream of nitrogen. Residues were reconstituted with 50 μL of acetonitrile/water/formic acid (20:80:0.0025 vol/vol/vol) and transferred to glass vials.

The LC-MS/MS analysis was carried out with an Agilent 1290 Infinity LC system (Agilent, Waldbronn, Germany) coupled to a hybrid triple quadrupole linear ion trap mass spectrometer QTRAP 6500+ (Sciex, Darmstadt, Germany) equipped with a Turbo-V-source operating in negative electrospray ionization mode. Chromatographic separation was carried out with a Synergi Hydro-RP Column (150 × 2 mm, 4-μm particle size, and 80-Å pore size; Phenomenex, Aschaffenburg, Germany). A linear gradient was used at a flow rate of 300 μL/min. Mobile phase A was water/formic acid (100:0.0025 vol/vol), and mobile phase B was acetonitrile/formic acid (100:0.0025 vol/vol). Analytes were separated under gradient conditions within 16 minutes. The injection volume was 20 μL. The gradient program started with 90% mobile phase A for 1 minute, and then mobile phase A was decreased to 60% within 1 minute, held for 1 minute, further decreased to 50% within 1 minute, and held for 2 minutes. Within 2 minutes, mobile phase A was further decreased to 10% and held for 1 minute. Within 1 minute, the initial conditions were restored and the column was re-equilibrated for 6 minutes. Mass spectrometric parameters were set as follows: ionspray voltage, −4500 V; source temperature, 500°C, curtain gas, 40 psi; nebulizer gas, 40 psi; and Turbo heater gas, 60 psi. Both quadrupoles were running at unit resolution.

For analysis and quantification, Analyst Software 1.6 and MultiQuant Software 3.0 (both from Sciex) were used. The following precursor-to-product ion transitions were used for quantification: m/z 351.2 → m/z 315.0 for PGE_2, m/z 351.2 → m/z 233.3 for PGD_2, m/z 353.2 → m/z 193.0 for $PGF_{2\alpha}$, m/z 369.2 → m/z 163.0 for 6-keto $PGF_{1\alpha}$, m/z 369.2 → m/z 195.0 for TXB_2, m/z 279.1 → m/z 178.9 for 12-hydroxyheptadecatrenoic acid, m/z 353.2 → m/z 309.2 for 11β-$PGF_{2\alpha}$, m/z 351.2 → m/z 333.2 for 13,14-dh-15-keto-PGD_2, m/z 333.2 → m/z 233.3 for PGJ_2, and m/z 333.2 → m/z 233.3 for Δ^{12}-PGJ_2. The peak area of each analyte was corrected by the peak area of the corresponding internal standard. Calibration curves were constructed by using linear regression with $1/x^2$ weighting. The coefficient of correlation was at least 0.99. Variations in accuracy were less than 15% over the whole range of calibration, except for the lowest limit of quantification, where a variation in accuracy of 20% was accepted. The calibration ranges were 0.02 to 10 ng/mL plasma for PGE_2, PGD_2, TXB_2, 13,14-dh-15keto-PGD_2, PGJ_2, and Δ^{12}-PGJ_2; 0.04 to 20 ng/mL plasma for $PGF_{2\alpha}$; 0.08 to 40 ng/mL plasma for 6-keto $PGF_{1\alpha}$; 0.1 to 10 ng/mL plasma for 11β-$PGF_{2\alpha}$; and 0.2 to 10 ng/mL plasma for 12-hydroxyheptadecatrenoic acid.

RT-qPCR

Total RNA from expanded tonsillar ILC2s was isolated with the RNeasy Micro Kit (Qiagen). The iScript cDNA Synthesis Kit (Bio-Rad Laboratories) was used for cDNA synthesis, and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories) was used for PCR reactions. Primers were the PrimePCR SYBR Green assay for human *PTGS2* (for COX-2), *HPGDS*, *HPGD*, *PTGDR* (for DP1), *GPR44* (for CRTH2), and *RPS18*; all primers were from Bio-Rad Laboratories. RT-qPCR was performed in the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories).

Statistical analyses

Differences between 2 groups were analyzed by using the matched-pairs *t* test, and 3 or more groups were compared by using 1-way ANOVA for repeated measurements and the Dunnett multiple comparisons test. Analyses were performed with GraphPad Prism 6 Software.

REFERENCES

E1. Konya V, Czarnewski P, Forkel M, Rao A, Kokkinou E, Villablanca EJ, et al. Vitamin D downregulates the IL-23 receptor pathway in human mucosal group 3 innate lymphoid cells. J Allergy Clin Immunol 2018;141:279-92.

E2. Gela A, Kasetty G, Jovic S, Ekoff M, Nilsson G, Morgelin M, et al. Eotaxin-3 (CCL26) exerts innate host defense activities that are modulated by mast cell proteases. Allergy 2015;70:161-70.

E3. Xiang Z, Moller C, Nilsson G. IgE-receptor activation induces survival and Bfl-1 expression in human mast cells but not basophils. Allergy 2006;61:1040-6.

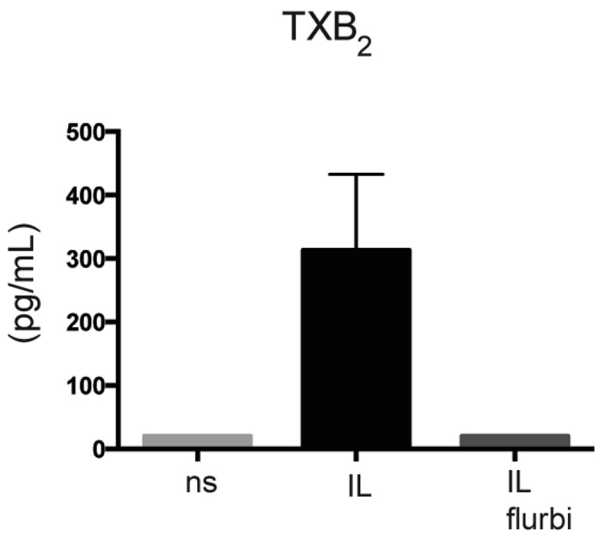


FIG E1. Stimulated ILC2s produce TXB_2. Sorted and expanded tonsillar ILC2s were stimulated as indicated for 24 hours. TXB_2 was detected in ILC2 supernatants by using LC-MS/MS. The graph shows mean + SEM concentrations (4 donors). *flurbi*, Flurbiprofen; *IL*, IL-33, IL-25, and TSLP; *ns*, nonstimulated.

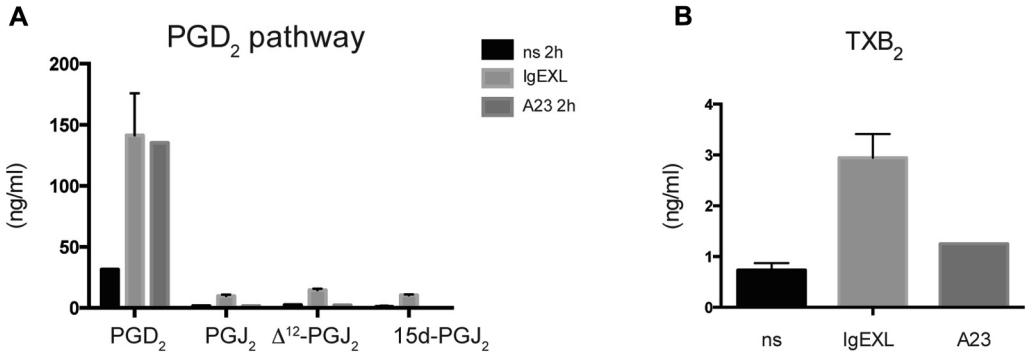


FIG E2. Metabolites of the PGD_2 pathway and TXB_2 are released by activated human mast cells. CBMCs were stimulated with IgEXL for 6 hours or A23 for 2 hours. Mediators of the PGD_2 pathways **(A)** and TXB_2 **(B)** were detected in mast cell supernatants by using LC-MS/MS. Graphs show mean + SEM concentrations (n = 1-3). *ns*, Nonstimulated.

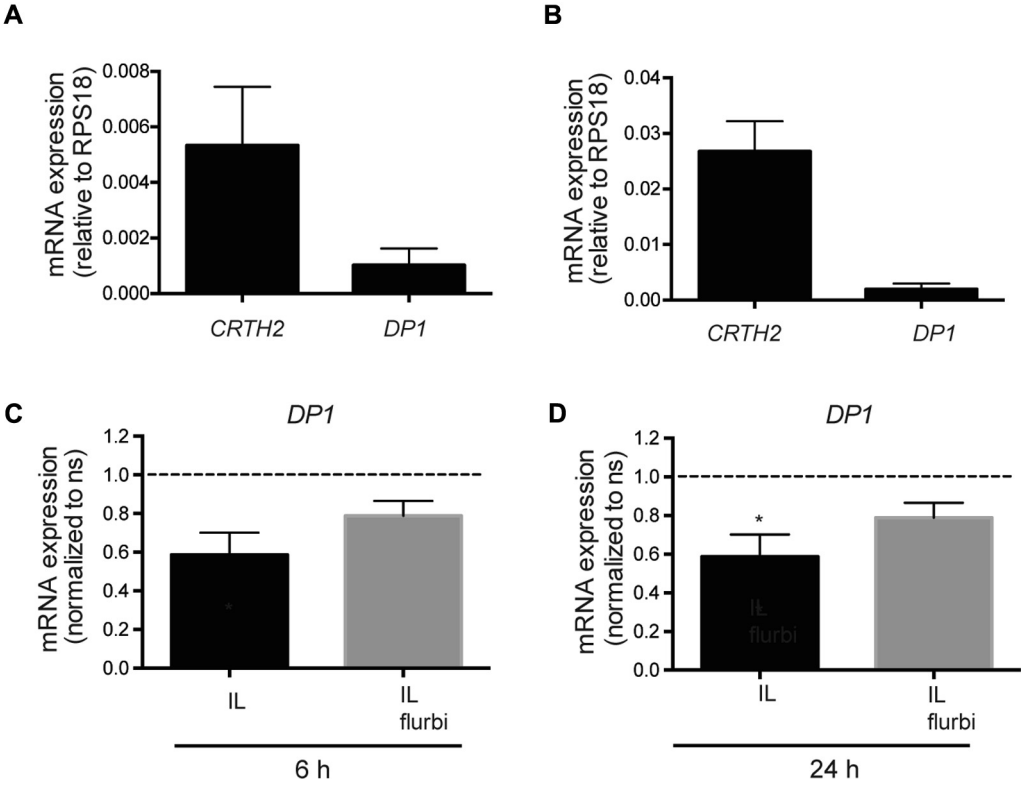


FIG E3. ILC2s express lower mRNA levels of *DP1* than *CRTH2* receptor. Sorted and expanded tonsillar ILC2s were stimulated as indicated for 6 (**A** and **C**) and 24 (**B** and **D**) with or without flurbiprofen (1 μmol/L, 40 minutes before treatment). Basal mRNA expression of *CRTH2* and *DP1* after 6 (Fig E3, *A*) and 24 (Fig E3, *B*) hours of culture were analyzed by using PCR. Data are shown as means + SEMs (2 donors for DP1 data for the 6-hour time point, 3 donors for CRTH2 data for 6 hours, and 4 donors for 24 hours of culture for both receptors). Changes of *DP1* mRNA expression normalized to nonstimulated cells after 6 (2 donors; Fig E3, *C*) and after 24 (**P* < .05, 4 donors; Fig E3, *D*) hours of stimulation are shown as means + SEMs. *flurbi*, Flurbiprofen; *IL*, IL-33, IL-25, and TSLP; *ns*, nonstimulated.