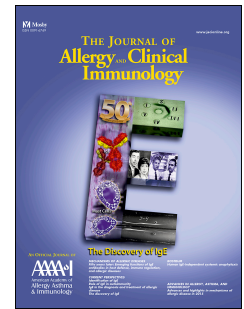


Accepted Manuscript

PGE₂ suppresses human group 2 innate lymphoid cell function

Jovana Maric, MSc, Avinash Ravindran, MSc, Luca Mazzurana, MSc, Åsa K. Björklund, PhD, Aline Van Acker, PhD, Anna Rao, PhD, Danielle Friberg, MD PhD, Sven-Erik Dahlén, Prof. MD PhD, Akos Heinemann, Prof. MD, Viktoria Konya, PhD, Jenny Mjösberg, PhD



PII: S0091-6749(17)31877-8

DOI: [10.1016/j.jaci.2017.09.050](https://doi.org/10.1016/j.jaci.2017.09.050)

Reference: YMAI 13143

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 7 November 2016

Revised Date: 30 August 2017

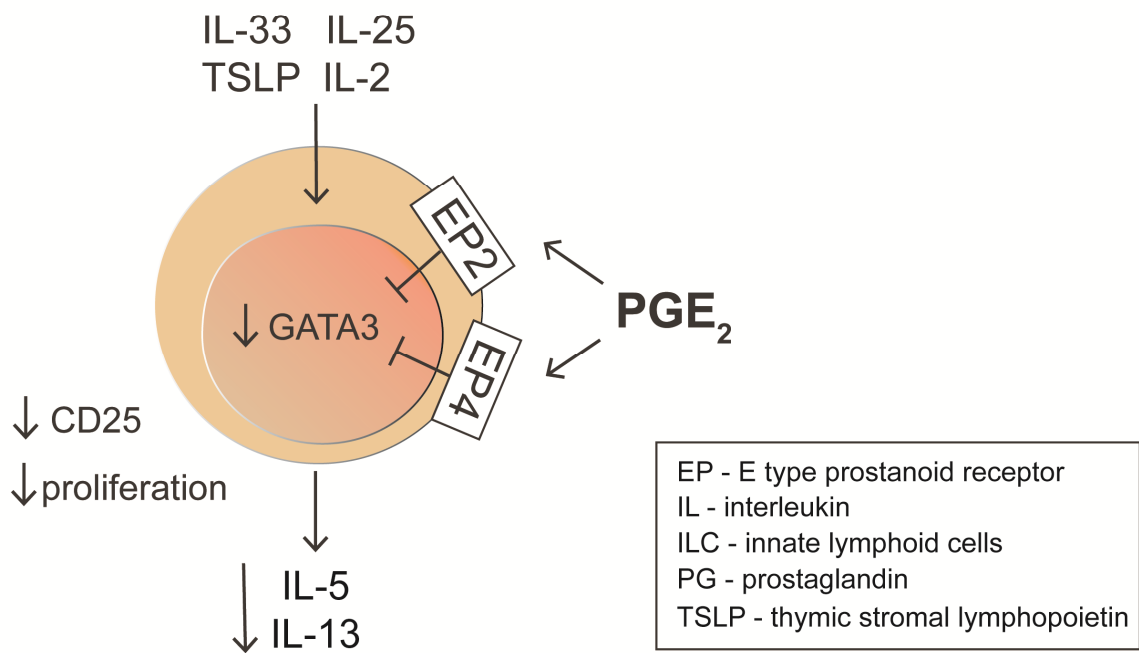
Accepted Date: 11 September 2017

Please cite this article as: Maric J, Ravindran A, Mazzurana L, Björklund ÅK, Van Acker A, Rao A, Friberg D, Dahlén S-E, Heinemann A, Konya V, Mjösberg J, PGE₂ suppresses human group 2 innate lymphoid cell function, *Journal of Allergy and Clinical Immunology* (2018), doi: 10.1016/j.jaci.2017.09.050.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



PGE_2 suppresses human blood and tonsil ILC2 function



1 PGE₂ suppresses human group 2 innate lymphoid cell function

2

3 Jovana Maric, MSc^{1,2}, Avinash Ravindran, MSc³, Luca Mazzurana, MSc², Åsa K.
4 Björklund, PhD⁴, Aline Van Acker, PhD², Anna Rao, PhD², Danielle Friberg, MD
5 PhD⁵, Sven-Erik Dahlén, Prof. MD PhD⁶, Akos Heinemann, Prof. MD¹, Viktoria
6 Konya, PhD^{1,2*} and Jenny Mjösberg, PhD^{2,7*}

7 * Contributed equally

8

9 ¹Institute of Experimental and Clinical Pharmacology, Medical University of Graz,
10 Graz, Austria

11 ²Center for Infectious Medicine, Department of Medicine Huddinge, Karolinska
12 Institutet, Stockholm, Sweden

13 ³Immunology and Allergy Unit, Department of Medicine, Solna, Karolinska Institutet,
14 Stockholm, Sweden

15 ⁴Science for Life Laboratory, Department of Cell and Molecular Biology, Uppsala
16 University, Uppsala, Sweden

17 ⁵Department of Oto-Rhino-Laryngology, Karolinska University Hospital and
18 CLINTEC, Karolinska Institutet, Stockholm, Sweden

19 ⁶Experimental Asthma and Allergy Research, Institute of Environmental Medicine,
20 Karolinska Institutet, Stockholm, Sweden

21 ⁷Department of Clinical and Experimental Medicine, Linköping University, Sweden

22

23 * Corresponding authors:

24 Jenny Mjösberg, PhD, Center for Infectious Medicine, F59, Karolinska Institutet, S-

25 14186 Stockholm, Sweden. jenny.mjoberg@ki.se, Phone: +46-70 547 29 56, Fax:

26 +46-8-31 1101

27 Viktoria Konya, PhD, Institute of Experimental and Clinical Pharmacology, Medical
28 University of Graz, Graz, Austria. viktoria.konya@medunigraz.at, Phone: +43-316
29 380-4512, Fax: 43-316-380-9645

30

31 Abstract

32 **Background:** Group 2 innate lymphoid cells (ILC2) are involved in the initial phase
33 of type 2 inflammation and can amplify allergic immune responses by orchestrating
34 other type 2 immune cells. PGE₂ is a bioactive lipid that plays protective roles in the
35 lung, particularly during allergic inflammation.

36 **Objective:** We set out to investigate how PGE₂ regulates human ILC2 function.

37 **Methods:** The effects of PGE₂ on human ILC2 proliferation, intracellular cytokine
38 and transcription factor expression were assessed by flow cytometry. Cytokine
39 production was measured by ELISA and real-time quantitative PCR was performed to
40 detect PGE₂ receptor expression.

41 **Results:** PGE₂ inhibited the expression of GATA3, as well as the production of type 2
42 cytokines, IL-5 and IL-13, from human tonsil and blood ILC2 in response to
43 stimulation with a combination of IL-25, IL-33, TSLP and IL-2. Furthermore, PGE₂
44 downregulated the expression of IL-2R α (CD25). In line with that observation, PGE₂
45 decreased ILC2 proliferation. These effects were mediated by the combined action of
46 the EP2 and EP4 receptors, which were specifically expressed on ILC2.

47 **Conclusion:** Our findings reveal that PGE₂ limits ILC2 activation and propose that
48 selective EP2 and EP4 receptor agonists might serve as promising therapeutic
49 approach in treating allergic diseases by suppressing ILC2 function.

50

Key messages:

- PGE₂ inhibits cytokine production and proliferation of stimulated human ILC2 via the EP2 and EP4 receptors
- PGE₂ interferes with CD25 and GATA3 expression in stimulated ILC2
- EP2 and EP4 agonists could prove beneficial for targeting ILC2 function in allergy therapy

Capsule summary

Our study has demonstrated mechanisms underlying the PGE₂-induced abolishment of ILC2 function, suggesting EP2/EP4 receptors as potential therapeutic targets in allergy and asthma.

Keywords: ILC2, allergy, prostaglandin E₂, EP2, EP4

Abbreviations: CRTH2 – chemoattractant receptor-homologous molecule expressed on Th2 cells, EP – E type prostanoid receptor, IL – interleukin, ILC – innate lymphoid cells, NF-κB – nuclear factor kappa B, PG – prostaglandin, RT-qPCR – quantitative reverse transcription polymerase chain reaction, TSLP – thymic stromal lymphopoietin

Acknowledgements

This project has 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 Je.M. and the Austrian Science Fund FWF (grant P25531-B23) to V.K. We thank the Department of Medicine, Huddinge Flow Cytometry Facility at Karolinska Institutet for allowing us to use the cell sorters.

Author Contribution

Jo.M. designed the study, performed experiments, interpreted data and wrote the manuscript. Av.R., L.M., A.V. and An.R. performed experiments. Å.K.B. performed the scRNA-seq data analysis. D.F. provided clinical samples. S.D. and A.H. contributed to study design and revised the manuscript. V.K. designed the study, performed experiments, interpreted data and wrote the manuscript. Je.M. designed the study, interpreted data and wrote the manuscript. All authors contributed to manuscript writing.

88 Introduction

89 Group 2 innate lymphoid cells (ILC2) were first described in mice as lymphocytes
90 lacking expression of B and T cell markers and being a rich source of type 2
91 cytokines^{1, 2}. ILC2 are crucial in driving type 2 immune responses even in the
92 absence of adaptive T cell activation³⁻⁶. Human ILC2 have been identified in the
93 lung, gut, nasal polyps, thymus, tonsils, skin and also in peripheral blood⁷⁻⁹. In the
94 mouse, ILC2 development is dependent on retinoic acid receptor-related orphan
95 receptor alpha and GATA3¹⁰. Additionally, GATA3 plays a key role in both human
96 and mouse ILC2 maintenance and function¹⁰. IL-2 and IL-7 are also necessary for
97 ILC2 maintenance and can contribute to ILC2 activation¹¹. Upon allergen
98 stimulation, activated airway epithelial cells and alveolar macrophages produce the
99 innate cytokines IL-33, IL-25 and thymic stromal lymphopoietin (TSLP)¹², which
100 activate nuclear factor kappa B (NF- κ B) and STAT-5 signaling pathways in ILC2¹¹.
101 These events lead to GATA3 upregulation and GATA3 activation triggers production
102 of the type 2 cytokines IL-5 and IL-13^{13, 14}. Consequently, eosinophils, mast cells,
103 Th2 cells and dendritic cells are activated that further amplify the type 2 immune
104 response characteristic for allergy and asthma^{15, 16}.
105 Pharmacological targeting of ILC2 is desirable due to the crucial role that ILC2 play
106 in the pathogenesis of allergy. Although several activators of ILC2 have been already
107 described, we still know relatively little about factors that restrict ILC2 responses.
108 Lipid mediators are key molecules in controlling allergic immune responses¹⁷ and
109 there is clear evidence for an immunoregulatory role of prostaglandin (PG) D₂¹⁸ and
110 other lipid mediators in ILC2 activation¹⁹.
111 One highly abundant lipid mediator in the lung is PGE₂ that exerts its effects through
112 four different G protein-coupled E-type prostanoid receptors (EP1-4). PGE₂ has been

shown to be protective in mouse models of asthma and allergy^{20, 21}, as well as in the human lung, where inhaled PGE₂ inhibits allergen-induced bronchoconstriction²². Furthermore, PGE₂ shows inhibitory effects on Th2 cells, macrophages, mast cells, neutrophils and eosinophils²³⁻²⁷. Importantly, the role of PGE₂ in regulating ILC2 function has not been described yet. Here we reveal PGE₂ as a potent suppressor of human ILC2 cytokine production and proliferation, most likely via downregulating the expression of GATA3 and IL-2R α (CD25). Furthermore, ILC2 exclusively express the EP2 and EP4 receptors and PGE₂ utilizes both these receptors to suppress ILC2 function. Our findings indicate that targeting the PGE₂ - EP2/EP4 pathway might be an efficient way to suppress ILC2 function as a therapeutic strategy for allergy and asthma.

Methods

Detailed description of methods and materials is provided in the Methods section in this article's Online Repository.

Isolation and flow cytometric sorting of tonsil and blood ILC2

ILC2 were obtained from human tonsils and buffy coats. The cells were isolated by flow cytometry sorting using antibodies against specific cell-surface markers as previously described²⁸. Detailed description of antibodies used for ILC2 isolation can be found in the Methods section in this article's Online Repository.

Culture, expansion and treatments of ILC2

Freshly sorted ILC2 were expanded for two weeks in culture with a feeder cell mixture as previously described²⁹. ILC2 were treated with PGE₂ (30 nM) for 10 min before adding IL-2 (10 U/mL) with or without IL-33, TSLP and IL-25 (50 ng/mL each). EP2 receptor antagonist, PF-04418948 and EP4 receptor antagonist, ONO AE3-208 were added 20 min before PGE₂. In some experiments, ILC2 were treated with EP2 receptor agonist, butaprost and EP4 receptor agonist, L-902, 688, 10 min before the cytokine stimulation. Expanded ILC2 were incubated for 24 hours or 72 hours and freshly sorted ILC2 for 5 days.

Flow cytometry staining and analysis of intracellular cytokines, transcription factors and proliferation

Cell surface staining of expanded tonsil ILC2 was assessed by using specific antibodies. CellTrace violet dye was used for analyzing cell proliferation. Intracellular cytokine detection and GATA3 expression were determined after specific fixation and

permeabilization protocols. After the staining, cells were acquired immediately on a LSR Fortessa flow cytometer.

ELISA

Collected cell supernatants were analyzed by using IL-5 ELISA Duo-Set kit (R&D) and IL-13 ELISA kit (Sanquin).

RT-qPCR

Total RNA from expanded tonsil ILC2 cells was isolated using RNeasy micro kit (Qiagen). After cDNA synthesis, qPCR reactions were performed using SsoAdvanced™ Universal SYBR® Green supermix (Bio-Rad) and primers for human EP1-EP4 receptors and house-keeping gene RPS18 (all primers were from Bio-Rad). RT-qPCRs were performed in CFX Connect Real-Time PCR Detection System (Bio-Rad).

RNA sequencing analysis

Single cell RNA-seq expression patterns were obtained as reads per kilobase gene model and million mappable reads (RPKM) from the Björklund, Forkel et al.²⁸ expression matrix. Expression levels of the *PTGER* genes in the different ILC subsets and NK cells were interpreted as violin plots using the R software.

Statistical analyses

In all experiments, 'n' represents the number of individual donors used for ILC2 isolation. Differences between two groups were analyzed by using matched-pairs t-test and three or more groups were compared by one-way ANOVA for repeated

175 measurements and Dunnett's multiple comparisons test. Analyses were performed
176 using GraphPad Prism 6 Software.

177

ACCEPTED MANUSCRIPT

Results

PGE₂ suppresses IL-5 and IL-13 production in human tonsil ILC2

ILC2 were sort-purified from tonsil mononuclear cells as Lin⁻ CD127⁺CD161⁺CRTH2⁺ lymphocytes (Figure 1A). Due to the relative rareness of ILC2 in tonsils, we expanded freshly sorted ILC2 by culturing the cells with irradiated feeder cells for two weeks in the presence of IL-2 (100 U/mL), IL-4 (25 ng/mL) and PHA (1 µg/mL). As compared to the freshly isolated ILC2 (see Figure E1A in the Online Repository), expanded ILC2 maintained their phenotype and expressed high levels of CD161 and CRTH2 while lacking CD3 and CD56 expression (see Figure E1B in the Online Repository). To assess how PGE₂ regulates ILC2 function, we incubated tonsil ILC2 with cytokines known to drive human ILC2 function: IL-33, TSLP, IL-25 and IL-2, in the presence or absence of PGE₂ for 24 hours. Control treatment of ILC2 contained only IL-2. We chose this time point as there is no detectable ILC2 proliferation occurring in these cultures within 24 hours, allowing us to assess proliferation-independent effects of PGE₂ on ILC2 (see Figure E2A in the Online Repository). As expected, the cytokine-activated ILC2 secreted high amounts of IL-5 and IL-13, as determined by ELISA (Figure 1B, C). Incubation of ILC2 with PGE₂ alone did not induce any changes in the cytokine production (Figure 1D), however PGE₂ inhibited cytokine production of activated ILC2 in a concentration-dependent manner (see Figure E3A, B in the Online Repository). In all subsequent experiments, we used 30 nM PGE₂, which caused on average 70% inhibition of cytokine secretion (Figure 1B, C). Similar results were obtained when we assessed the IL-5 and IL-13 production on a per-cell basis, as PGE₂ significantly reduced the percentage of ILC2 expressing intracellular IL-5 and IL-13 following cytokine-stimulation (Figure 1E-G).

Of note, as previously demonstrated²⁹, we observed that the combination of all three cytokines (IL-33, IL-25 and TSLP) generated higher ILC2-responses than the individual cytokines alone (see Figure E3C, D in the Online Repository). Interestingly, IL-33, and to a lesser extent IL-25 and TSLP, alone could induce only moderate IL-5 and IL-13 production. Importantly, PGE₂ inhibited ILC2 activation in response to the combination of all cytokines, as well as in response to IL-33, IL-25 and TSLP alone (see Figure E3C, D in the Online Repository).

The transcription factor GATA3 is crucial in ILC2 function, as it regulates production of type 2 cytokines^{13,30}. Intracellular protein detection of GATA3 revealed that it is highly expressed in resting ILC2 and further enhanced after 24 hours of cytokine stimulation (Figure 1H-J). We show that addition of PGE₂ caused statistically significant decrease in the GATA3 expression (Figure 1H-J). Cell viability was not affected by PGE₂ (see Figure E2B in the Online Repository). Taken together, PGE₂ reduces the production and release of IL-5 and IL-13 by stimulated ILC2, likely via the downregulation of GATA3.

PGE₂ reduces CD25 expression and cell proliferation of ILC2

We observed that cytokine-stimulated ILC2 upregulated the expression of IL-2R α (CD25) (Figure 2A-F). After co-incubation with PGE₂ for 24 hours, ILC2 showed significantly reduced expression of CD25 as compared to cells stimulated only with the cytokines (Figure 2A-C). Upon culturing ILC2 for 72 hours we could observe an even more pronounced inhibitory effect of PGE₂ on the CD25 expression (Figure 2D-F). As IL-2 strongly stimulates ILC2 proliferation³¹, we set out to assess the effects of PGE₂ on ILC2 proliferation. Cytokine stimulation of ILC2 for 72 hours induced up to 4 rounds of cell division. Furthermore, as predicted by the PGE₂-induced

suppression of CD25 expression, PGE₂ blocked cytokine-driven ILC2 proliferation, with the majority of ILC2 remaining in resting phase or in the 1st round of cell division, similarly to non-stimulated control (Figure 2G, H). PGE₂ treatment did not affect cell viability after 72 hours of incubation (Figure 2I).

Longer culture time generated higher concentrations of released IL-5 and IL-13 in the ILC2 supernatants as compared to 24 hours (Figure 3A, B), which could be due to the intensive cell proliferation seen at this time point. Strikingly, PGE₂ significantly suppressed the cytokine-stimulated IL-5 and IL-13 secretion of ILC2 after 72 hours of culture (Figure 3A, B). Furthermore, expression of GATA3 was also significantly inhibited by PGE₂ treatment after 72 hours incubation similarly to after 24 hours (Figure 3C-E and Figure 1H-J).

In summary, these data indicate that PGE₂ does not only act on the “per cell basis”, i.e. by suppressing cytokine production of individual ILC2. In addition, PGE₂ limits ILC2 function also by inhibiting cell proliferation most likely via negatively affecting CD25 and GATA3 expression.

PGE₂ reduces IL-5 and IL-13 secretion from freshly sorted blood and tonsil ILC2

As our initial experiments were performed using *in vitro* expanded ILC2, we wanted to confirm the suppressive effect of PGE₂ on freshly sorted human ILC2. For this purpose, we analyzed freshly sorted ILC2 from buffy coats (Figure 4A-E) and tonsils (Figure 4F, G). As we have previously observed that freshly isolated ILC2 produce less cytokines on a per cell basis²⁹, we incubated ILC2 for 5 days. Freshly isolated blood ILC2 (Figure 4A, B) and tonsil ILC2 (Figure 4F, G) released comparable amounts of IL-5 and IL-13 after 5 days of culture. PGE₂ significantly reduced IL-5

and IL-13 production of blood ILC2 (Figure 4A, B). Additionally, PGE₂ significantly reduced IL-13 production of tonsil ILC2 to approximately 60%, and also slightly decreased IL-5 release (Figure 4F, G).

Furthermore, cytokine stimulation upregulated CD25 expression in freshly sorted blood ILC2, which was significantly decreased by PGE₂ treatment (Figure 4C, D). To assess effects of PGE₂ on ILC2 proliferation, we captured images of ILC2 after 5 days of culture. These images, evaluated by eye, revealed that ILC2 cultured in the presence of PGE₂ formed a smaller cell mass as compared to ILC2 cultured in the presence of only cytokines (Figure 4E).

These results indicate that freshly isolated blood and tonsil ILC2 also respond to PGE₂ with repressed activity and confirm the inhibitory effects of PGE₂ we observed on expanded tonsil ILC2.

PGE₂ suppresses IL-5 and IL-13 production of ILC2 by acting on EP2 and EP4 receptors

PGE₂ exerts its physiological effects by acting on four different G protein-coupled receptors: EP1-EP4. To determine which receptors mediate the PGE₂-induced inhibitory effects on ILC2, we assessed expression of the EP receptors on human tonsil ILC2. In our previously published single-cell RNA sequencing data generated from freshly isolated tonsil ILC subsets²⁸ we detected the EP2 and EP4 receptor encoding transcripts *PTGER2* and *PTGER4* in ILC2. In contrast, ILC2 lacked expression of transcripts for EP1 and EP3 receptors (Figure 5A). RT-qPCR experiments performed with expanded tonsil ILC2 revealed that cultured ILC2 retain the same EP receptor expression pattern as fresh ILC2, and exclusively express EP2

and EP4 receptors (Figure 5A, B). Of note, expression of EP2 and EP4 mRNA was not affected by cytokine stimulation of expanded tonsil ILC2 (Figure 5C).

Having identified the expression of EP2 and EP4 receptor mRNAs in tonsil ILC2, we set out to determine if these receptors were responsible for the inhibitory effects of PGE₂. We pre-treated ILC2 with selective EP2 (PF-04418948) and EP4 (ONO AE3-208) receptor antagonists prior to cytokine and PGE₂ treatments (Figure 6). Assessment of IL-5 and IL-13 production revealed that selective antagonism of EP2 or EP4 receptor only slightly prevented the suppressive effects of PGE₂. Interestingly, simultaneous EP2 and EP4 blockade largely abolished the PGE₂-induced reduction of IL-5 and IL-13 release after 24 hours of culture (Figure 6A, B). Since antagonists used separately in 1 μ M concentration did not reverse the effect of PGE₂, we examined whether higher concentrations would be more efficient. Using antagonists in the concentration range of 0.1-3 μ M, we demonstrated that concentrations higher than 1 μ M did not have more profound reversal effect on PGE₂-induced inhibition of ILC2 function (see Figure E4A, B in the Online Repository).

We observed the same effects on the per-cell basis, revealing that only dual EP2 and EP4 antagonism reversed the PGE₂-induced reduction of IL-5 and IL-13 intracellular expression (Figure 6C-E). Joint antagonism of EP2 and EP4 receptors also reversed the PGE₂-induced reduction of GATA3 expression after incubation for 24 hours (Figure 6F, G).

Additionally, we addressed the role of selective activation of EP2 and EP4 receptors in regulating the cytokine production of ILC2 by pre-incubation with specific agonists butaprost (EP2) and L-902, 688 (EP4) in the concentration range of 10-1000 nM (see Figure E4C, D in the Online Repository). By using this approach we confirmed that dual engagement of EP2 and EP4 is required for mimicking the inhibitory effects of

PGE₂ on the ILC2 cytokine production. We observed a tendency for more pronounced inhibition by selectively engaging the EP4 receptor as compared to EP2, although these effects did not reach statistical significance. In direct comparison with PGE₂, we show that the selective EP2 and EP4 agonists in 100 nM concentration only partly reduced cytokine production, while their simultaneous activation completely blocked ILC2 stimulation, comparably to the effect of PGE₂ (Figure 6H, I).

Next, we aimed to determine whether antagonism of EP2 and EP4 receptors is able to effectively reverse the long-term ILC2 blocking effects of PGE₂ after 72 hours incubation. As PGE₂ almost completely abolished proliferation of ILC2 induced by the cytokine stimulation, we examined the role of EP2 and EP4 receptors in ILC2 proliferation. Indeed, combined EP2 and EP4 antagonism reversed the PGE₂-suppressed ILC2 proliferation, by keeping a large proportion of ILC2 into the first and second division phases (Figure 7A, B). Dual EP2 and EP4 blockade also reversed the PGE₂-caused suppression of ILC2 cytokine production (Figure 7C, D) and GATA3 expression at this time point (Figure 7E, F), further confirming the role of both receptors in mediating the long-term inhibitory effects of PGE₂ in ILC2 function. Taken together, PGE₂ suppresses IL-5 and IL-13 production and blocks ILC2 proliferation via engagement of both EP2 and EP4 receptors.

Discussion

In this study we describe PGE₂ as a potent negative regulator of human ILC2 function by inhibiting IL-5 and IL-13 production as well as the proliferation of ILC2. The cellular mechanism of the PGE₂-caused ILC2 inhibition involved suppression of CD25 and GATA3 expression in ILC2. Furthermore, we found that PGE₂ mediated these effects via engagement of both EP2 and EP4 receptors, which are expressed by ILC2.

ILC2 are important in initiating the type 2 inflammation, as shown in mouse models of asthma and allergy^{3, 16}. In line with these observations, studies of human subjects showed increased numbers of ILC2 in peripheral blood and sputum of patients with allergic asthma^{32, 33} and lung fibrosis³⁴, in nasal tissue of patients with chronic rhinosinusitis³⁵ and in the skin of atopic dermatitis patients³⁶. PGE₂ is abundantly present in the human lung, produced by alveolar epithelial and endothelial cells as well as by macrophages and dendritic cells³⁷, while under inflammatory conditions PGE₂ levels are further elevated³⁸. Our data, therefore, firstly indicate that PGE₂ might serve as an endogenous messenger for restriction of uncontrolled signals mediated by ILC2. Secondly, the findings support the proposal that pharmacological targeting of the PGE₂ - EP2/EP4 pathway might be an efficient novel way to modulate ILC2 function as a therapeutic strategy in allergy and asthma.

Importantly, earlier clinical studies showed that inhalation of PGE₂ prevented bronchoconstriction in healthy and asthmatic subjects^{22, 39-41}. Additionally, PGE₂ inhibited human Th2 cell activation,⁴² eosinophil trafficking^{24, 43} and mast cell-dependent constriction of human bronchi⁴⁴. In mouse models of asthma and allergy, PGE₂ has been shown to be protective by preventing airway sensitization^{21, 45}. As

ILC2 are important initiators of allergic responses, we now extend these findings by showing that PGE₂ directly suppresses ILC2 function.

Our single-cell RNA sequencing and RT-qPCR data demonstrated that human tonsil ILC2 exclusively express EP2 and EP4 receptors. Interestingly, selective blockade of EP2 or EP4 receptors resulted in only partial reversion of the inhibitory effect of PGE₂, whereas their combined antagonism abolished the inhibitory effect of PGE₂. Accordingly, joint EP2 and EP4 receptor agonism was necessary to completely inhibit ILC2 activation. Selective EP4 receptor activation appeared to have more profound suppressive effects than EP2. This might be due to differences in expression levels of the EP2 and EP4 receptors as we observed that freshly isolated and *in vitro* expanded ILC2 expressed higher levels of *PTGER4* than *PTGER2* mRNA. However, only simultaneous engagement of EP2 and EP4 receptors could mimic the inhibitory effect of PGE₂ in ILC2 function. This suggests an interesting mechanism, where PGE₂ requires engagement of both EP2 and EP4 receptors to exert its full inhibitory effect on ILC2 function. In line with our findings, previous studies described anti-inflammatory roles of the G α s protein-coupled EP2 and EP4 receptors^{21, 25, 46, 47}. Furthermore, PGE₂ was shown to control immunological responses, where such co-operation of EP2 and EP4 receptors was essential, for instance in the PGE₂-induced inhibition of antigen-specific T cell responses of human peripheral blood Th2 cells⁴². Similarly, EP2 and EP4 receptor engagement suppressed human alveolar macrophages⁴⁸. In contrast, the PGE₂-induced inhibition of mast cells and the consequent bronchoprotection⁴⁹ as well as the cytokine production of human nasal polyp cells⁴⁶ were mediated only by the EP2 receptor. Besides, the PGE₂-EP2 signaling was impaired in patients with aspirin-exacerbated respiratory disease^{50, 51}. Another study showed that whereas EP2 receptor activation induced bronchodilation

in several animal models, only EP4 receptor was responsible for inducing relaxation of human isolated bronchi⁵². Bronchorelaxation, together with EP2- and EP4-induced inhibition of the immune cells involved in allergic response, would be additional beneficial effect of treatments with these agonists. Beside these protective effects, EP2 and EP4 receptor activation induces vasodilation and decreases blood pressure⁵³,⁵⁴. Activation of these receptors additionally upregulates production of the vascular endothelial growth factor (VEGF)^{55, 56}. Therefore, local administration of EP2 and/or EP4 agonists in the airways might be beneficial in order to minimize systemic side effects.

Although both EP2 and EP4 receptors are G α s protein-coupled receptors and activate cAMP/PKA pathways, the EP4 receptor can trigger pathways independent of the cAMP signaling. It was shown that EP4 activation can inhibit NF- κ B activation in human macrophage⁵⁷, and that EP4 engagement attenuates human eosinophil migration through cAMP independent pathway²⁶. Thus, these previously described mechanisms support our findings about the dual requirement of EP2 and EP4 receptors in mediating the PGE₂-induced suppression of ILC2 function.

In addition to PGE₂, some other lipid mediators are emerging as novel inhibitors of ILC2 function²⁸. Human ILC2 were inhibited by the pro-resolving lipid mediator lipoxin A4⁵⁸ and PGI₂ was shown to reduce mouse lung ILC2 activity⁵⁹. In contrast, PGD₂ activates ILC2 by binding to CRTH2 receptor¹⁸. CRTH2 is G α i protein-coupled receptor and inhibits cAMP-mediated signaling. Previously it was shown that PGD₂, via activating the CRTH2 receptor, stimulates other immune cells like Th2 cells, basophils and eosinophils^{60, 61}. As we previously mentioned, EP2 and EP4 have been shown to act in an inhibitory manner during allergic inflammation. We demonstrate here that PGE₂ also negatively regulates human ILC2 function.

We observed that PGE₂ inhibited ILC2 response to each of the stimulatory cytokines: IL-33, IL-25 and TSLP, as well as to their combination, which was necessary for complete ILC2 stimulation. Therefore, we assessed the mechanism of the PGE₂-induced inhibition of ILC2 function by using the combination of these activating cytokines. Importantly, we demonstrated that PGE₂ suppressed both freshly isolated blood and tonsil ILC2 function, although the inhibitory effects of PGE₂ were more pronounced in expanded ILC2.

As PGE₂ reduced cytokine production of ILC2, we investigated whether PGE₂ could affect signaling pathways essential for ILC2 activation. The transcription factor GATA3 was shown to be crucial for ILC2 development, function and activation¹³. In our work, PGE₂ reduced upregulation of GATA3 upon stimulatory cytokine treatment, suggesting that PGE₂ interferes with GATA3 induction. To the best of our knowledge, we provide the first evidence for PGE₂ modulating GATA3 expression in immune cells. Additionally, we observed that PGE₂ inhibited ILC2 proliferation. IL-2 is an essential co-activator of human ILC2³². A recent study showed that IL-2 is required for ILC2 function in type 2 lung inflammation in the mice⁶². As IL-2 up-regulates CD25 expression via a positive feedback mechanism, it might serve as an indicator of ILC2 activation. CD25 expression was increased upon cytokine stimulation, while PGE₂ prevented the cytokine-induced CD25 upregulation, indicating the impact of this lipid mediator on ILC2 activation and at later time point also on ILC2 proliferation. These data are in line with previous reports demonstrating that PGE₂ reduces CD25 expression and consequently IL-2 signaling in human primary peripheral blood T cells⁶³ and bovine CD4⁺ T cells⁶⁴. Similarly, PGE₂ was previously shown to induce genes that block the cell cycle and proliferation of human primary CD4⁺ T cells^{42, 65}.

In conclusion, our findings reveal that PGE₂ hampers fresh and expanded ILC2 activity by reducing production of the type 2 cytokines IL-5 and IL-13. We demonstrated that both EP2 and EP4 receptors are expressed on ILC2 and are critical in mediating the PGE₂-caused abolishment of ILC2 activation. We showed that PGE₂ interferes with molecular pathways involved in type 2 cytokine production, preventing upregulation of CD25 and GATA3 expression. As ILC2 are essential in the initiation and amplification of type 2 inflammation, our data propose PGE₂ and selective or even dual EP2/EP4 agonists as promising therapeutic tools for allergic diseases by directly limiting human ILC2 activation.

430 References

431

- 432 1. Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25 induces IL-
433 4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity* 2001;
434 15:985-95.
- 435 2. Hurst SD, Muchamuel T, Gorman DM, Gilbert JM, Clifford T, Kwan S, et al.
436 New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo
437 function of the novel cytokine IL-25. *J Immunol* 2002; 169:443-53.
- 438 3. Halim TY, Krauss RH, Sun AC, Takei F. Lung natural helper cells are a
439 critical source of Th2 cell-type cytokines in protease allergen-induced airway
440 inflammation. *Immunity* 2012; 36:451-63.
- 441 4. Barlow JL, Bellosi A, Hardman CS, Drynan LF, Wong SH, Cruickshank JP, et
442 al. Innate IL-13-producing nuocytes arise during allergic lung inflammation
443 and contribute to airways hyperreactivity. *J Allergy Clin Immunol* 2012;
444 129:191-8 e1-4.
- 445 5. Bartemes KR, Iijima K, Kobayashi T, Kephart GM, McKenzie AN, Kita H.
446 IL-33-responsive lineage- CD25+ CD44(hi) lymphoid cells mediate innate
447 type 2 immunity and allergic inflammation in the lungs. *J Immunol* 2012;
448 188:1503-13.
- 449 6. Chang YJ, DeKruyff RH, Umetsu DT. The role of type 2 innate lymphoid
450 cells in asthma. *J Leukoc Biol* 2013; 94:933-40.
- 451 7. Monticelli LA, Sonnenberg GF, Abt MC, Alenqhat T, Ziegler CG, Doering
452 TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after
453 infection with influenza virus. *Nat Immunol* 2011; 12:1045-54.
- 454 8. Smith SG, Chen R, Kjarsgaard M, Huang C, Oliveria JP, O'Byrne PM, et al.
455 Increased numbers of activated group 2 innate lymphoid cells in the airways
456 of patients with severe asthma and persistent airway eosinophilia. *J Allergy
457 Clin Immunol* 2016; 137:75-86 e8.
- 458 9. Mjösberg J, Eidsmo L. Update on innate lymphoid cells in atopic and non-
459 atopic inflammation in the airways and skin. *Clin Exp Allergy* 2014; 44:1033-
460 43.
- 461 10. Mjösberg J, Bernink J, Peters C, Spits H. Transcriptional control of innate
462 lymphoid cells. *Eur J Immunol* 2012; 42:1916-23.
- 463 11. Duerr CU, Fritz JH. Regulation of group 2 innate lymphoid cells. *Cytokine*
464 2016; 87:1-8.
- 465 12. Hazenberg MD, Spits H. Human innate lymphoid cells. *Blood* 2014; 124:700-
466 9.
- 467 13. Mjösberg J, Bernink J, Golebski K, Karrich JJ, Peters CP, Blom B, et al. The
468 transcription factor GATA3 is essential for the function of human type 2
469 innate lymphoid cells. *Immunity* 2012; 37:649-59.
- 470 14. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol* 2015;
471 16:45-56.
- 472 15. Li BW, Hendriks RW. Group 2 innate lymphoid cells in lung inflammation.
473 *Immunology* 2013; 140:281-7.
- 474 16. Halim TY, Hwang YY, Scanlon ST, Zaghouani H, Garbi N, Fallon PG, et al.
475 Group 2 innate lymphoid cells license dendritic cells to potentiate memory
476 TH2 cell responses. *Nat Immunol* 2016; 17:57-64.

- 477 17. Fanning LB, Boyce JA. Lipid mediators and allergic diseases. *Ann Allergy*
478 *Asthma Immunol* 2013; 111:155-62.
- 479 18. Xue L, Salimi M, Panse I, Mjösberg JM, McKenzie AN, Spits H, et al.
480 Prostaglandin D2 activates group 2 innate lymphoid cells through
481 chemoattractant receptor-homologous molecule expressed on TH2 cells. *J*
482 *Allergy Clin Immunol* 2014; 133:1184-94.
- 483 19. Konya V, Mjösberg J. Lipid mediators as regulators of human ILC2 function
484 in allergic diseases. *Immunol Lett* 2016.
- 485 20. Machado-Carvalho L, Roca-Ferrer J, Picado C. Prostaglandin E2 receptors in
486 asthma and in chronic rhinosinusitis/nasal polyps with and without aspirin
487 hypersensitivity. *Respiratory research* 2014; 15:100-014-0100-7.
- 488 21. Zaslona Z, Okunishi K, Bourdonnay E, Domingo-Gonzalez R, Moore BB,
489 Lukacs NW, et al. Prostaglandin E(2) suppresses allergic sensitization and
490 lung inflammation by targeting the E prostanoid 2 receptor on T cells. *J*
491 *Allergy Clin Immunol* 2014; 133:379-87.
- 492 22. Pavord ID, Wong CS, Williams J, Tattersfield AE. Effect of inhaled
493 prostaglandin E2 on allergen-induced asthma. *Am Rev Respir Dis* 1993;
494 148:87-90.
- 495 23. Sreeramkumar V, Fresno M, Cuesta N. Prostaglandin E2 and T cells: friends
496 or foes? *Immunology and cell biology* 2012; 90:579-86.
- 497 24. Sturm EM, Schratl P, Schuligoi R, Konya V, Sturm GJ, Lippe IT, et al.
498 Prostaglandin E2 inhibits eosinophil trafficking through E-prostanoid 2
499 receptors. *Journal of immunology (Baltimore, Md.: 1950)* 2008; 181:7273-83.
- 500 25. Konya V, Ullen A, Kampitsch N, Theiler A, Philipose S, Parzmair GP, et al.
501 Endothelial E-type prostanoid 4 receptors promote barrier function and inhibit
502 neutrophil trafficking. *J Allergy Clin Immunol* 2013; 131:532-40.e1-2.
- 503 26. Luschnig-Schratl P, Sturm EM, Konya V, Philipose S, Marsche G, Frohlich E,
504 et al. EP4 receptor stimulation down-regulates human eosinophil function.
505 *Cell Mol Life Sci* 2011; 68:3573-87.
- 506 27. MacKenzie KF, Clark K, Naqvi S, McGuire VA, Noehren G, Kristariyanto Y,
507 et al. PGE(2) induces macrophage IL-10 production and a regulatory-like
508 phenotype via a protein kinase A-SIK-CRTC3 pathway. *Journal of*
509 *immunology (Baltimore, Md.: 1950)* 2013; 190:565-77.
- 510 28. Bjorklund AK, Forkel M, Picelli S, Konya V, Theorell J, Friberg D, et al.
511 Corrigendum: The heterogeneity of human CD127(+) innate lymphoid cells
512 revealed by single-cell RNA sequencing. *Nature immunology* 2016; 17:740-a.
- 513 29. Mjösberg JM, Trifari S, Crellin NK, Peters CP, van Drunen CM, Piet B, et al.
514 Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined
515 by expression of CCR2 and CD161. *Nature immunology* 2011; 12:1055-62.
- 516 30. Hoyler T, Klose CS, Souabni A, Turqueti-Neves A, Pfeifer D, Rawlins EL, et
517 al. The transcription factor GATA-3 controls cell fate and maintenance of type
518 2 innate lymphoid cells. *Immunity* 2012; 37:634-48.
- 519 31. Mirchandani AS, Besnard AG, Yip E, Scott C, Bain CC, Cerovic V, et al.
520 Type 2 innate lymphoid cells drive CD4⁺ Th2 cell responses. *J Immunol*
521 2014; 192:2442-8.
- 522 32. Bartemes KR, Kephart GM, Fox SJ, Kita H. Enhanced innate type 2 immune
523 response in peripheral blood from patients with asthma. *J Allergy Clin*
524 *Immunol* 2014; 134:671-8.e4.

- 525 33. Nagakumar P, Denney L, Fleming L, Bush A, Lloyd CM, Saglani S. Type 2
526 innate lymphoid cells in induced sputum from children with severe asthma. *J*
527 *Allergy Clin Immunol* 2016; 137:624-6.e6.
- 528 34. Hams E, Armstrong ME, Barlow JL, Saunders SP, Schwartz C, Cooke G, et
529 al. IL-25 and type 2 innate lymphoid cells induce pulmonary fibrosis. *Proc*
530 *Natl Acad Sci U S A* 2014; 111:367-72.
- 531 35. Ho J, Bailey M, Zaunders J, Mrad N, Sacks R, Sewell W, et al. Group 2 innate
532 lymphoid cells (ILC2s) are increased in chronic rhinosinusitis with nasal
533 polyps or eosinophilia. *Clinical and experimental allergy : journal of the*
534 *British Society for Allergy and Clinical Immunology* 2015; 45:394-403.
- 535 36. Salimi M, Barlow JL, Saunders SP, Xue L, Gutowska-Owsiak D, Wang X, et
536 al. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic
537 dermatitis. *The Journal of experimental medicine* 2013; 210:2939-50.
- 538 37. Harizi H. Reciprocal crosstalk between dendritic cells and natural killer cells
539 under the effects of PGE2 in immunity and immunopathology. *Cellular &*
540 *molecular immunology* 2013; 10:213-21.
- 541 38. Rumzhum NN, Ammit AJ. Cyclooxygenase 2: its regulation, role and impact
542 in airway inflammation. *Clinical and experimental allergy : journal of the*
543 *British Society for Allergy and Clinical Immunology* 2016; 46:397-410.
- 544 39. Melillo E, Woolley KL, Manning PJ, Watson RM, O'Byrne PM. Effect of
545 inhaled PGE2 on exercise-induced bronchoconstriction in asthmatic subjects.
546 *American journal of respiratory and critical care medicine* 1994; 149:1138-41.
- 547 40. Szczeklik A, Mastalerz L, Nizankowska E, Cmiel A. Protective and
548 bronchodilator effects of prostaglandin E and salbutamol in aspirin-induced
549 asthma. *American journal of respiratory and critical care medicine* 1996;
550 153:567-71.
- 551 41. Gauvreau GM, Watson RM, O'Byrne PM. Protective effects of inhaled PGE2
552 on allergen-induced airway responses and airway inflammation. *American*
553 *journal of respiratory and critical care medicine* 1999; 159:31-6.
- 554 42. Okano M, Sugata Y, Fujiwara T, Matsumoto R, Nishibori M, Shimizu K, et al.
555 E prostanoid 2 (EP2)/EP4-mediated suppression of antigen-specific human T-
556 cell responses by prostaglandin E2. *Immunology* 2006; 118:343-52.
- 557 43. Konya V, Philipose S, Balint Z, Olschewski A, Marsche G, Sturm EM, et al.
558 Interaction of eosinophils with endothelial cells is modulated by prostaglandin
559 EP4 receptors. *Eur J Immunol* 2011; 41:2379-89.
- 560 44. Saffholm J, Manson ML, Bood J, Delin I, Orre AC, Bergman P, et al.
561 Prostaglandin E2 inhibits mast cell-dependent bronchoconstriction in human
562 small airways through the E prostanoid subtype 2 receptor. *J Allergy Clin*
563 *Immunol* 2015; 136:1232-9.e1.
- 564 45. Birrell MA, Maher SA, Dekkak B, Jones V, Wong S, Brook P, et al. Anti-
565 inflammatory effects of PGE2 in the lung: role of the EP4 receptor subtype.
566 *Thorax* 2015; 70:740-7.
- 567 46. Okano M, Fujiwara T, Haruna T, Kariya S, Makiyama S, Higaki T, et al.
568 Prostaglandin E(2) suppresses staphylococcal enterotoxin-induced
569 eosinophilia-associated cellular responses dominantly through an E-prostanoid
570 2-mediated pathway in nasal polyps. *J Allergy Clin Immunol* 2009; 123:868-
571 74.e13.
- 572 47. Konya V, Maric J, Jandl K, Luschnig P, Aringer I, Lanz I, et al. Activation of
573 EP4 receptors prevents endotoxin-induced neutrophil infiltration into the

- 574 airways and enhances microvascular barrier function. *British journal of*
575 *pharmacology* 2015.
- 576 48. Ratcliffe MJ, Walding A, Shelton PA, Flaherty A, Dougall IG. Activation of
577 E-prostanoid4 and E-prostanoid2 receptors inhibits TNF-alpha release from
578 human alveolar macrophages. *The European respiratory journal* 2007; 29:986-
579 94.
- 580 49. Kay LJ, Yeo WW, Peachell PT. Prostaglandin E2 activates EP2 receptors to
581 inhibit human lung mast cell degranulation. *British journal of pharmacology*
582 2006; 147:707-13.
- 583 50. Corrigan CJ, Napoli RL, Meng Q, Fang C, Wu H, Tochiki K, et al. Reduced
584 expression of the prostaglandin E2 receptor E-prostanoid 2 on bronchial
585 mucosal leukocytes in patients with aspirin-sensitive asthma. *J Allergy Clin*
586 *Immunol* 2012; 129:1636-46.
- 587 51. Cahill KN, Raby BA, Zhou X, Guo F, Thibault D, Baccarelli A, et al.
588 Impaired E Prostanoid2 Expression and Resistance to Prostaglandin E2 in
589 Nasal Polyp Fibroblasts from Subjects with Aspirin-Exacerbated Respiratory
590 Disease. *American journal of respiratory cell and molecular biology* 2016;
591 54:34-40.
- 592 52. Buckley J, Birrell MA, Maher SA, Nials AT, Clarke DL, Belvisi MG. EP4
593 receptor as a new target for bronchodilator therapy. *Thorax* 2011; 66:1029-35.
- 594 53. Norel X. Prostanoid receptors in the human vascular wall. *ScientificWorldJournal* 2007; 7:1359-74.
- 595 54. Audoly LP, Tilley SL, Goulet J, Key M, Nguyen M, Stock JL, et al.
596 Identification of specific EP receptors responsible for the hemodynamic
597 effects of PGE2. *Am J Physiol* 1999; 277:H924-30.
- 598 55. Bradbury D, Clarke D, Seedhouse C, Corbett L, Stocks J, Knox A. Vascular
599 endothelial growth factor induction by prostaglandin E2 in human airway
600 smooth muscle cells is mediated by E prostanoid EP2/EP4 receptors and SP-1
601 transcription factor binding sites. *J Biol Chem* 2005; 280:29993-30000.
- 602 56. Deacon K, Knox AJ. Human airway smooth muscle cells secrete amphiregulin
603 via bradykinin/COX-2/PGE2, inducing COX-2, CXCL8, and VEGF
604 expression in airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol*
605 2015; 309:L237-49.
- 606 57. Takayama K, Sukhova GK, Chin MT, Libby P. A novel prostaglandin E
607 receptor 4-associated protein participates in antiinflammatory signaling. *Circ*
608 *Res* 2006; 98:499-504.
- 609 58. Barnig C, Cernadas M, Dutile S, Liu X, Perrella MA, Kazani S, et al. Lipoxin
610 A4 regulates natural killer cell and type 2 innate lymphoid cell activation in
611 asthma. *Science translational medicine* 2013; 5:174ra26.
- 612 59. Zhou W, Toki S, Zhang J, Goleniewska K, Newcomb DC, Cephus JY, et al.
613 Prostaglandin I2 Signaling and Inhibition of Group 2 Innate Lymphoid Cell
614 Responses. *American journal of respiratory and critical care medicine* 2016;
615 193:31-42.
- 616 60. Xue L, Gyles SL, Wettestad FR, Gazi L, Townsend E, Hunter MG, et al.
617 Prostaglandin D2 causes preferential induction of proinflammatory Th2
618 cytokine production through an action on chemoattractant receptor-like
619 molecule expressed on Th2 cells. *Journal of immunology (Baltimore, Md.:*
620 *1950)* 2005; 175:6531-6.
- 621 61. Sedej M, Schroder R, Bell K, Platzer W, Vukoja A, Kostenis E, et al. D-type
622 prostanoid receptor enhances the signaling of chemoattractant receptor-
623

- 624 homologous molecule expressed on T(H)2 cells. *J Allergy Clin Immunol*
625 2012; 129:492-500. .e1-9.
- 626 62. Roediger B, Kyle R, Tay SS, Mitchell AJ, Bolton HA, Guy TV, et al. IL-2 is a
627 critical regulator of group 2 innate lymphoid cell function during pulmonary
628 inflammation. *J Allergy Clin Immunol* 2015; 136:1653-63.e1-7.
- 629 63. Kolenko V, Rayman P, Roy B, Cathcart MK, O'Shea J, Tubbs R, et al.
630 Downregulation of JAK3 protein levels in T lymphocytes by prostaglandin E2
631 and other cyclic adenosine monophosphate-elevating agents: impact on
632 interleukin-2 receptor signaling pathway. *Blood* 1999; 93:2308-18.
- 633 64. Maslanka T, Spodniewska A, Barski D, Jasiecka A, Zuska-Prot M, Ziolkowski
634 H, et al. Prostaglandin E(2) down-regulates the expression of CD25 on bovine
635 T cells, and this effect is mediated through the EP4 receptor. *Veterinary*
636 *immunology and immunopathology* 2014; 160:192-200.
- 637 65. Chemnitz JM, Driesen J, Classen S, Riley JL, Debey S, Beyer M, et al.
638 Prostaglandin E2 impairs CD4+ T cell activation by inhibition of lck:
639 implications in Hodgkin's lymphoma. *Cancer research* 2006; 66:1114-22.
- 640
641

642

Figure legends

Figure 1. PGE₂ suppresses IL-5 and IL-13 production of human tonsil ILC2. A.

Gating strategy for sort-purifying human tonsil ILC2. Sorted and expanded tonsil ILC2 in the presence of IL-2 (10 U/mL) were stimulated with IL-33, IL-25 and TSLP (50 ng/mL each), shortened as IL, or left non-stimulated (ns) for 24 hours. PGE₂ (30 nM) was added 10 min before the cytokines. Concentrations of released IL-5 (**B, D**) and IL-13 (**C, D**) in ILC2 supernatants were assessed by ELISA, graphs show individual concentrations (****p<0.0001, n=9-10). **E.** Intracellular cytokine expression of IL-5 and IL-13 was determined by flow cytometry. **F** and **G.** The graphs present individual percentages of IL-5- (**F**) and IL-13- (**G**) positive cells (**p<0.01, ***p<0.001, n=6). **H.** Expression of GATA3 was analyzed by flow cytometry. Graphs show individual geometric mean fluorescence intensity (**I**) and geometric mean fluorescence intensity normalized to non-stimulated cells (**J**), of GATA3 expression (*p<0.05, n=5).

Figure 2. PGE₂ prevents cytokine-induced CD25 upregulation and proliferation

of ILC2. ILC2 were stimulated as indicated for 24 hours (**A, B, C**) or 72 hours (**D-I**).

A. and **D.** Expression of CD25 was analyzed by flow cytometry, representative histograms are shown. Graphs show individual geometric mean fluorescence intensity of CD25 expression after 24 hours (**B**) (*p<0.05, n=8) and 72 hours (**E**) (*p<0.05, n=6) and geometric mean fluorescence intensity normalized to non-stimulated cells of CD25 expression after 24 hours (**C**) (*p<0.05, n=8) and 72 hours of culture (**F**) (**p<0.01, n=6). **G.** Proliferation of CellTrace violet dye-loaded ILC2 after 72 hours incubation was analyzed by flow cytometry. **H.** Data are shown as percentage of ILC2

in different division phases (D0-D4) ($***p < 0.001$, $n=4$). **I.** Cell viability was determined as percentage of singlet cells which are negative for dead cell marker. Data are shown as mean $\pm$ SEM ($n=4$).

Figure 3. PGE₂ reduces GATA3 upregulation. ILC2 were stimulated as indicated for 72 hours. Concentrations of IL-5 (**A**) and IL-13 (**B**) in ILC2 supernatants were determined by ELISA, data are shown as individual concentrations ($***p < 0.001$, $****p < 0.0001$, $n=10$). **C.** Expression of GATA3 was analyzed by flow cytometry. Graphs show individual geometric mean fluorescence intensity (**D**) and geometric mean fluorescence intensity normalized to non-stimulated cells (**E**), of GATA3 expression ($**p < 0.01$, $n=8$).

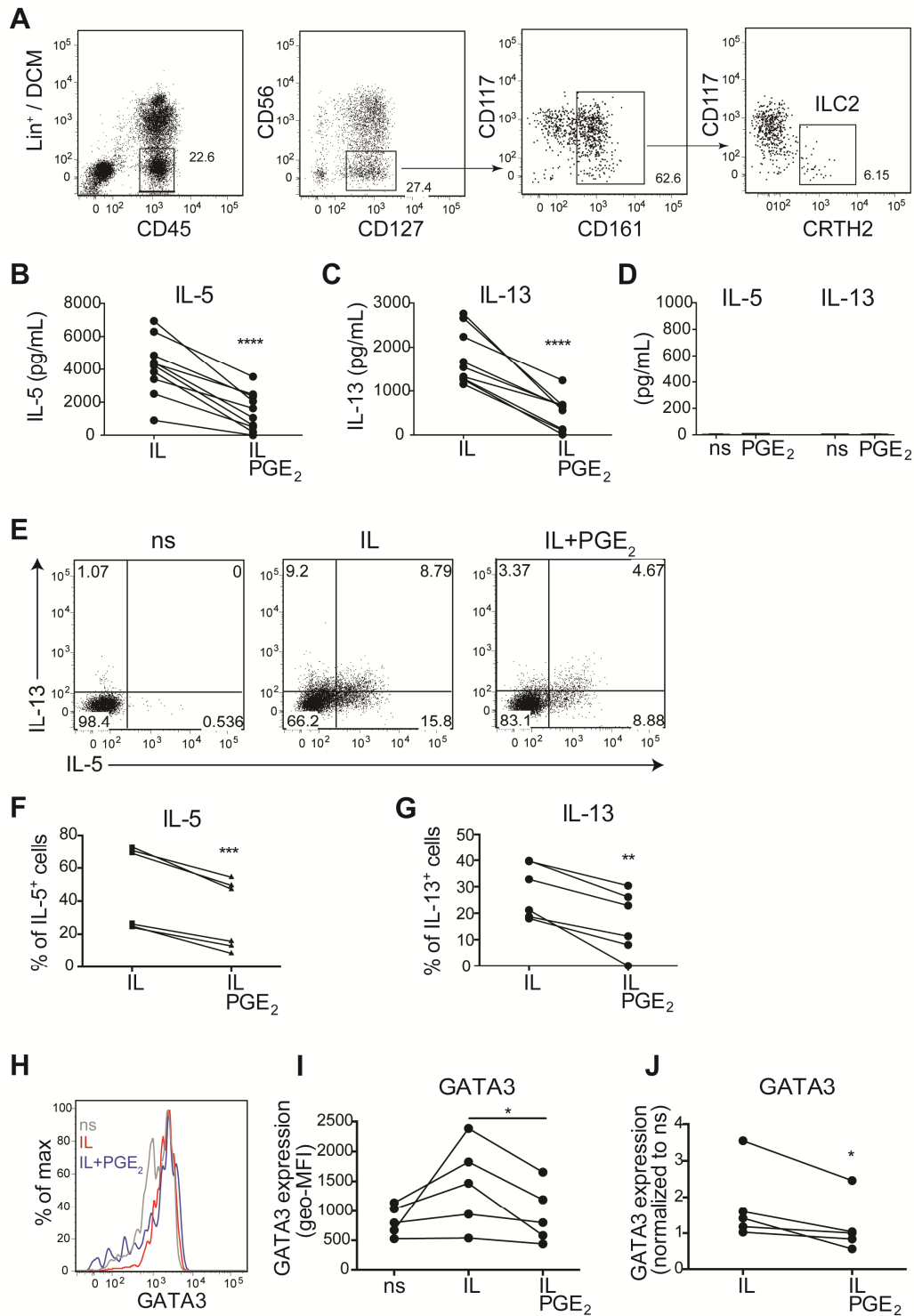
Figure 4. PGE₂ reduces IL-5 and IL-13 secretion from fresh blood and tonsil ILC2. Freshly sorted human blood (**A-E**) and tonsil (**F, G**) ILC2 were incubated as indicated for 5 days. Concentrations of released IL-5 from blood ILC2 (**A**) and tonsil ILC2 (**F**) and IL-13 from blood ILC2 (**B**) and tonsil ILC2 (**G**) were measured by ELISA, graphs show individual concentrations ($*p < 0.05$, $**p < 0.01$; $n=6$ for blood ILC2 and $n=4$ for tonsil ILC2). **C.** Expression of CD25 in freshly sorted blood ILC2 was analyzed by flow cytometry, representative histograms are shown. **D.** Graph shows individual geometric mean fluorescence intensity of CD25 expression in blood ILC2 after 5 days of culture ($*p < 0.05$, $n=4$). **E.** Images of freshly sorted blood ILC2 after 5 days of treatments; 10 x magnification, scale bar = 100 μ m.

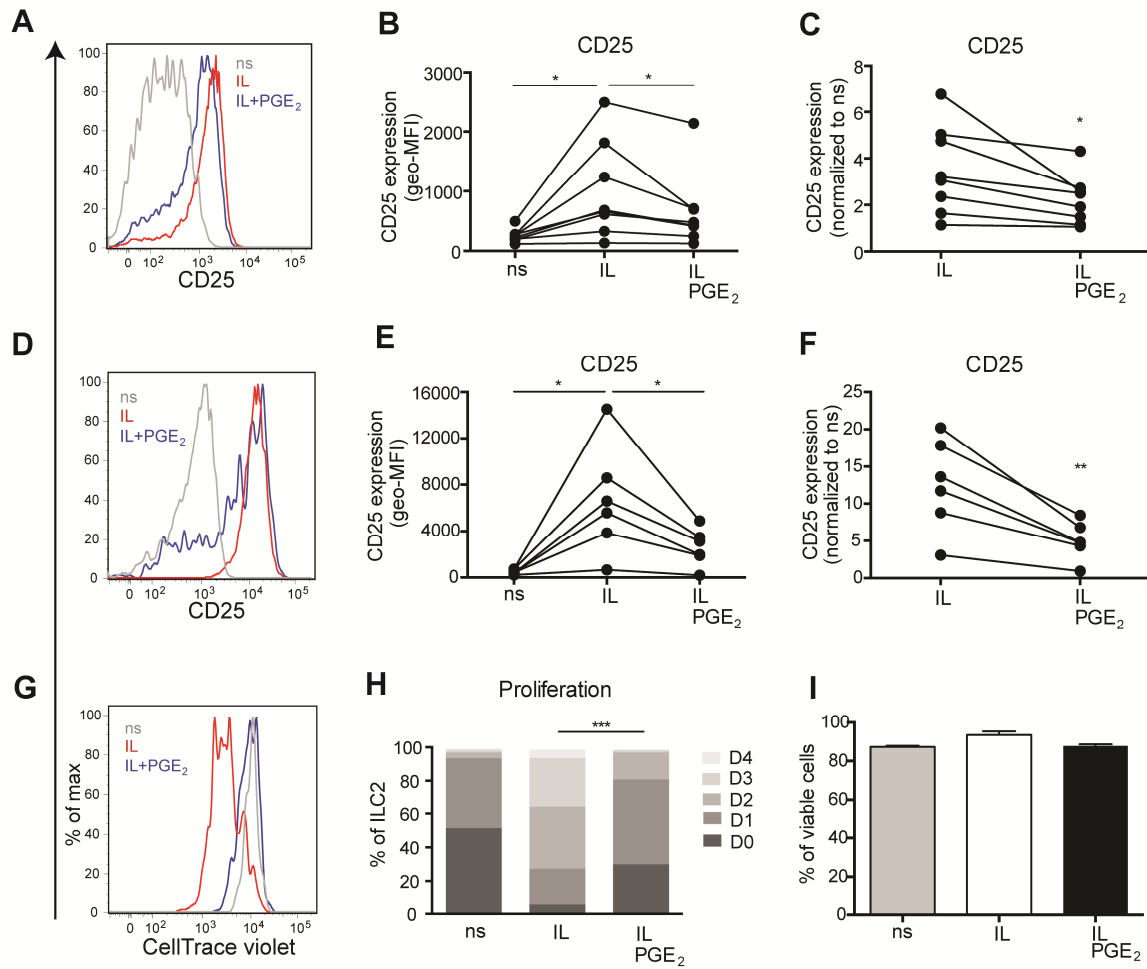
Figure 5. Human tonsil ILC2 selectively express EP2 and EP4 receptors. **A.** EP receptor transcripts of freshly sorted human tonsil ILC2 were detected by single cell

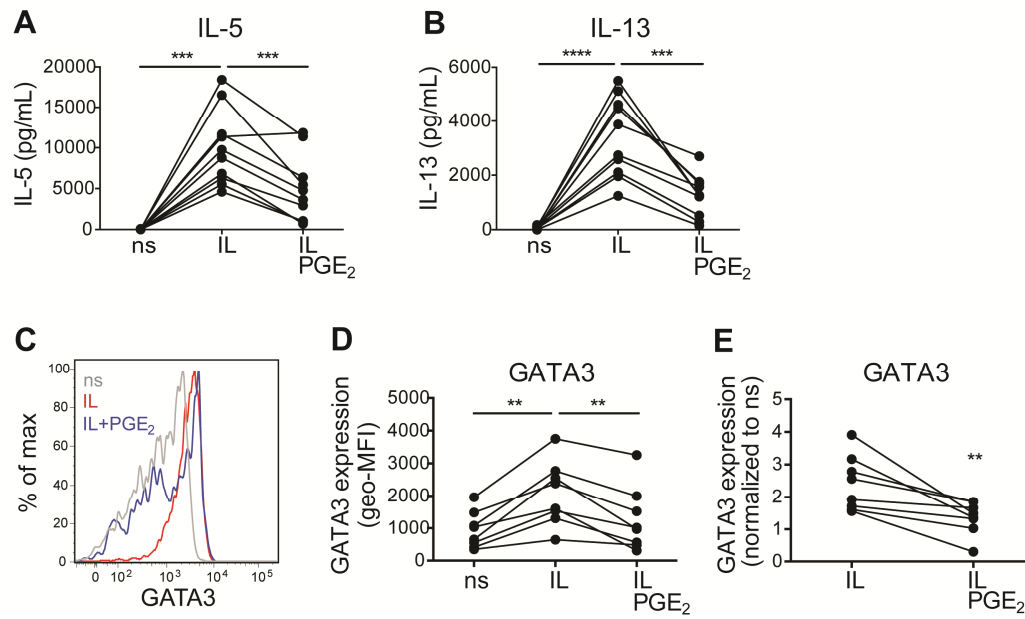
RNA sequencing. Expression distribution (violin plots) are shown for genes of PGE₂ receptors (*PTGER1-4*) in each ILC subset (ILC1-3 and NK cells), where expression patterns were obtained as reads per kilobase gene model and million mappable reads (RPKMs) **B.** mRNA expression of EP receptors (*PTGER1-4*) in sorted and expanded tonsil ILC2 was determined by RT-qPCR (n=5). **C.** mRNA expression of EP2 and EP4 receptor after 24 hours stimulation normalized to non-stimulated cells. Graphs show mean expression +SEM (n=5).

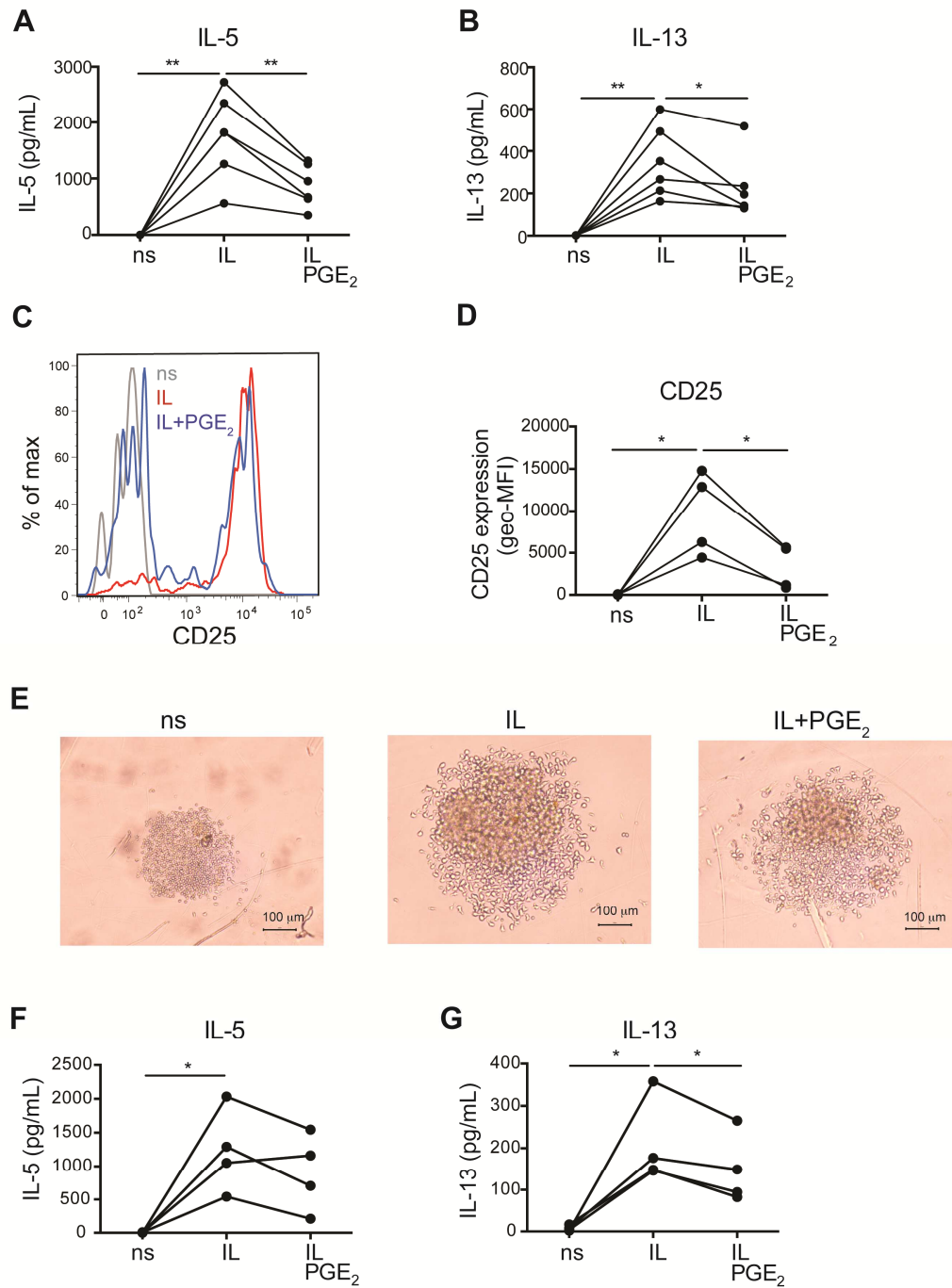
Figure 6. PGE₂ reduces IL-5 and IL-13 production in ILC2 through activation of EP2 and EP4 receptors. Sorted and expanded tonsil ILC2 were stimulated as indicated for 24 hours. EP2 receptor antagonist, PF-04418948 (PF, 1 μ M) and EP4 receptor antagonist, ONO AE3-208 (ONO, 1 μ M) were added separately or together, 20 min before PGE₂. **A** and **B.** Concentrations of released IL-5 (**A**) and IL-13 (**B**) in ILC2 supernatants were determined by ELISA. Bar graphs show means +SEM (*p<0.05, **p<0.01, n=6-7) **C.** Representative dot plots show intracellular expression of IL-5 and IL-13 assessed by flow cytometry. **D.** and **E.** Graphs show mean percentage +SEM of IL-5- (**D**) and IL-13- (**E**) positive ILC2 (*p<0.05, **p<0.01, n=6). Expression of GATA3 was analyzed by flow cytometry. Graphs show geometric mean fluorescence intensity +SEM (**F**) and geometric mean fluorescence intensity normalized to non-stimulated cells +SEM (**G**), of GATA3 expression (*p<0.05, n=5). Released IL-5 (**H**) and IL-13 (**I**) are shown after 10 min pretreatment with EP2 receptor agonist, butaprost (But) and EP4 receptor agonist, L-902, 688 (L9), separately or together in 100 nM concentration. Concentrations were determined by ELISA and are shown as means $\pm$ SEM (*p<0.05, **p<0.01, n=4; compared to IL treatment).

Figure 7. Activation of EP2 and EP4 receptors inhibits ILC2 cytokine release likely via preventing ILC2 proliferation. Sorted and expanded tonsil ILC2 were stimulated as indicated for 72 hours. Additionally, EP2 receptor antagonist, PF-04418948 (PF, 1 μ M) and EP4 receptor antagonist, ONO AE3-208 (ONO, 1 μ M) were added separately or together, 20 min before PGE₂. **A.** Proliferation of CellTrace violet dye-loaded ILC2 after 72 hours of incubation was analyzed by flow cytometry. **B.** Data are shown as percentage of ILC2 in different division phases (D0-D4) (* p <0.05, *** p <0.001, n =4). Concentrations of released IL-5 (**C**) and IL-13 (**D**) in ILC2 supernatants were detected by ELISA, bars show means +SEM (* p <0.05, ** p <0.01, n =7). Expression of GATA3 was analyzed by flow cytometry. Graphs show geometric mean fluorescence intensity +SEM (**E**) and geometric mean fluorescence intensity normalized to non-stimulated cells +SEM (**F**), of GATA3 expression (* p <0.05, ** p <0.01, n =7).

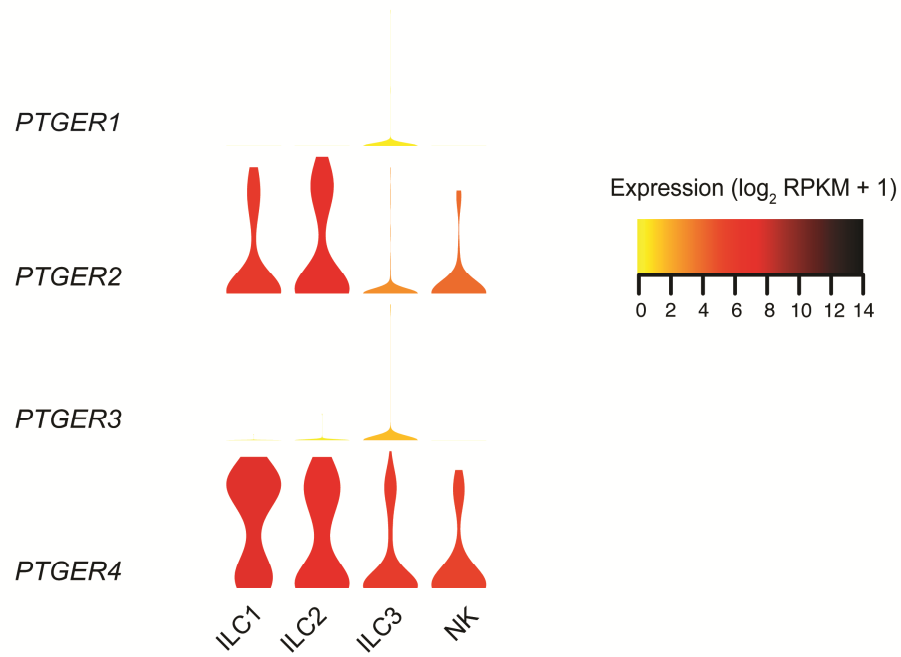




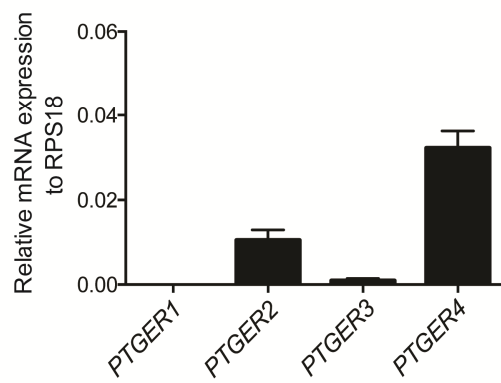




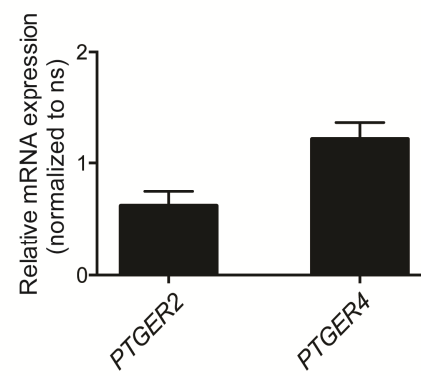
A

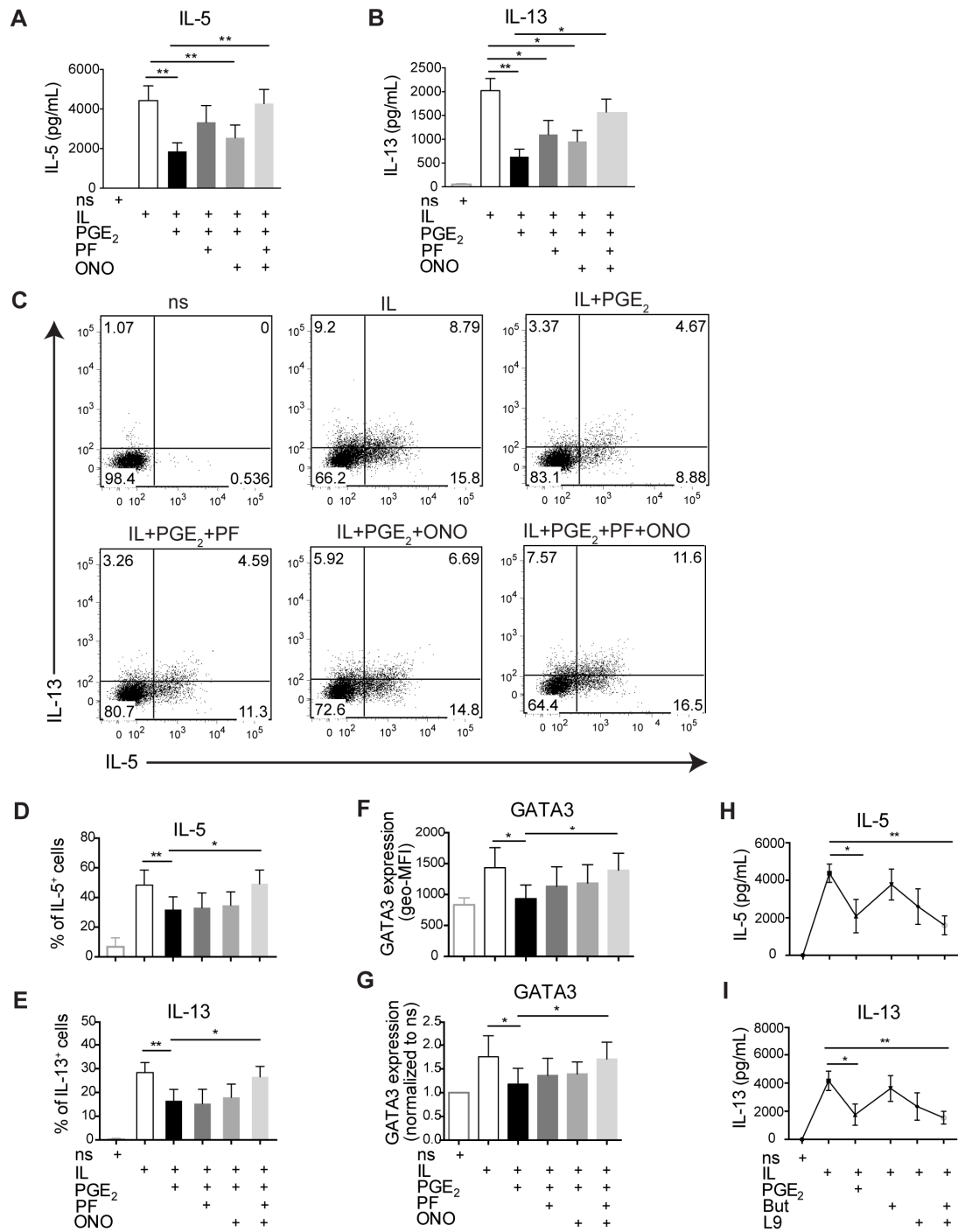


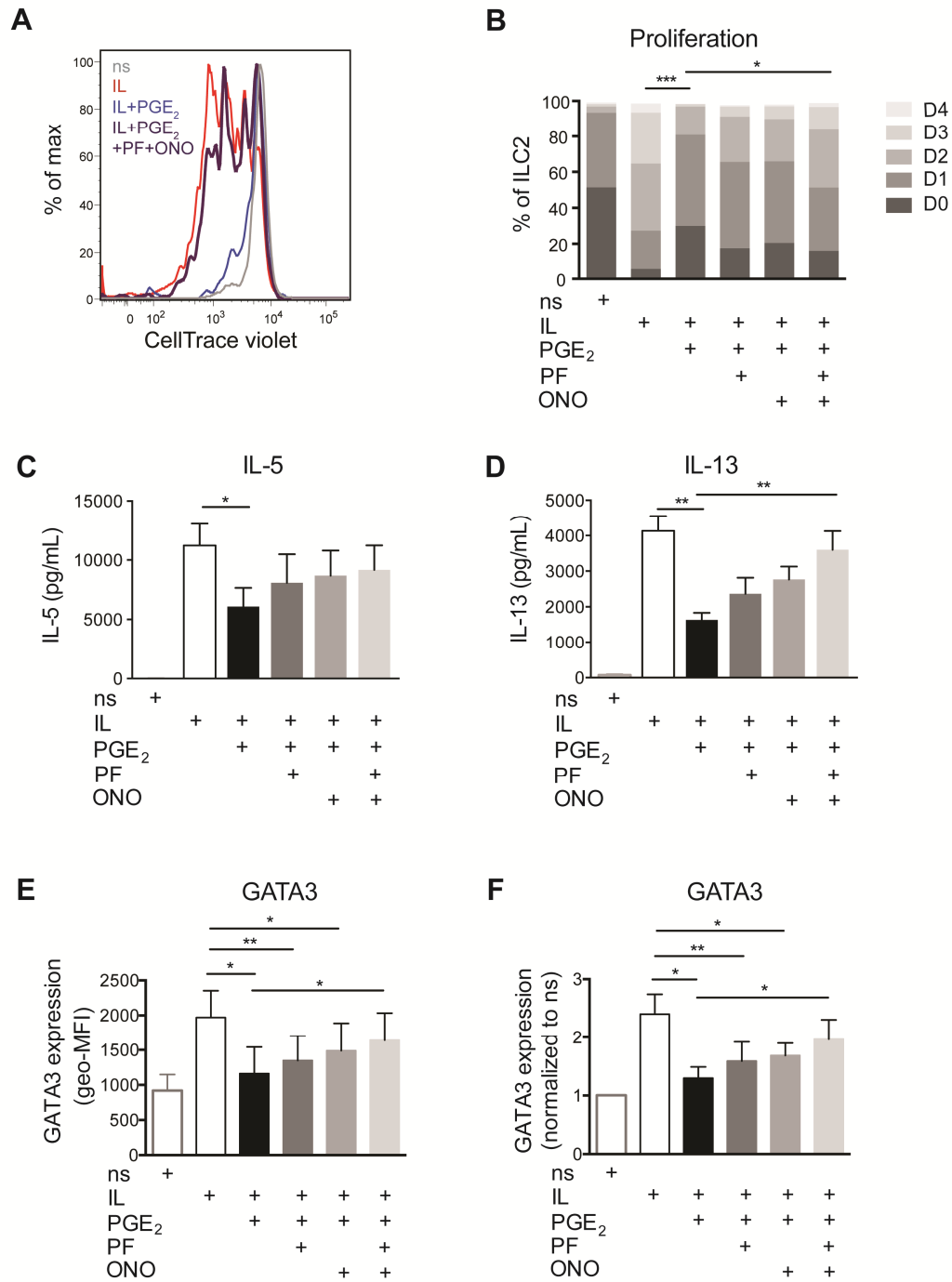
B



C







1 ONLINE REPOSITORY MATERIAL**2 PGE₂ suppresses human group 2 innate lymphoid cell function**

3
4 Jovana Maric, MSc^{1,2}, Avinash Ravindran, MSc³, Luca Mazzurana, MSc², Åsa K.
5 Björklund, PhD⁴, Aline Van Acker, PhD², Anna Rao, PhD², Danielle Friberg, MD
6 PhD⁵, Sven-Erik Dahlén, Prof. MD PhD⁶, Akos Heinemann, Prof. MD¹, Viktoria
7 Konya, PhD^{1,2*} and Jenny Mjösberg, PhD^{2,7*}

8 * Contributed equally

9
10 ¹Institute of Experimental and Clinical Pharmacology, Medical University of Graz,
11 Graz, Austria

12 ²Center for Infectious Medicine, Department of Medicine Huddinge, Karolinska
13 Institutet, Stockholm, Sweden

14 ³Immunology and Allergy Unit, Department of Medicine, Solna, Karolinska Institutet,
15 Stockholm, Sweden

16 ⁴Science for Life Laboratory, Department of Cell and Molecular Biology, Uppsala
17 University, Uppsala, Sweden

18 ⁵Department of Oto-Rhino-Laryngology, Karolinska University Hospital and
19 CLINTEC, Karolinska Institutet, Stockholm, Sweden

20 ⁶Experimental Asthma and Allergy Research, Institute of Environmental Medicine,
21 Karolinska Institutet, Stockholm, Sweden

22 ⁷Department of Clinical and Experimental Medicine, Linköping University, Sweden

23
24 *Corresponding authors:

25 Jenny Mjösberg, PhD, Center for Infectious Medicine, F59, Karolinska Institutet, S-

26 14186 Stockholm, Sweden. jenny.mjosberg@ki.se, Phone: +46-70 547 29 56, Fax:
27 +46-8-31 1101
28 Viktoria Konya, PhD, Institute of Experimental and Clinical Pharmacology, Medical
29 University of Graz, Graz, Austria. viktoria.konya@medunigraz.at, Phone: +43-316
30 380-4512, Fax: 43-316-380-9645
31

METHODS

Isolation and flow cytometric sorting of tonsil and blood ILC2

Human tonsils were freshly obtained from tonsillectomies of patients with obstructive sleep apnea syndrome at the Ear Nose Throat Clinic at the Karolinska University Hospital Huddinge, Sweden. The collection and use of tonsils were approved by the regional ethical board at 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. Cell isolation from tonsils was performed as previously described¹. Briefly, tonsils were dissected into small pieces, ground through filter and centrifuged. Mononuclear cells were isolated by lymphoprep centrifugation and depleted from lineage positive cells using directly conjugated anti-CD3, CD19 and CD14 microbeads (markers for T cells, B cells and monocytes, respectively) and LD MACS columns (Miltenyi). Lineage-depleted cells from buffy coats were additionally enriched using CD127 MicroBead Kit (Miltenyi). Lineage-depleted cells were incubated with the following specific monoclonal antibodies: FITC-conjugated anti-CD14 (TÜK4, Dako); anti-CD3 (SK7), anti-FcεR1α (AER-37 CRA-1), anti-CD34 (581), anti-CD123 (6H6), anti-CD1a (HI149), anti-TCRαβ (IP26), anti-TCRγδ (B1), anti-CD94 (DX22) (all Biolegend); anti-BDCA2 (AC144, Miltenyi Biotech), anti-CD19 (4G7, BD Biosciences). Additionally we used Brilliant Violet 605-conjugated anti-CD161 (HP-3G10), Brilliant Violet 711-conjugated anti-CD56 (HCD56) (both Biolegend); PE-CF594-conjugated anti-CRTH2 (BM16), V500-conjugated anti-CD45 (HI30) (both BD Biosciences); PE-Cy7-conjugated anti-CD127 (R34.34), PE-Cy5.5-conjugated anti-CD117 (104D2D1), PE-Cy5-conjugated anti-NKp44 (Z231) (all Beckman Coulter). In addition, cells were

stained with the LIVE/DEAD Fixable Green Dead Cell Stain Kit (Life Technologies). ILC2 were sort-purified based on their specific phenotype as Lin⁻ CD127⁺ CD161⁺ CRTH2⁺ cells by using FACS Aria Fusion cell sorter (BD Bioscience) equipped with the FACSDiva software version 8.

Culture and expansion of ILC2

Freshly sorted ILC2 were cultured in IMDM medium (Gibco) with Yssel's supplement and 1% normal human serum (NHS) (Invitrogen) with penicillin (100 U/mL, HyClone) and streptomycin (0.1 mg/mL, HyClone). Sorted tonsil ILC2 were expanded for two weeks in culture with a feeder cell mixture of peripheral blood mononuclear cells irradiated at 25 Gy and JY cells irradiated at 50 Gy, with IL-2 (100 U/mL), IL-4 (25 ng/mL) and PHA (1 µg/mL) as previously described².

Freshly sorted ILC2 were kept in Yssel's medium/1% NHS with IL-2 (10 U/mL) in 96-well plate (2000 cells/sample) and treated the following day.

Treatments of ILC2

ILC2 were expanded for two weeks and then rested in Yssel's 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 5x10⁴ cells/well. ILC2 were treated with PGE₂ (30 nM; Cayman Chemicals) for 10 min before adding IL-2 (10 U/mL) with or without IL-33 (PeproTech), TSLP (PeproTech) and IL-25 (R&D) at 50 ng/mL each. EP2 receptor antagonist, PF-04418948 and EP4 receptor antagonist, ONO AE3-208 (both from Cayman Chemicals) were added 20 min before PGE₂ in 1 µM concentration. ILC2 were incubated at 37°C for 24 hours or 72 hours. For addressing concentration-dependent effects of PGE₂, following concentrations were used: 3nM, 10 nM, 30 nM,

100 nM and 300 nM. Dose-response effects of EP2 and EP4 receptors antagonists were analyzed using following concentrations: 100 nM, 300 nM, 1 μ M, 3 μ M. To confirm involvement of EP2 and EP4 receptors in suppression of ILC2 function, we used EP2 receptor specific agonist, butaprost and EP4 receptor specific agonist, L-902, 688 (both from Cayman Chemicals), in the following concentrations: 10 nM, 30 nM, 100 nM, 300 nM and 1 μ M.

For RT-qPCR, expanded ILC2 were seeded 1×10^5 cells/well and incubated for 24 hours with IL-2 (10 U/mL) and with or without combination of IL-33, TSLP and IL-25 (50 ng/mL each).

Freshly sorted ILC2 were kept in the presence of IL-2 (10 U/mL) and were control-treated or with the combination of IL-33, TSLP and IL-25 (50 ng/mL each) for 5 days. PGE₂ (100 nM) was added 10 min before the cytokines. Supernatants were collected after 5 days.

Flow cytometry staining and analysis of intracellular cytokines and transcription factors

Cell surface staining of expanded tonsil ILC2 was assessed 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 using LIVE/DEAD Fixable Green Dead Cell Stain Kit (Life Technologies). CD25 cell surface expression was determined by using Brilliant Violet 650-conjugated anti-CD25 (BC96, Biolegend).

For intracellular cytokine detection, ILC2 were stimulated for 24 hours as described above, with Golgi plug and Golgi stop added after 18 hours. ILC2 were thereafter fixed (2% paraformaldehyde), permeabilized (FACS Permeabilizing Solution 2, BD

Bioscience) and stained for intracellular IL-13 (APC-conjugated anti-IL-13; JES10-5A2) and IL-5 (PE-conjugated anti-IL-5; TRFK5) with antibodies from BD Biosciences.

Intracellular expression of GATA3 was determined after fixation and permeabilization (Foxp3/transcription factor staining buffer set, eBioscience) by using APC-conjugated anti-GATA3 (clone TWAJ, eBioscience).

All samples were measured on a LSR Fortessa flow cytometer (BD Bioscience), equipped with FACSDiva software version 8 and data were analyzed using FlowJo software (Tree Star Inc.).

Proliferation assay

For assessing cell proliferation, ILC2 were incubated in 500 μ L IMDM with 1 μ L of CellTrace violet dye (Invitrogen) for 20 min at 37°C. Cells were washed twice with IMDM/10% FCS. Cells were thereafter resuspended in Yssel's medium/1% NHS and seeded into 96-well plates. Cells were treated with PGE₂ (30 nM) for 10 min and thereafter with IL-2 (10 U/mL) with or without IL-33, TSLP and IL-25 (50 ng/mL each) for 72 hours. EP2 and EP4 receptor antagonists (1 μ M) were added 20 min before PGE₂. All samples were measured on a LSR Fortessa flow cytometer as described above. Cell viability was determined as the percentage of all singlet cells lacking staining of the LIVE/DEAD cell marker.

ELISA

IL-5 and IL-13 concentrations in ILC2 supernatants after the indicated treatments were assessed by ELISA. Human IL-5 ELISA Duo-Set kit was purchased from R&D systems, and the human IL-13 ELISA kit was from Sanquin.

RT-qPCR

Total RNA from expanded tonsil ILC2 cells was isolated using RNeasy micro kit (Qiagen). iScriptTM cDNA Synthesis Kit (Bio-Rad) was used for cDNA synthesis, and SsoAdvancedTM Universal SYBR[®] Green supermix (Bio-Rad) for PCR reactions. Primers were PrimePCRTM SYBR[®]Green assay: *PTGER1-PTGER4*, for human EP1-EP4 receptors and PrimePCRTM SYBR[®]Green assay: human *RPS18*, for house-keeping gene RPS18 (all primers were from Bio-Rad). RT-qPCRs were performed in CFX Connect Real-Time PCR Detection System (Bio-Rad).

RNA sequencing analysis

Single cell RNA-seq expression patterns were obtained as reads per kilobase gene model and million mappable reads (RPKM) from the Björklund, Forkel et al.¹ expression matrix (Gene Expression Omnibus, GSE70580). Expression levels of the *PTGER* genes in the different ILC subsets and NK cells were interpreted as violin plots using the R software.

Statistical analyses

In all experiments, "n" represents the number of individual donors used for ILC2 isolation. Differences between two groups were analyzed by using matched-pairs t-test and three or more groups were compared by one-way ANOVA for repeated measurements and Dunnett's multiple comparisons test. Analyses were performed using GraphPad Prism 6 Software.

References

1. 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. *Nature immunology* 2016;
17:740-a.
2. 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 CCR4 and CD161. *Nature immunology* 2011;
12:1055-62.

SUPPLEMENTAL FIGURE LEGENDS**Figure E1. Expanded human tonsil ILC2 retain their phenotype.**

A. Gating of ILC2 from lineage-depleted alive freshly isolated human tonsil mononuclear cells. **B.** Sorted and expanded ILC2 retain their phenotype after two weeks of culture expressing CD161 and CCR2 and lacking expression of CD3 and CD56 as analyzed by flow cytometry.

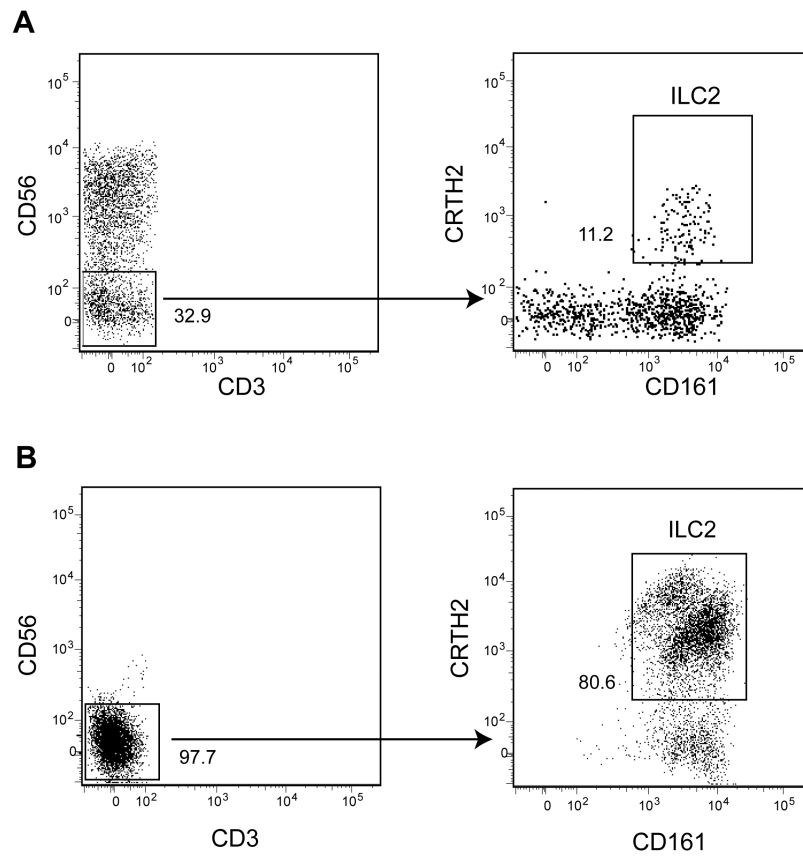
Figure E2. PGE₂ does not affect ILC2 viability and proliferation after 24 hours.

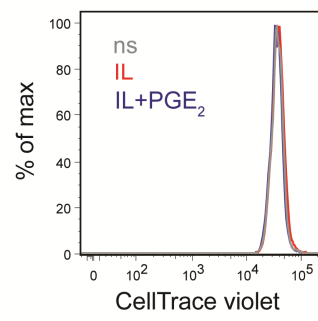
ILC2 were stimulated as indicated for 24 hours. **A.** Proliferation of CellTrace violet dye-loaded ILC2 after 24 hours was analyzed by flow cytometry. Representative histograms are shown (n=2). **B.** Cell viability was determined as percentage of singlet cells, which are negative for dead cell marker. Data are shown as mean +SEM (n=4).

Figure E3. PGE₂ concentration-dependently inhibits cytokine production of

ILC2. Sorted and expanded tonsil ILC2 were stimulated with combination of cytokines (IL-33, TSLP, IL-25 plus IL-2) (**A, B**) for 24 hours. PGE₂ was added in different concentrations (3, 10, 30, 100 and 300 nM) 10 min before the cytokines. Concentrations of IL-5 (**A**) and IL-13 (**B**) in ILC2 supernatants were determined by ELISA, data are shown as mean concentrations ±SEM (*p<0.05, **p<0.01, ***p<0.001, n=4). ILC2 were stimulated with a cocktail of cytokines or individual cytokines in addition to IL-2 (**C, D**). Graphs show individual IL-5 (**C**) and IL-13 (**D**) concentrations, n=3.

Figure E4. Dual engagement of EP2 and EP4 receptors is essential for the PGE₂-induced reduction of ILC2 cytokine production. Sorted and expanded tonsil ILC2 were stimulated as indicated for 24 hours. Additionally, EP2 receptor antagonist, PF-04418948 (PF) and EP4 receptor antagonist, ONO AE3-208 (ONO) were added separately or together in different concentrations (100 nM, 300 nM, 1 μ M, 3 μ M) 20 min before PGE₂. **A** and **B**. Concentrations of released IL-5 (**A**) and IL-13 (**B**) in ILC2 supernatants were determined by ELISA and shown as means $\pm$ SEM (n=4). **C** and **D**. Sorted and expanded tonsil ILC2 were treated with EP2 receptor agonist, butaprost (But) and EP4 receptor agonist, L-902, 688 (L9), separately or together in different concentrations (10 nM, 30 nM, 100 nM, 300 nM and 1 μ M) 10 min before the stimulatory cytokines. Concentrations of released IL-5 (**C**) and IL-13 (**D**) in ILC2 supernatants were determined by ELISA and are shown as means $\pm$ SEM (*p<0.05, **p<0.01, n=4; compared to IL treatment).



A**B**