

Dissertation

**Role of prostaglandins in regulation of
human group 2 innate lymphoid cell function**

Submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz”.

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Abbreviations

AC – adenylate cyclase

AD – atopic dermatitis

APC - allophycocyanin

BAL – bronchoalveolar lavage

cAMP – cyclic adenosine monophosphate

CCR10 – C-C chemokine receptor type 10

cDNA – complementary deoxyribonucleic acid

CLP - common lymphoid progenitor

COPD - chronic obstructive pulmonary disease

COX - cyclooxygenase

CRTH2 – chemoattractant receptor-homologous molecule expressed on Th2 cells

CysLT – cysteinyl leukotriene

DC – dendritic cell

DK-PGD₂ – 13,14 dihydro-15-keto PGD₂

DP1 – prostaglandin D₂ receptor 1

EDTA – ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

EP – E type prostanoid receptor

EPRAP – EP4-associated protein

FACS – fluorescence-activated cell sorting

FBS – fetal bovine serum

FC – flow cytometry

FcεR1 – FC epsilon R1, high-affinity receptor for IgE

FITC – fluorescein isothiocyanate

GM-CSF – granulocyte-macrophage colony-stimulating factor

HDM – house dust mite

HPGD – 15-hydroxyprostaglandin dehydrogenase

HPGDS – hematopoietic prostaglandin D₂ synthase

ICOS – inducible costimulator

ICOS-L – ICOS ligand

IFN – interferon
Ig – immunoglobulin
IL – interleukin
ILC – innate lymphoid cell
LC-MS/MS – liquid chromatography-tandem mass spectrometry
LPGDS – lipocalin-type prostaglandin D₂ synthase
LPS – lipopolysaccharide
LT – leukotriene
LTi – lymphoid tissue inducers
LX – lipoxin
MAPK – mitogen-activated protein kinase
MDSCs – myeloid-derived suppressor cells (MDSCs)
MHCII – major histocompatibility complex class II
mRNA – messenger ribonucleic acid
NCR – natural cytotoxicity receptor
Nfil3 – nuclear factor IL-3 dependent
NF- κ B – nuclear factor kappa B
NK – natural killer
NSAID – non-steroidal anti-inflammatory drug
OVA – ovalbumin
PBMCs – peripheral blood mononuclear cells
PBS – phosphate buffered saline
PE – phycoerythrin
PG – prostaglandin
PHA – phytohemagglutinin
PKC – protein kinase C
PLZF – promyelocytic leukemia zinc finger (PLZF)
PPAR- γ – peroxisome proliferator-activated receptor- γ
RAG – recombination activating gene
ROR α – retinoic acid receptor-related orphan receptor alpha
RPS18 – ribosomal protein S18
RT-qPCR – quantitative reverse transcription polymerase chain reaction
Sca-1 – stem cell antigen-1
TCF1- T-cell factor 1

TCR – T-cell receptor

TLR – Toll-like receptor

TNF – tumor necrosis factor

TOX - thymocyte selection-associated high-mobility group box

Tregs – Regulatory T cells

TSLP – thymic stromal lymphopoietin

TX - thromboxane

γ c - common γ chain of IL-2 receptor

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Abstract

Innate lymphoid cells (ILCs) are a recently identified family of lymphoid cells lacking expression of lineage markers and antigen receptors. Group 2 innate lymphoid cells (ILC2) are involved in the initiation and amplification of type 2 immune responses.

Upon allergen or pathogen stimulation, the airway epithelium-derived innate cytokines IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) activate ILC2. These events lead to production of type 2 cytokines IL-5 and IL-13 by ILC2, which contribute to asthma and allergic inflammation.

Lipid mediators have been shown to play important roles in ILC2 biology. Prostaglandin E₂ (PGE₂), a bioactive lipid abundantly present in the lung, mediates protective effects in allergic responses. Thus, in the first part of my thesis, the aim was to reveal the role of PGE₂ in regulating human ILC2 function.

I demonstrated that PGE₂ inhibits production and release of IL-5 and IL-13 from human tonsil and blood ILC2 in response to stimulation with activating cytokines IL-33, IL-25, TSLP plus IL-2. PGE₂ affects molecular pathways important for ILC2 activation, as it reduces upregulation of GATA3 and CD25 expression and blocks ILC2 proliferation. Additionally, I showed that the suppressive effects of PGE₂ are mediated through simultaneous engagement of EP2 and EP4 receptors, PGE₂ receptors which are specifically expressed on ILC2.

Another lipid mediator, PGD₂, has been shown to induce cytokine production and migration of ILC2 via CRTH2 receptor activation. In the second part of the thesis, my objective was to determine whether ILC2 are capable of prostaglandin, in particular PGD₂ production, on their own and what would be the impact of such a process in ILC2 function. I showed that ILC2 constitutively express hematopoietic PGD₂ synthesis (HPGDS) and highly upregulate COX-2 upon stimulation with activating cytokines. Consequently, PGD₂ pathway is induced, as PGD₂ and its metabolites are detected in the supernatants of stimulated ILC2. Importantly, I revealed that, upon cytokine stimulation, endogenously produced PGD₂, acting in an autocrine manner via CRTH2 receptor, is crucial for ILC2

responses. I proved this by demonstrating that blocking of COX-1/2 enzyme or the CRTH2 receptor inhibited cytokine-induced ILC2 activation.

Overall, these data elucidate new mechanisms of ILC2 regulation on the one hand by showing the inhibitory PGE₂-EP2/EP4 axis and on the other hand by revealing the stimulatory function of the endogenous PGD₂-CRTH2 axis. These novel findings provide potential therapeutic approaches by targeting ILC2 function in the treatment of allergic inflammatory diseases.

Zusammenfassung

Innate lymphoid cells (ILCs) gehören zu einer kürzlich entdeckten Familie von Immunzellen, die durch das Fehlen von Abstammungsmarkern und Antigenrezeptoren charakterisiert sind. Die ILCs der Gruppe 2 sind an der Einleitung und der Verstärkung von Typ 2 Immunreaktionen beteiligt. Bei einer Stimulation durch ein Allergen oder Pathogen kommt es durch das vom respiratorischen Epithel exprimierte Zytokin Interleukin (IL)-33, IL-25 und dem Thymic stromal lymphopoietin (TSLP) zu einer Aktivierung der ILC2. Diese Aktivierung bewirkt in den ILC2 eine Produktion der Typ 2 Zytokine IL-5 und IL-13, die am Asthma bronchiale und an allergischen Reaktionen beteiligt sind.

Es wurde gezeigt dass Lipid-Mediatoren eine wichtige Rolle in der Biologie der ILC2 spielen. Prostaglandin (PG) E₂, ein bioaktives Lipid das in hohem Maße in der Lunge gebildet wird, vermittelt protektive Effekte bei der allergischen Immunantwort. Daher war das Ziel im ersten Teil der Dissertation aufzuzeigen, welche Rolle PGE₂ in der Steuerung der Funktionen humaner ILC2 hat.

Ich habe gezeigt, dass PGE₂ die Produktion und Freisetzung von IL-5 und IL-13 von ILC2 - isoliert aus humanen Tonsillen und Blut - hemmt, wenn diese mit proinflammatorischen Zytokinen (IL-33, IL-25, TSLP und IL-2) stimuliert werden. PGE₂ beeinflusst molekulare Mechanismen, die wichtig für die ILC2 Aktivierung sind, da es die Hochregulierung von GATA3 und CD25 Expression reduziert und die ILC2 Proliferation hemmt. Außerdem konnte ich zeigen, dass die suppressiven Effekte von PGE₂ durch die simultane Bindung an EP2 und EP4 Rezeptoren vermittelt werden, PGE₂ Rezeptoren, die spezifisch von ILC2 exprimiert werden.

Für einen anderen Lipid-Mediator, PGD₂, wurde gezeigt, dass es über den CRTH2 Rezeptor die Produktion von Zytokinen und die Migration von ILC2 stimuliert. Im zweiten Teil meiner Dissertation war es mein Ziel zu klären, ob ILC2 von sich aus Prostaglandine, besonders das PGD₂ produzieren können und was der Auslöser eines solchen Prozesses in der ILC2 Funktion sein könnte.

Ich habe gezeigt, dass ILC2 eine ständige Expression der hämatopoetischen PGD₂ Synthese (HPDGS) aufweisen und bei Stimulation mit aktivierenden Zytokinen COX-2 stark hochregulieren. Folglich wird der PGD₂ Weg induziert und PGD₂ sowie seine

Metabolite werden in dem Überstand von stimulierten ILC2 detektiert. Ich konnte darlegen, dass durch Zytokin Stimulation PGD_2 , welches essentiell für die ILC2 Antwort ist, über den CRTH2 Rezeptor in autokriner Weise wirkt. Das konnte ich beweisen indem ich zeigte, dass eine Hemmung des COX-1/2 Enzyms oder eine Blockade des CRTH2 Rezeptors die Zytokin-induzierte ILC2 Aktivierung hemmte.

Insgesamt beleuchten diese Daten neue Mechanismen der ILC2 Regulation durch die Darstellung der hemmenden PGE_2 -EP2/EP4-Achse und andererseits durch das Aufzeigen der stimulierenden Funktion der endogenen PGD_2 -CRTH2-Achse. Diese neuen Erkenntnisse liefern einen möglichen therapeutischen Ansatz durch die Blockade der ILC2 Funktion in der Behandlung von allergischen Erkrankungen.

1. Introduction

Immune system enables defense of the host organisms against various dangerous factors from environment. Immune system consists of innate and adaptive immune responses which together make three major types of cell-mediated immunity: type 1, 2 and 3 ². Type 1 immunity is important for defense against intracellular pathogens. It involves IFN- γ -producing innate lymphoid cells type 1 (ILC1), natural killer (NK) cells, CD8+ cytotoxic T cells (Tc1) and CD4+ helper T cells (Th1 cells), which can activate mononuclear phagocytes. Type 2 immunity provides protection against parasites. Cells which participate in these responses are Th2 cells, Tc2 cells and ILC2 cells which produce IL-4, IL-5 and IL-13, which stimulate eosinophils, mast cells, basophils and IgE production from B cells. Type 3 immunity enables protection against extracellular bacteria and fungi and includes the lymphocytes which produce IL-17 and/or IL-22, such as Th17 cells, Tc17 cells and ILC3.

Innate immune system enables fast response to different stimuli and initiation of the subsequent processes, whereas adaptive immune responses require recognition of specific antigens and provide immune memory.

Innate lymphoid cells (ILCs) are a novel group of mononuclear hematopoietic cells with lymphoid morphology which lack expression of lineage markers and recombination activating gene (RAG)-dependent rearranged antigen receptors which are expressed on T and B cells ³. ILCs reside in tissues, play roles in barrier epithelial integrity and respond to different stress signals upon damage of the tissue, mainly by producing cytokines ⁴⁻⁶. Hence, ILCs initiate early immune responses.

1.1. Classification and development of ILCs

Classification of ILCs is based on specific transcription factors which they express and cytokines that they produce ^{5, 6}.

Prototypes of the ILC family are natural killer (NK) cells and lymphoid tissue inducers (LTi), cells considered also as cytotoxic innate lymphoid cells. NK cells were discovered in mice in 1975 as cells which do not express specific antigen receptors but are able to kill target cells ⁷. NK cells play important role in defense against viruses and also can target

cancer cells⁸. LTi cells were identified in 1997, as the cells crucial for the formation of lymph nodes during embryogenesis but are also present in postnatal gut. Both NK and LTi cells require common γ chain (γc) of IL-2 receptor and transcriptional repressor inhibitor of DNA binding 2 (Id2) for their development⁹.

Recently identified group of cells, ILCs, also require γc and Id2 for their development, and additionally, express IL-7 receptor α (IL-7R α or CD127) and use IL-7 signaling for their development and maintenance^{5,6}.

Group 1 ILCs depend on the transcription factor T-bet and produce IFN- γ , reflecting Th1 cells characteristics. Helper ILC1, referred as ILC1, express CD127 and participate in type 1 inflammation in both mice and humans, producing IFN- γ in response to IL-12 and IL-18 stimulation. The cytotoxic ILC subsets: conventional NK cells and intraepithelial type 1 innate lymphoid cells (ieILC1) are also part of group 1 ILCs^{6,10,11}. Helper ILC1 are found in inflamed ileum of Crohn's disease patients, possibly contributing to gut inflammation^{10,12}.

Group 2 ILCs (ILC2) require transcription factors GATA3¹³ and retinoic acid receptor-related orphan receptor alpha (ROR α)^{14,15} for their development. GATA3 is important for their activation, and additionally, ILC2 need IL-7 signaling⁵ for optimal function. ILC2 are activated by innate cytokines: IL-25, IL-33, TSLP and produce type 2 cytokines, mostly IL-5 and IL-13, but also IL-4, IL-9 and IL-6^{13,16-18}. ILC2 initiate type 2 immune responses and contribute to allergic inflammation, but also provide defense against parasites⁶. ILC2 in addition produce amphiregulin, which has an important role in tissue repair^{19,20}.

Group 3 ILCs produce IL-17A and/or IL-22 in response to IL-1 β and IL-23 stimulation. They express and depend on the transcription factors ROR γt , Notch and Tcf1 for their development^{21,22}. Human ILC3 are further divided based on expression of the natural cytotoxicity receptor (NCR) NKp44. NKp44⁻ ILC3 produce mostly IL-17 and little IL-22, whereas NKp44⁺ ILC3 produce IL-22. NKp44⁺ ILC3 are absent in human peripheral blood under healthy conditions²³, and are present in the healthy human intestine, where they are crucial in the maintenance of gut barrier by producing IL-22²⁴. LTi cells are also classified into group 3 ILC¹¹. Recent study from Dr. Mjösberg's group based on single cell RNA sequencing of sorted tonsil ILCs revealed three subpopulations of ILC3 (NKp44⁺, CD62L⁺ and HLA-DR⁺)²⁵.

In mice, various studies clarified the signals necessary for the development of ILCs. In mice, ILCs derive from the common lymphoid progenitor (CLP) in the bone marrow, which further gives rise to the early lymphoid progenitor under the impact of transcription factors: thymocyte selection-associated high-mobility group box (TOX)²⁶, nuclear factor IL-3 dependent (NFIL3)²⁷ and T-cell factor 1²⁸ (TCF1). This precursor can develop to all so far known ILC populations. Transcription factors Id2 and GATA3 are necessary for the commitment to so-called common helper ILC progenitor^{29,30}. This progenitor further can, through promyelocytic leukemia zinc finger (PLZF)-precursor³¹, give rise to helper ILCs subsets, under the influence of T-bet for ILC1, GATA3/Bcl11b/ROR α for ILC2 and ROR γ t for ILC3^{30,32-34}.

Human ILCs can be differentiated *in vitro* from CD34⁺ progenitor isolated from bone marrow, thymus, peripheral blood or cord blood³⁵. Precursors of only ILCs and NK cells can only be found in the tissues such as tonsils and spleen, but not in bone marrow or blood. This finding indicates that ILCs can develop also from local precursors^{36,37}. Human ILCs and NK cells can be differentiated *in vitro* from CD34⁺ progenitor which expresses ROR γ t, CD34, CD45A, CD117, CD161, integrin β 7, and IL1R1. The development of human ILCs is still not completely understood and further research is required to clarify the mechanism behind this process.

1.2. Discovery of ILC2

ILCs were first discovered in mice and afterwards in different human tissues and in peripheral blood. For the first time, studies in 2001 and 2002 demonstrated presence of the novel innate lymphocytes which were able to produce type 2 cytokines. This new cell type was shown to be dependent on γ c, and to respond to IL-25 stimulation in *RAG-knockout* mice even in the absence of T and B cells^{3,38}. In subsequent studies these cells were described as non T/B cells, c-Kit⁺, Fc ϵ R1⁻, Sca-1⁺, being important source of IL-4, IL-5 and IL-13 upon allergen challenge or infection with parasite *Nippostrongylus brasiliensis*^{39,40}. Afterwards, parallel studies showed that these IL-5 and IL-13 producing cells were responsive to IL-25 and IL-33 and were crucial in the immune response against *N. brasiliensis* in adipose tissue¹⁶ and in lung and gut^{17,18}. These cells were defined as cells

which lack expression of many lineage markers but express c-Kit, inducible costimulator (ICOS), CD127, IL-2 receptor α (CD25), ST2 (ILRL1, IL-33 receptor), IL17RB (IL-25 receptor), and respond to IL-25 and IL-33¹⁸. They were found in different mouse tissues as lymph nodes, spleen, liver, lung¹⁷. Initially, several names were suggested for these cells as natural helper cells,¹⁶ innate helper type 2 cells¹⁷ and nuocytes¹⁸.

Human ILC2 were first identified by Mjösberg et al.⁴¹. Afterwards, these cells were also characterized in different human tissues as tonsils, liver, skin, nasal polyps, adipose tissue and also in peripheral blood^{19, 42-46}. Human ILC2 are defined by high expression of CRTH2⁴¹. Human ILC2 lack markers of progenitor cells (CD34), T cells (CD3, TCR $\alpha\beta$, TCR $\gamma\delta$), B cells (CD19), NK cells (CD94/NKG2A), dendritic cells-monocytes/macrophages (CD14, CD123, BDCA2, CD1a) and mast cells (Fc ϵ R1 α), thus they are characterized as Lin⁻; and additionally ILC2 are CD45⁺, CD127⁺, CD161⁺, CRTH2⁺ cells^{25, 41}.

All these above described cells are considered as one type of cells and the term group 2 innate lymphoid cells (ILC2) is now widely accepted¹¹.

1.3. Stimulating cytokines of ILC2

ILC2 are crucial in the initiation of type 2 immune responses^{6, 47}. Type 2 immunity is characterized by production of type 2 cytokines: IL-4, IL-5, IL-9 and IL-13, which lead to IgE production by B cells, activation of mast cells and eosinophils, mucus production and airway hyperreactivity. The Th2 cell-derived IL-4 and IL-13 promote IgE production of B cells. Allergen-specific IgE bind to Fc ϵ R1 on mast cells, eosinophils and basophils, subsequently inducing their degranulation and release of pro-inflammatory mediators. Independently of IgE, IL-5 and IL-9 can cause eosinophilia and mast cell hyperplasia, and IL-13 leads to airway hyperreactivity⁴⁸.

The type 2 immune response provides defense against extracellular parasites, which is a beneficial role of this process. However, dysregulation of these responses leads to different pathologic conditions such as allergic inflammation and asthma.

Upon exposure to pathogens or allergens, epithelial cells produce innate cytokines: IL-33, IL-25 and TSLP. IL-33 belongs to the IL-1 cytokine family, it is released by damaged endothelial and epithelial cells, and is considered as an 'alarmin'^{49, 50}. Increased level of

IL-33 was detected in lung or nasal epithelium of patients with allergic asthma and allergic rhinitis^{51, 52}. Under inflammatory conditions, IL-33 is also expressed by immune cells such as macrophages, dendritic cells, B cells and mast cells⁵⁰. IL-33 binds to ST2 receptor on ILC2, which is also expressed on other immune cells^{16, 41}. Signaling through ST2 triggers nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) activation in target cells^{49, 53}. TSLP is mostly produced by epithelial cells. TSLP binds to a receptor complex consisting of TSLP receptor and CD127 on ILC2. TSLP has been shown to upregulate GATA3 expression through STAT5 activation in human ILC2¹³. IL-25 (IL-17E) belongs to the IL-17 cytokine family and has been shown to induce type 2 cytokine production from human and mouse ILC2^{41, 54}. Beside epithelial cells, IL-25 can also be produced by eosinophils, mast cells and Th2 cells⁵⁵. These three cytokines: IL-33, TSLP and IL-25 have been shown to have synergistic effects on ILC2 activation and their impact depends on the tissue and stimulation^{41, 53, 56}.

ILC2 constitutively express CD25, and IL-2 signaling is important for ILC2 maintenance and activation^{44, 57-59}. As other ILCs subsets, ILC2 express CD127 and depend on IL-7 signaling for their development, maintenance and function^{6, 53}. Both IL-2 and IL-7 can activate STAT5 pathway upon stimulation of their receptors.

TL1A (TNFSF15) is part of the tumor necrosis factor (TNF) cytokine superfamily and binds to death receptor (DR) 3, which was recently detected on ILC2^{48, 60}. It was shown that TL1A induces IL-5 and IL-13 cytokine production from both human and mouse ILC2^{48, 61}.

1.4. Role of ILC2 in airways

1.4.1. Lung

Earlier studies demonstrated an important role of ILC2 in the progress of lung inflammation. First indication of ILC2 existence appeared after observation of non-T/B, type 2 cytokine producing cells which contribute to lung eosinophilia and mucus production upon IL-25 injection. Subsequent studies confirmed involvement of this cell type in response to various allergens, as protease papain¹⁴, chitin⁶², *Alternaria alternata*^{63, 64}, ovalbumin (OVA)⁶⁵ or house dust mite (HDM)⁶⁶ exposure. Upon stimulation with

different allergens, ILC2 produce IL-5 and IL-13, which mediate effects of ILC2 in airway inflammation. IL-5 and IL-13 increase survival rate of eosinophils^{67, 68}. IL-13 can also trigger eotaxin production in the airway and smooth muscle cells and promotes eosinophil infiltration and accumulation^{45, 69}. Moreover, IL-13 induces goblet cell hyperplasia, mucus production, contraction of smooth muscle cells and airway hyperreactivity. ILC2 and mast cell co-interaction is additionally crucial in the development of airway inflammation. ILC2-derived cytokines IL-5 and IL-9 can activate mast cell degranulation and mast cells produce PGD₂⁷⁰ and IL-33, which can further stimulate ILC2^{71, 72}. In human lungs, ILC2 were detected in the proximity of mast cells, implicating importance of interaction between these cell types⁴³. Previously described findings demonstrated ILC2-induced non-adaptive allergic response. Further studies provided evidences for the role of these cells in linking innate and adaptive immunity in lung allergic reactions. ILC2-deficient mice exerted impaired type 2 response to stimulation with allergen papain⁷³, showing less eosinophils in bronchoalveolar lavage (BAL) and lung, decreased number of dendritic cells (DCs) and CD4+ cells and lower IgE levels than in wild type mice. This study also demonstrated that ILC2-derived IL-13 is crucial for pulmonary dendritic cell migration to the draining lymph node and recruitment of Th2 cells⁷³. Similarly, it was shown that lack of ILC2 led to reduced type 2 cytokines and IgE level after HDM exposure additionally confirming role of ILC2 in activation of adaptive immune response⁷⁴. Direct interactions between ILC2 and B cells are still not completely understood. Recently it was observed that mouse lung ILC2 promote production of IgE, IgM, IgA and IgG1 *in vitro* through soluble factors, independently of T cells⁷⁵.

Asthma is a chronic inflammatory airway disease characterized by mucus production, chest tightness, shortness of breath and airway remodeling⁷⁶. Asthmatic responses can be triggered by allergens, but also non-specific stimuli. Dysregulated type 2 immune responses lead to the features of asthma as increased levels of type 2 cytokines, Th2 cell activation, IgE production by B cells, activation of mast cells and eosinophils.

Previously described mouse models showed a role for ILC2 in asthmatic inflammation *in vivo*. Several studies detected higher levels of IL-33, TLSP, IL-25, PGD₂ and cysteinyl leukotrienes (CysLTs) in asthmatic individuals⁵¹, implicating a possible role of ILC2 activation⁴⁸. Genome studies of asthmatic patients detected associations of genes, such as *RORA*, *IL33*, *IL1R1*, *IL13* with asthma prevalence⁷⁶. Studies showed higher number of ILC2 in the peripheral blood and elevated production of type 2 cytokines by ILC2 from asthmatic patients^{44, 77}. Increased number of IL-5 and IL-13 producing ILC2 were also

found in BAL and sputum of patients with severe eosinophilic asthma ^{45, 77}. Furthermore, ILC2 number was elevated in blood of children with asthma compared to healthy ones ⁷⁸. In addition, upregulation of transcription factors important for cell migration and proliferation (FOS, FOSB and JUN) was detected in blood ILC2 from allergic patients ⁷⁹. This study also showed higher expression of the chemokine receptor CCR10 on the ILC2 surface of allergic asthmatic patients pointing to the potential of circulating ILC2 to migrate into the lung.

Treatment with corticosteroids, which suppress activation of inflammatory cells, can control the majority of asthma patients. However, 5-10 % of asthmatics do not respond to corticosteroid therapy and are diagnosed with severe asthma. One report showed that mouse ILC2 were resistant to the corticosteroid dexamethasone due to TSLP and STAT5 activation ⁸⁰. Recently, a human study confirmed these findings, reporting that ILC2 failed to respond to dexamethasone upon stimulation with TSLP and IL-7 ⁸¹. In contrast, IL-33- and IL-25- stimulated ILC2 were sensitive to dexamethasone. This study additionally showed that TSLP levels were increased in BAL fluid from asthmatic patients.

ILC2 can be involved in the responses to respiratory viruses where they might have both beneficial and detrimental effects. Interestingly, upon influenza virus infection ILC2 have protective role in the recovery phase. Producing amphiregulin, ILC2 helped to retain lung integrity and contributed to the resolution of inflammation ¹⁹. Another study showed that IFN- γ induced by pandemic H1N1 (pH1N1) virus suppressed ILC2 activation in mice and disabled beneficial effects of amphiregulin ²⁰. In contrast, influenza subtype H3N1 and pH1N1 infection stimulated ILC2 to produce IL-5 and IL-13 and further promote accumulation of eosinophils in the lung and cause airway hyperreactivity ^{82, 83}. Similarly, respiratory syncytial virus initiated IL-13 production by ILC2 in the early phase of infection ⁸⁴. One human study also showed that pH1N1 infection can cause asthmatic symptoms in patients ⁸³. Furthermore, increased IL-5, IL-13 and IL-33 levels were detected in the BAL and nasal fluid of asthmatic patients after rhinovirus challenge compared to baseline and healthy individual ⁸⁵. This finding may provide explanation for virus-induced asthma exacerbations.

Beside allergen-induced asthma, ILC2 have been shown to play a role in non-allergic asthma ⁸⁶.

An earlier study showed that ILC2, producing IL-13 could induce airway hyperreactivity in the absence of adaptive immunity in the lung of mice treated with glycolipid antigen ⁸⁷.

Furthermore, numbers of ILC2 and ILC3 were increased in the lungs of high-fat fed mice comparing to standard diet in a model of obesity-induced asthma ⁸⁸.

Recently was revealed that ILC2 play a role in early inflammatory response in lung after systemic injury in a mouse model of hemorrhagic shock with tissue trauma through IL-33-ILC2-IL-5-neutrophil axis ⁸⁹.

Moreover, increased ILC2 frequencies were observed in patients with idiopathic pulmonary fibrosis ⁹⁰.

1.4.2. Upper airways

Allergic rhinitis is a response to inhaled allergens and involves activation of the cells characteristic for type 2 inflammation. A few recent human studies investigated the role of ILC2 in allergic rhinitis and detected higher levels of ILC2 in blood of rhinitis patients after nasal challenge ^{91,92}. Non-invasive techniques such as nasal curettage for cell analysis revealed an increase of ILC2 after allergen challenge, which correlated to elevated eosinophil numbers, and serum IgE and IL-5 levels ⁹³.

Chronic rhinosinusitis is a complex disorder characterized by inflammation of the paranasal sinuses. Recent human studies demonstrated increased percentage of ILC2 producing type 2 cytokines in nasal polyps of patients with chronic rhinosinusitis compared with healthy nasal mucosa ⁹⁴⁻⁹⁷, along with elevated levels of IL-5 and IL-13 also being detected ⁹⁶.

In addition, ILC2 can contribute to aspirin-exacerbated respiratory disease, as they are recruited to nasal mucosa of these patients ⁹⁸.

1.5. Role of ILC2 in skin

Atopic dermatitis (AD) is a chronic inflammatory skin disorder, characterized by dysregulated type 2 responses and disrupted epithelial barrier ⁹⁹. The levels of ILC2-stimulating cytokines IL-25, IL-33 and TSLP, as well as ILC2 numbers are elevated in lesional skin from AD patients. Circulating ILC2 express CCR10 and ILC2 can infiltrate into the skin of AD patients ¹⁰⁰.

ILC2 are resident in healthy human and mice skin. Human skin ILC2 express KLRG1, which maintains homeostatic inhibition of ILC2 via interaction with E-cadherin ¹⁰⁰. In the skin of AD patients, E-cadherin is downregulated, and thereby facilitating the activation of ILC2 as well as production and release of type 2 cytokines. Accumulated mast cells are also characteristic of lesional AD skin and implicate their interaction with ILC2. A recent study showed increased ILC2 and basophil numbers and levels of IL-4 and IL-13 in the skin of AD patients compared to psoriatic ones ¹⁰¹. Human ILC2, which express NKp30, can be activated by tumor-associated surface molecule B7-H7, which is upregulated in the skin of AD patients ¹⁰². In addition, by producing amphiregulin, skin ILC2 may contribute to tissue repair ¹⁰⁰.

1.6. ILC2 and helminth infections

ILC2 activation can be beneficial in the defense against parasite infection. Previous studies which identified ILC2 in mice, demonstrated a role of these cells in response to *N. brasiliensis*^{16, 18}. Later it was shown that crosstalk between ILC2 and T cells is essential for *N. brasiliensis* worm expulsion¹⁰³. ILC2 are also important for wound healing. Although mouse studies clearly showed involvement of ILC2 in response to parasite infections, there are only a few human studies. Analysis of ILC2 and ILC3 showed increased number of these cells and among them IL-13-producing cells in the peripheral blood of filarial infected individuals¹⁰⁴. Another report examined ILC2 in Zimbabwean children infected with *Schistosoma haematobium*¹⁰⁵. Infected younger children had lower ILC2 level in the peripheral blood than healthy children, whereas infected older children had same ILC2 numbers as healthy ones. Exact role of ILC2 in human helminth infections remains to be explored in greater detail.

1.7. ILC2 in other tissues

1.7.1. Gastrointestinal tract

Beside a potential beneficial role in the clearance of parasitic intestinal infection, ILC2 can also mediate pro-inflammatory effects in other conditions of the gastro-intestinal tract.

Eosinophilic gastrointestinal disorders were considered to be dependent on Th2 cell-derived production of IL-5 and IL-13. Recent studies indicate a role of ILC2 in the pathogenesis of these diseases. Increased IL-33, as well as IL-25 levels and ILC2 frequencies were detected in the esophageal biopsy samples from patients with active eosinophilic esophagitis¹⁰⁶.

Breakdown of the tolerance to food components can lead to food allergy. Contribution of ILC2 in IgE-mediated food allergy by producing IL-13 has been demonstrated in mouse models^{107, 108}. Additionally it was shown that ILC2-derived IL-4 blocks specific regulatory T cells (Tregs) and promotes food allergy¹⁰⁸.

1.7.2. ILC2 in adipose tissue

Although ILC2 showed pro-inflammatory effects in the airways, beneficial roles of these cells have been demonstrated in the adipose tissue. IL-33- and IL-25-stimulated ILC2 maintain metabolic homeostasis and promote energy expenditure of adipocytes¹⁰⁹⁻¹¹¹. A human study showed lower percentage of ILC2 in white adipose tissue from obese donors compared to non-obese controls¹¹⁰. Additionally, it was reported that IFN- γ , induced by certain infections, can inhibit ILC2, which causes dysregulation of visceral adipose tissue¹⁰⁹.

1.7.3. ILC2 in cancer

ILC2 play different roles in cancer progression depending on the type and the microenvironment of the tumor. Increased number of ILC2 was found in gastric cancer patients where they can contribute to immune suppression by producing IL-13 and activating myeloid-derived suppressor cells (MDSCs) and M2 macrophages¹¹². ILC2-derived IL-13 contributed to biliary carcinogenesis inducing proliferation of cholangiocytes and additionally led to liver metastases¹¹³. Furthermore, the ILC2/IL-13 axis modulates T cell to MDSC ratio in patients with bladder cancer, and this mechanisms is now proposed as a new target for immunotherapy¹¹⁴. In another study, via activating eosinophil migration to the lung, ILC2 promoted killing of the tumor cells in a mouse lung tumor model¹¹⁵.

1.8. Interactions of ILC2 with other immune cells

ICOS is related to the CD28 family and it is known to be expressed on activated T cells. It has been shown that both human and mouse ILC2 express ICOS and ICOS ligand (ICOS-L)¹¹⁶. This study revealed that ICOS:ICOS-L interaction is important for ILC2 function, likely via activating the STAT5 signaling pathway¹¹⁶. Other studies confirmed this finding, additionally demonstrating a role of ICOS:ICOS-L interaction on ILC2 homeostasis, activation and proliferation^{117, 118}. In contrast, interaction with Tregs through ICOS:ICOSL may decrease ILC2 activation, since Tregs occupy ICOS-L and disturb the

communication between ILC2¹¹⁹. Tregs additionally can suppress ILC2 function by producing TGF- β and IL-10¹²⁰.

Interaction of ILC2 with Th2 cells can be direct or indirect. Both human and mouse ILC2 express major histocompatibility complex class II (MHCII) and activate Th2 cells by processing and presenting antigens¹⁰³. Besides, ILC2 express co-stimulatory molecules CD80 and CD86 which could contribute to ILC2 stimulation of Th2 cells. This study additionally showed that IL-2, produced by both ILC2 and CD4⁺ T cells is crucial for the crosstalk of these cells. ILC2 also express OX40L and its interaction with OX40 on CD4⁺ T cells was shown to be important for type 2 cytokine production¹²¹.

ILC2 indirectly affect B cells through production of IL-13 and IL-5 and activation of other cells. Furthermore, IL-4 and IL-13 released from activated Th2 cells and ILC2 promote IgE production from B cells. Additionally, ILC2 may also interact with B cells through ICOS:ICOS-L interaction, but this process remains to be investigated. Recently it was shown that human ILC2 upregulate CD154 upon IL-25/IL-33/TSLP or TLR stimulation and subsequently regulate IgE production from B cells¹²².

ILC2 can also interact with eosinophils. Human ILC2 activate and increase the survival rate of eosinophils through production of IL-5 and IL-13¹²³. On the other side, eosinophil-derived IL-4 co-stimulates ILC2 and increases IL-5 production from ILC2.

Mast cells are one of important players in allergy responses. Releasing PGD₂ and LTD₄, mast cells can activate ILC2 and stimulate cytokine production and migration.⁷⁰

IL-5 and IL-9 produced by ILC2 activate mast cells. Interaction between ILC2, mast cells and Th9 cells has recently been shown in cystic fibrosis model¹²⁴. Mast cells, upon activation by IL-33 and ILC2- and Th9-derived IL-9 and IL-2 and can contribute to ILC2 activation.

It has also been shown that IL-4 released by basophils enhances IL-5 and IL-13 production from ILC2¹²⁵⁻¹²⁷. Furthermore, basophils and ILC2 levels were both increased in correlation in the skin of AD patients¹⁰¹.

Beside known IL-13-mediated stimulation, ILC2 can also directly interact with DCs through ICOS-L:ICOS binding. Although there is a positive co-interaction between DCs

and ILC2, plasmacytoid DCs, which produce type I interferons and induce Tregs can suppress ILC2-induced airway hyperreactivity¹²⁸.

Overall, ILC2 clearly mediate both innate and adaptive responses in type 2 inflammation.

1.9. ILC plasticity

ILCs show high potential for plasticity, and can transdifferentiate to the other subsets depending on the stimulation⁶. ILC2, after stimulation with IL-1 β and IL-12 can differentiate to ILC1 and produce IFN- γ ¹²⁹. IL-1 β in the presence of IL-2 induces proliferation and IL-5 and IL-13 production of ILC2. Combination of these cytokines also induces production of IL-10 and GM-SCF from ILC2. On the other hand, IL-1 β upregulates IL-12 receptor, so that IL-1 β -treated ILC2 have higher capacity to respond to IL-12 and conversion to ILC1 after co-stimulation with IL-12. Stimulation of ILC2 with IL-12 decreases IL-5 and IL-13 producing cells and induces IFN- γ production, converting ILC2 to ILC1 phenotype^{123, 130}. These cells can be reversed to ILC2 by IL-4 stimulation.

Patients with chronic obstructive pulmonary disease (COPD) have higher levels of IL-1 β , IL-12 and IL-18 and IFN- γ . Number of ILC2 is decreased, whereas total ILC number is elevated in the lungs of COPD patients¹³⁰. Changes of lung microenvironment in COPD patients promote differentiation of ILC2 to ILC1. In addition, elevated number of NKp44⁺ ILC3 and levels of IL-17A and IL-17F were detected in the lungs of COPD patients. Additionally, a mouse model demonstrated that ILC2 can transdifferentiate to ILC1 upon influenza virus infection¹³⁰. Moreover, ILC2 producing IFN- γ were also found in the intestine of patients with Crohn's disease¹³¹.

1.10. Lipid mediators in ILC2 regulation

A class of lipid molecules, the so called bioactive lipids are important players of various physiological and pathological processes¹³². Recent studies have revealed the crucial role of lipid mediators in ILC2 function¹³³. CTRH2 is one of defining surface markers of human ILC2. It was shown that PGD₂, via activation of the CTRH2 receptor, induces IL-13 production in human blood ILC2, acting synergistically with IL-25, IL-33 and IL-2⁴³.

PGD₂ contributes to chemotaxis of human blood ILC2¹³⁴. Furthermore, PGD₂ stimulates IL-5 and IL-13 production and migration of human skin ILC2⁷⁰. Beside type 2 cytokines, PGD₂ also induces the production of additional cytokines such as IL-3, IL-9, GM-CSF which also mediate pro-inflammatory roles⁷⁰. Additionally, CRTH2 antagonist reduced stimulating effects induced by the supernatants of IgE-stimulated mast cells⁷⁰. The PGD₂-caused migration and recruitment of ILC2 was found to be more prominent in ILC2 from asthmatic patients than healthy individuals¹³⁴. It was also demonstrated that PGD₂ triggered accumulation of ILC2 into the mouse lung during helminth infection¹³⁵.

Furthermore, cysteinyl leukotrienes affect ILC2 function. Leukotriene D₄ (LTD₄) activated both murine and human ILC2¹⁰⁶, and LTE₄ potentiated the stimulating effect of PGD₂ and other cytokines in activation of human ILC2⁵⁹.

In contrast, other lipid mediators have suppressive roles on ILC2 function. The pro-resolving mediator lipoxin A₄ (LXA₄) reduced IL-13 secretion from human blood activated ILC2 through ALX/FPR2 receptor⁴³.

Additionally, PGI₂ was demonstrated to reduce cytokine production and proliferation of mouse ILC2¹³⁶.

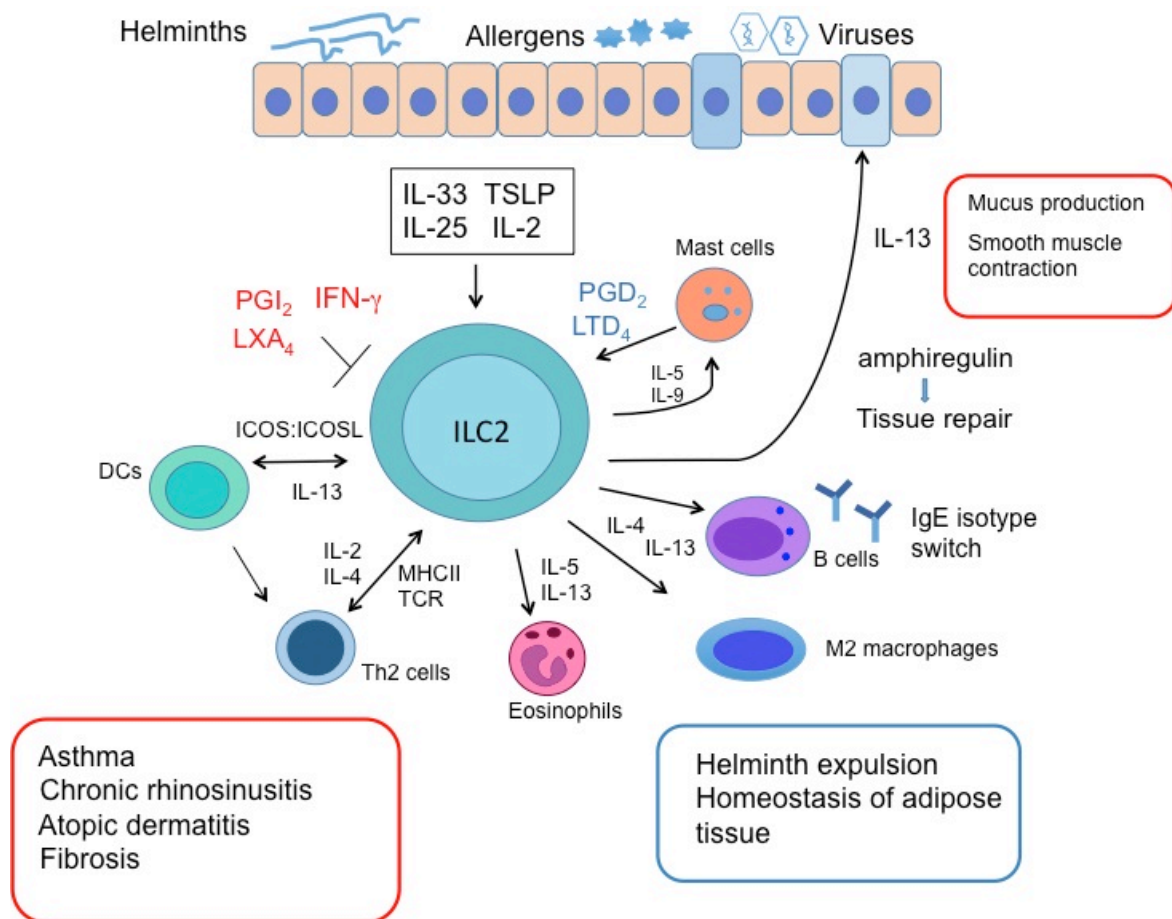


Figure 1. Roles of ILC2 in homeostasis and inflammation.

Upon different stimuli, such as allergens, viruses, helminthes, or injuries, epithelial cells produce innate cytokines: IL-33, IL-25 and TSLP. These cytokines, in addition to IL-2, activate ILC2 to produce type 2 cytokines IL-5 and IL-13, and to a lesser extent IL-4 and IL-9. IL-5 and IL-13 promote eosinophil survival and activation and cause tissue eosinophilia. Producing IL-13, ILC2 activate dendritic cells, which then induce migration of Th2 cells. ILC2 and DCs may also co-interact through ICOS:ICOS-L interaction. ILC2 and Th2 can co-interact via IL-2 and IL-4. Besides, ILC2 express MHCII and present antigens to Th2 cells. IL-4 and IL-13 induce isotype switch to IgE production by B cells and activate M2 macrophages. Mast cell-derived PGD_2 and LTD_4 positively regulate ILC2 function, and IL-5 and IL-9 produced by ILC2 stimulate mast cells. IL-13 also stimulates goblet cells and mucus production, as well as smooth muscle cell contraction. These events lead to asthma, allergic inflammation, atopic dermatitis, tissue fibrosis. Also beneficial effects of these processes are helminth clearance and homeostasis of adipose tissue. In addition, ILC2 produce amphiregulin which may contribute to tissue regeneration. There

are a few known negative regulators of ILC2 function such as IFN- γ and lipid mediators LXA₄ and PGI₂.

1.11. Prostaglandins in immune response

Prostanoids are a group of eicosanoids which include prostaglandins, thromboxanes (TX) and prostacyclin. As all eicosanoids, prostanoids are derived from arachidonic acid which is synthesized from membrane phospholipids via phospholipase A₂ (PLA₂)¹³⁷.

Activation of cyclooxygenase enzymes COX-1 and COX-2 leads to conversion of arachidonic acid to PGG₂ which then subsequently forms PGH₂ (Figure 2). COX-1 is constitutively expressed in various cell types, whereas COX-2 is upregulated upon different stimuli¹³⁸. PGH₂ is substrate for specific synthases which produce PGD₂, PGE₂, PGF_{2 α} , PGI₂ or TXA₂ (Figure 2).

Prostaglandins are present in most of the tissues and play different regulatory roles. Studies have shown importance of prostaglandins in mediating effects on different immune cells during inflammatory processes¹³⁹.

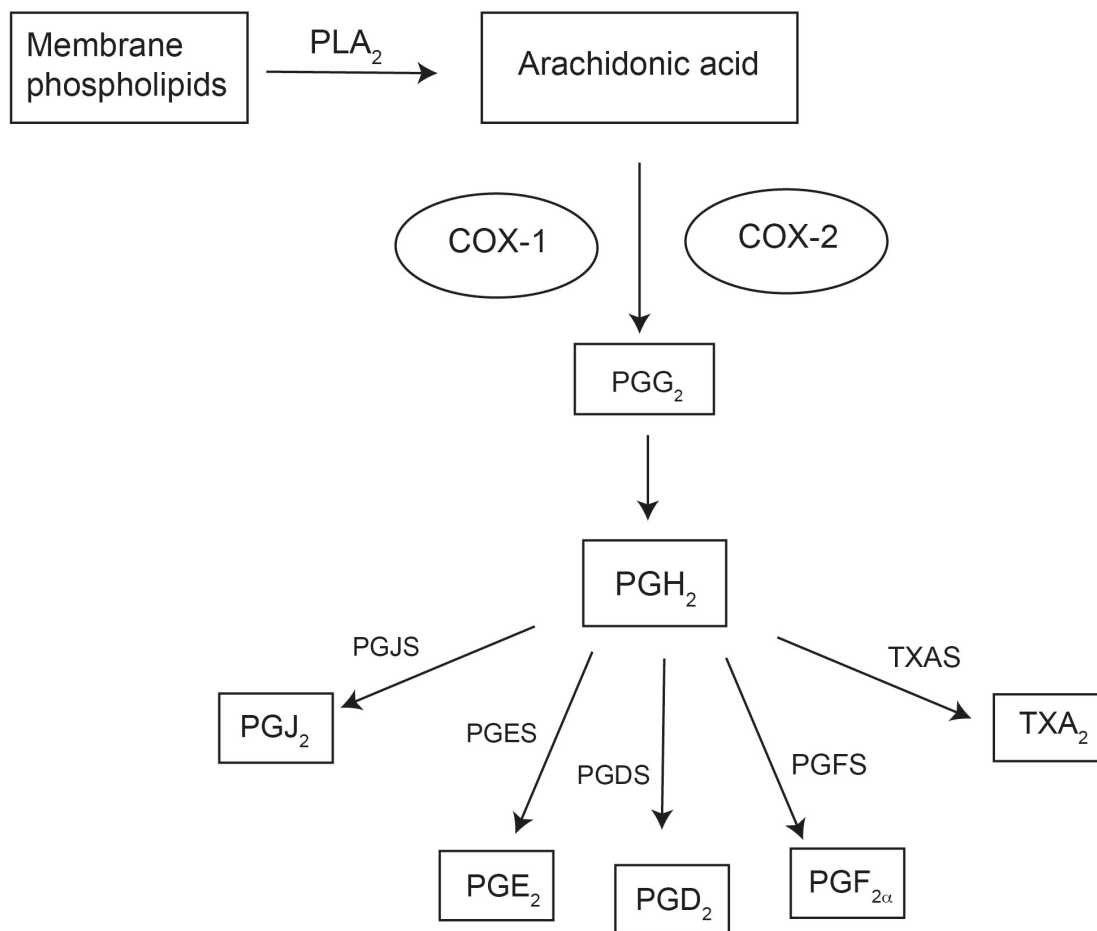


Figure 2. COX biosynthetic pathway.

Arachidonic acid is released from membrane phospholipids by activation of PLA₂. Action of COX-1 and COX-2 further convert arachidonic acid to PGG₂ and PGH₂, which is the substrate for synthases specialized to generate different prostanoids.

1.11.1. Immuregulatory roles of PGE₂

PGE₂ is one of the most abundant PGs in the organism and has different physiological roles. PGH₂ is converted into PGE₂ by the action of PGE synthases: membrane/microsomal-bound (mPGES-1 and mPGES-2) and cytosolic PGE synthase (cPGES). COX-1/cPGES is involved in PGE₂ production in homeostasis, whereas COX-2/mPGES-1 pathway is activated by different stimuli ¹⁴⁰. In lung, PGE₂ is produced by endothelial and epithelial cells, smooth muscle cells, alveolar macrophages and fibroblasts ¹⁴¹. PGE₂ exerts its effects through four different G protein-coupled receptors: EP1-EP4

(Figure 3). EP1 is a Gq coupled receptor and leads to increase of intracellular calcium (Ca^{2+}) level. EP2 and EP4 are Gs coupled receptors which trigger activation of adenylate cyclase (AC) and production of cAMP. Signaling of EP3 is mediated by Gi protein, leading to inhibition of cAMP formation and elevation of Ca^{2+} levels.

PGE_2 mediates both pro- and anti-inflammatory roles depending on the tissue and the receptors it binds to.

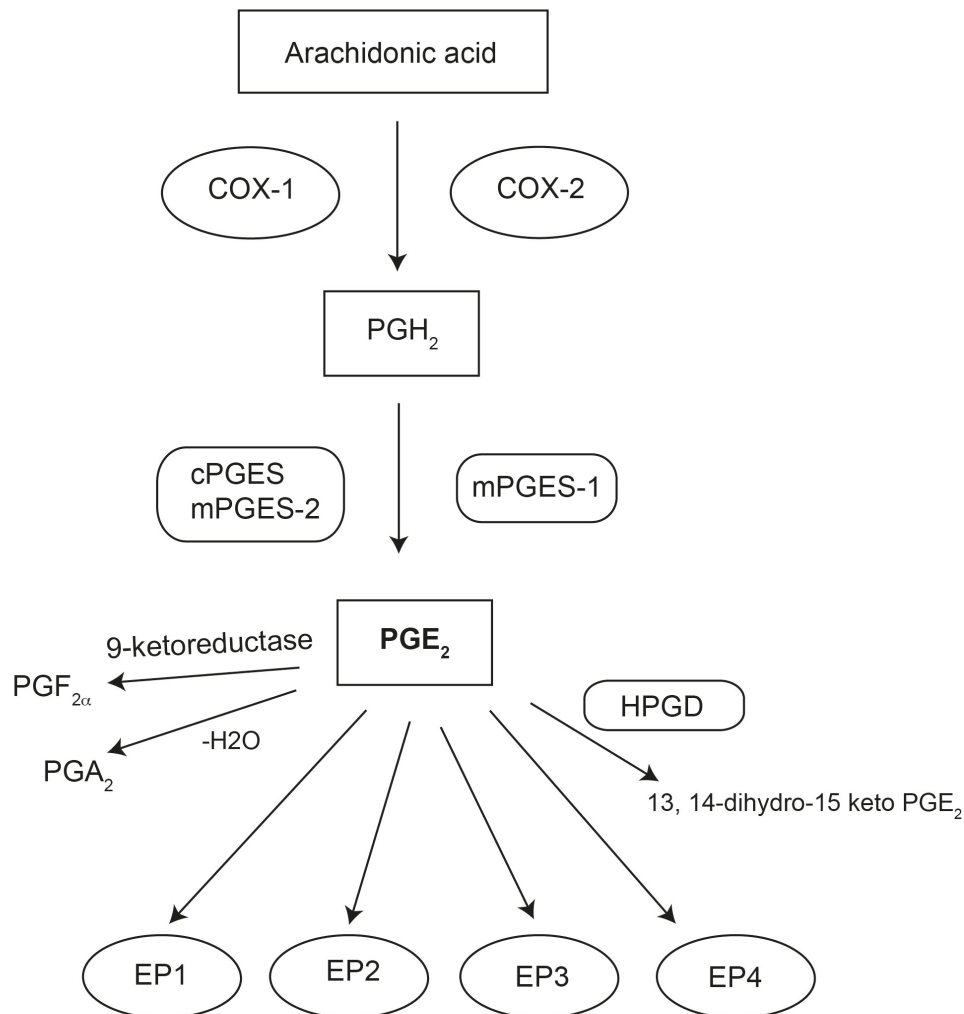


Figure 3. PGE_2 pathway

PGE_2 is derived from arachidonic acid via activation of COX-1/COX-2, which lead to formation of PGH_2 . Specific PGE synthetases: constitutive cPGES/mPGES-2 and inducible mPGES-1 further produce PGE_2 . PGE_2 can be metabolized by enzyme HPGD to 13, 14-dihydro-15-keto- PGE_2 , and via 9-ketoreductase to $\text{PGF}_{2\alpha}$. PGE_2 can also be converted to PGA_2 . PGE_2 exerts its effects through activation of four receptors: EP1-EP4.

PGE₂ is known to be involved in pain and can directly act on nociceptors and additionally sensitizes to other pain mediators through activation of EP1 and EP4 receptors ^{142, 143}. PGE₂ is also related to central nervous functions, where it contributes to thermogenesis and fever.

Although PGE₂ can cause inflammation, it plays protective roles in different organs, among them in the respiratory system ¹³⁹. PGE₂ reduces phagocytosis and production of pro-inflammatory cytokines from alveolar macrophages ¹⁴⁴ and impairs recruitment of neutrophils into the lung ¹⁴⁵. Furthermore, PGE₂ shows inhibitory effects on other immune cells, reducing cytokine production from dendritic cells and Th2 cells and degranulation of mast cells and eosinophils ¹⁴⁶⁻¹⁴⁹. These effects were mediated through EP2 and/or EP4 receptor activation. Moreover, PGE₂ promotes lung microvascular endothelial barrier by EP4 receptor ¹⁵⁰.

Several animal studies confirmed beneficial effects of PGE₂. In OVA model, PGE₂ negatively regulated eosinophil activation ¹⁴⁹ and suppressed airway sensitization via EP2 receptor ¹⁵¹. Other studies demonstrated involvement of EP4 receptor activation in reducing airway inflammation and improving lung function ^{152, 153}.

PGE₂ is additionally protective in asthma and allergy, as it inhibits bronchial hyperresponsiveness. Even earlier clinical studies showed beneficial effects of PGE₂ inhalation in asthmatic individuals. PGE₂ inhibited bronchoconstriction induced by allergens, physical exercises or aspirin ¹⁵⁴⁻¹⁵⁶. PGE₂ also caused cough and this effect was later shown to be mediated through EP3 receptor ¹⁵⁷, suggesting a selective EP receptor targeting approach.

PGE₂ production is enhanced in inflammatory conditions, as during stimulation with pro-inflammatory cytokines or lipopolysaccharide (LPS) ^{150, 158}. Higher levels of PGE₂ were also found in airways of asthmatic individuals ¹⁵⁹ and in sputum of patients with eosinophilic bronchitis ¹³⁹. Another study showed inverse relation between PGE₂ and eosinophil number in airways of asthmatic patients ¹⁶⁰.

Although inhibitory effects of PGE₂ on different immune cells were shown, its impact on ILC2 function has not yet been elucidated.

1.11.2. PGD₂ in allergic inflammation

Another prostaglandin, PGD₂, has been demonstrated to mediate pro-inflammatory roles in allergic diseases. There are two types of PGD₂ synthases: hematopoietic PGD₂ synthase (HPGDS), expressed in hematopoietic-derived cells in blood, which requires glutathione (GSH -) for its activation, and lipocalin PGD₂ synthase (LPGDS) which is GSH independent and found in central nervous system, heart, retina and genital organs¹⁶¹. These PGD₂ synthases selectively convert PGH₂ to PGD₂. HPGDS has been detected in mast cells, Th2 cells, dendritic cells, eosinophils and macrophages¹⁶²⁻¹⁶⁴. Recently, it was demonstrated in Dr. Mjösberg's group by single cell RNA sequencing that human tonsil ILC2 express high level of *HPGDS*²⁵.

PGD₂ is rapidly degraded enzymatically and non-enzymatically and it can be metabolized to PGJ₂, which is further converted to delta-12-PGJ₂ (Δ^{12} -PGJ₂) and 15-deoxy-delta 12, 14-PGJ₂ (15d-PGJ₂)¹⁶⁵. Enzymatic degradation is mediated through 15-hydroxyprostaglandin dehydrogenase (HPGD) to formation of 13,14 dihydro-15-keto PGD₂ (DK-PGD₂), which is a natural selective agonist of CTRH2 receptor¹⁶⁶ (Figure 4).

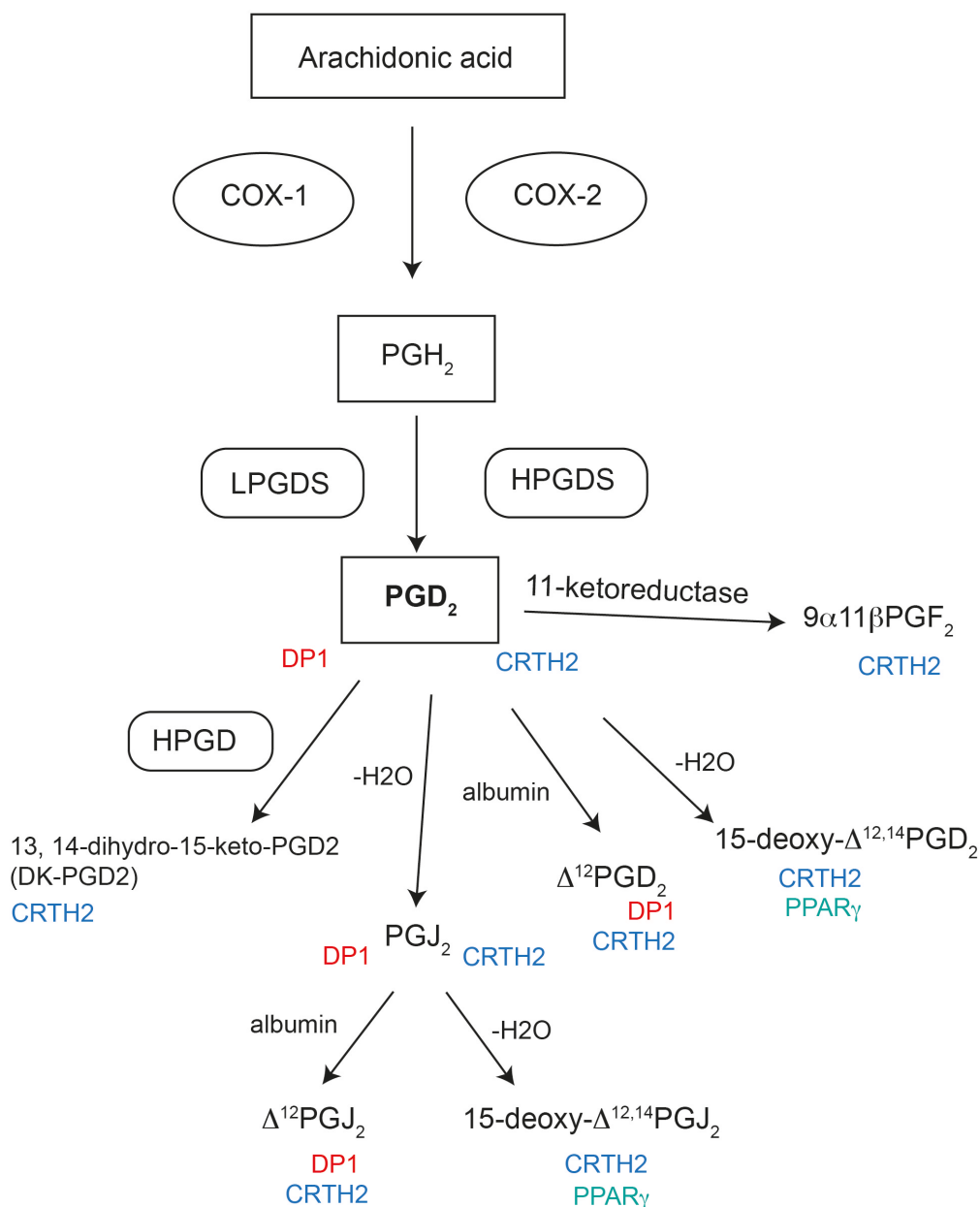


Figure 4. PGD_2 metabolism

PGD_2 is synthesized from arachidonic acid via action of COX-1/COX-2, which generate PGH_2 . There are two PGD_2 synthases which produce PGD_2 : HPGDS and LPGDS.

PGD_2 can be metabolized enzymatically, via HPGD to 13, 14-dihydro-15-keto PGD_2 and via 11-ketoreductase to $9\alpha11\beta\text{PGF}_2$. Non-enzymatically PGD_2 can be converted to PGJ_2 , $\Delta^{12}\text{-PGJ}_2$, 15d- PGJ_2 , $\Delta^{12}\text{-PGD}_2$, 15d- PGD_2 . PGD_2 and its metabolites bind to DP1 and CRTH2 receptors, and some of them (15d- PGJ_2 and 15d- PGD_2) also to PPAR_γ .

PGD_2 mediates its physiological effects via binding to CRTH2 and DP1 receptors. CRTH2 is a G_i protein-coupled receptor and its triggering leads to inhibition of cAMP and increase

of intracellular Ca^{2+} level. Roles of CRTH2 in allergic diseases have been in focus of interest in recent years ¹⁴¹. Increased PGD₂ levels were detected in BAL of patients with severe asthma ^{167, 168}. These data correlated with elevated eosinophils number in blood. One study reported genetic variations in *CRTH2* gene which may have impact on allergic diseases ¹⁶⁹. PGD₂ has been shown to mediate pro-inflammatory effects on different immune cells. Acting via CRTH2 receptor, PGD₂ recruited and activated Th2 cells inducing IL-4, IL-5 and IL-13 production, even independently of co-stimulation with antigen ¹⁷⁰. PGD₂ is an important activator of eosinophils ¹⁴¹. Through CRTH2 signaling, PGD₂ stimulates eosinophil migration, priming, respiratory burst and secretion of cationic proteins ^{166, 171, 172}. It was also shown *in vivo* that PGD₂ and CRTH2 agonists enhance allergic responses in mouse lung and skin ^{173, 174}. Additionally, CRTH2 activation induces activation of basophils and macrophages ^{173, 175}. Mast cells are also activated by CRTH2 stimulation and accordingly, increased numbers of CRTH2 expressing macrophages and mast cells were detected in nasal mucosa of allergic patients ¹⁷⁶. It was recently shown that PGD₂ also activates ILC2 through CRTH2 receptor ⁷⁰.

Besides PGD₂, its metabolites: PGJ₂, Δ^{12} -PGJ₂, Δ^{12} -PGD₂, 15d-PGJ₂ and DK-PGD₂ can also bind to the CRTH2 receptor and mimic the effects of PGD₂ ^{165, 166, 172}.

Due to the role of CRTH2 in promoting asthmatic inflammation, CRTH2 antagonists are promising tools for controlling these conditions. CRTH2 antagonism reduced asthmatic pathology in experimental mouse models of allergic airway inflammation ^{177, 178}. Additionally, there is clinical evidence of beneficial effects of CRTH2 antagonists in patients with asthma and allergies in phase II clinical studies ^{179, 180}. However, in the study with stable asthmatic patients, CRTH2 antagonist, AZD1981, did not have significant beneficial effect ¹⁸¹. With uncontrolled asthma patients, this CRTH2 antagonist showed some improvement in asthma control and forced expiratory volume.

DP1 receptors is more widely distributed, and beside in immune cells, expressed by platelets, in the vasculature, central nervous system, retina, intestine and lung ¹⁴¹. DP1 stimulates Gs protein, and subsequent activation of AC increases cAMP level. Both pro- and anti-inflammatory effects of DP1 receptor activation have been described. Activation of DP1 receptor was shown to inhibit chemotaxis of eosinophils, Th2 cells and basophils, and to reduce mast cell degranulation ¹⁷¹. Besides, DP1 suppresses platelet, neutrophil and dendritic cell function ^{141, 182, 183}. In contrast, DP1 receptor can stimulate mucus production from epithelial cells. In some studies DP1 deficient animals were protected from allergic

lung inflammation ^{184, 185}, while other studies demonstrated beneficial effects of DP1 agonists ¹⁸⁶. Furthermore, DP1 activation induces vasodilatation and bronchodilatation ¹⁶⁴. Cooperation of DP1 and CRTH2 was shown to mediate some of PGD₂ effects. DP1 receptor activation enhanced responsiveness of CRTH2 in human eosinophils and HEK293 cells ¹⁶⁶. However, dual DP1/CRTH2 antagonist, AMG 853, did not show significant improvement in lung function of asthma patients. On the other hand, DP1 antagonist, laropiprant, reduced PGD₂-induced nasal congestion ¹⁸⁷, but it was ineffective in phase II clinical trials with allergic rhinitis and asthmatic patients ¹⁸⁸. Further investigations are required to elucidate the therapeutic potential of CRTH2 and/or DP1 antagonists in the treatment of asthma and allergic diseases.

1.12. Aims of the thesis

ILC2 are crucial in the initiation and amplification of type 2 immune responses and hence contribute to asthma and allergic inflammation. Due to the crucial role of ILC2 in allergic diseases, it is important to identify the factors which might modulate the function of these cells.

As PGE₂ was shown to inhibit activation of other immune cells involved in type 2 responses such as Th2 cells and eosinophils, the hypothesis of the first part of the thesis was that PGE₂ might also act as a negative regulator of ILC2. The aim was to examine whether PGE₂ suppresses type 2 cytokine production from stimulated ILC2 and to unravel the exact mechanisms behind its actions. Furthermore, we set out to determine which receptors mediate the effects of PGE₂.

Another prostaglandin, PGD₂, activates ILC2 through CRTH2 receptor. In the second part of the thesis, I aimed to determine whether ILC2 have the potential to produce prostaglandins, of particular interest PGD₂, by their own action and what would be the impact of endogenously produced PGD₂ on ILC2 function.

Overall, the aim was to extend our current knowledge about regulation of ILC2 function and to identify novel mechanisms for potential pharmacological targeting of ILC2.

2. Material and methods

2.1. Isolation and flow cytometric sorting of tonsil and blood ILC2

Human tonsils were freshly obtained after 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 described ²⁵. Tonsils were dissected into small pieces, ground through 100 µm filter with a plunger of a plastic syringe using phosphate buffered saline (PBS) and then centrifuged. Mononuclear cells were isolated by lymphoprep centrifugation where cell suspension with 35 mL of PBS was added on a top of 15 mL of lymphoprep. Mononuclear cell layer was recovered in PBS and resuspended in MACS buffer containing PBS with 2 mM EDTA and 0.5 % fetal bovine serum (FBS). Cells were 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). Briefly, lineage depleted PBMCs were incubated with CD127-biotin antibody for 10 min, and then anti-biotin microbeads were added and incubated 15 more minutes. After being washed in MACS buffer, positive selection of CD127 cells was performed using MACS separator and LS columns. 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 FACSARIA Fusion cell sorter (BD Bioscience) equipped with the FACSDiva software version 8. Cell sorting was done at the Department of Medicine, Huddinge Flow Cytometry Facility at Karolinska Institutet, Stockholm.

2.2. 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 (mixed from three donors) irradiated at 25 Gy and JY cells (B cell line) irradiated at 50 Gy, with IL-2 (100 U/mL), IL-4 (25 ng/mL) and phytohemagglutinin (PHA) (1 µg/mL, Oxoid) as previously described¹³. Medium was changed every second day. First week, the cells were cultured in 96 well U bottom plates, 200 µL volume, afterwards, the cells were transferred to 24 well plate in the volume 1 mL and cultured one more week.

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

2.3. 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 5×10^4 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 at 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. Concentration-response effects of EP2 and EP4 receptors antagonists were analyzed using following concentrations: 100 nM, 300 nM, 1 µM and 3 µM. To confirm involvement of EP2 and EP4 receptors in suppression of ILC2 function, EP2 receptor specific agonist, butaprost and EP4 receptor specific agonist, L-

902, 688 (both from Cayman Chemicals), were used in the following concentrations: 10 nM, 30 nM, 100 nM, 300 nM and 1 μ M.

For the second part of the thesis when I was analyzing the role of endogenous prostaglandins in ILC2 function, ILC2 were incubated 40 min with flurbiprofen (1 μ M) or 20 min with CRTH2 antagonist, CAY10471 (1 μ M) before adding stimulating cytokines: IL-33, TSLP and IL-25 at 50 ng/mL each plus IL-2 10 U/mL.

For LC-MS/MS experiments, expanded ILC2 were seeded in a density of 2.5×10^5 cells/well in IMDM/1%NHS without Yssel's supplement and incubated 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 μ M) was used as 40 min pre-treatment.

For RT-qPCR, expanded ILC2 were seeded 1×10^5 cells/well and incubated for 6 hours or 24 hours with IL-2 (10 U/mL) and with or without combination of IL-33, TSLP and IL-25 (50 ng/mL each), with or without a 40 min pre-treatment with flurbiprofen (1 μ M).

Freshly sorted ILC2 were kept in the presence of IL-2 (10 U/mL) and were treated with or without 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. As pre-treatments, flurbiprofen (1 μ M) was added 40 min, and the CRTH2 antagonist, CAY10471 (1 μ M) 20 min before the cytokines. Flurbiprofen and CAY10471 treatments were repeated on days 2 and 4 of incubation. Microscopic images of freshly sorted blood ILC2 were captured after 5 days of culture, with 10 times magnification by using camera and Image View program. Supernatants were collected after 5 days and IL-5 and IL-13 concentrations were detected by ELISA. CD25 and CRTH2 protein expression was analysed by flow cytometry.

2.4. 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). The cells were incubated with antibodies at room temperature for 30 minutes.

For intracellular cytokine detection of IL-5 and IL-13, ILC2 were incubated with Golgi plug (Brefeldin A, BD Bioscience) and Golgi stop (Monensin, BD Bioscience) added after 18 hours to block protein transport and keep the cytokines inside the cells. ILC2 were thereafter fixed (2% paraformaldehyde, 10 min), permeabilized (FACS Permeabilizing Solution 2, BD Bioscience, 10 times diluted in water, 15 min) 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. Staining and washing of the cells was done in flow cytometry buffer (FC buffer: PBS supplemented with 2% FBS and 2 mM EDTA). 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.).

2.5. Proliferation assay

To analyze 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% FBS. Afterwards, the cells were resuspended in Yssel's medium/1% NHS and seeded into 96 well plates. Cells were treated with PGE₂ (30 nM) for 10 min before 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 as 20 min pre-treatments 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.

2.6. Mast cell culture and stimulation

Culture and stimulation of mast cells was performed by Maria Ekoff (Karolinska Institutet, Stockholm, Sweden). Cord blood-derived mast cells (CBMCs) were derived and then maintained as previously described^{189, 190}. CBMCs were pre-incubated with 10 ng/mL human IL-4 (Peprotech EC Ltd, London, UK) for 4 days and received 1 μ g/mL IgE

(Calbiochem, Merck Millipore, Darmstadt, Germany) overnight prior activation. For activation, cells (250.000 cells/well eq. to 1.25×10^6 cells/mL) were activated in IMDM medium supplemented with 1% normal human serum and penicillin (100 U/mL) and streptomycin (0.1 mg/mL) using either 2 μ g/mL anti-human IgE (Sigma, St. Louis, MO, USA) for 6 hours or 2 μ M calcium ionophore A23187 (Sigma) for 2 hours. Supernatants were collected and stored at -80°C.

2.7. Enzyme-linked immunosorbent assay (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 (Amsterdam, Netherlands).

2.8. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of prostanoids and metabolites

LC-MS/MS analysis were performed by Alexander Fauland and Craig E. Wheelock (both Karolinska Institutet, Stockholm, Sweden), or Nerea Ferreirós and Dominique Thomas (both University of Frankfurt, Germany).

The analysis of cell culture supernatants was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). All analytical standards and internal standards were purchased from Cayman Chemical (Ann Arbor, Michigan, USA). Solvents (LC-MS grade) were obtained from Carl Roth (Karlsruhe, Germany).

For the extraction of prostanoids and metabolites, 200 μ L of cell culture supernatant were spiked with isotopically labeled internal standards (PGE₂-d4, PGD₂-d4, TXB₂-d4, PGF_{2 α} -d4, 6-keto PGF_{1 α} -d4, 20-HETE-d6 and 13,14-dh-15-keto PGE₂), 100 μ L EDTA solution (0.15M) and 600 μ L ethyl acetate. Samples were vortexed and centrifuged at 20,000 g for 5 min. The organic phase was removed, and the extraction was repeated with 600 μ L ethyl acetate. The organic fractions were combined and evaporated at a temperature of 45°C under a gentle stream of nitrogen. The residues were reconstituted with 50 μ L of acetonitrile/water/formic acid (20:80:0.0025, v/v/v) and transferred to glass vials.

The LC-MS/MS analysis was carried out using 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 ESI mode. The chromatographic separation was carried out using a Synergi Hydro-RP column (150 × 2 mm, 4 µm particle size and 80 Å pore size; Phenomenex, Aschaffenburg, Germany). A linear gradient was employed at a flow rate of 300 µL/min. Mobile phase A was water/formic acid (100:0.0025, v/v) and mobile phase B was acetonitrile/formic acid (100:0.0025, v/v). The analytes were separated under gradient conditions within 16 min. The injection volume was 20 µL. The gradient program started with 90% A for 1 min, then mobile phase A was decreased to 60% within 1 min, held for 1 min, further decreased to 50% within 1 min and held for 2 min. Within 2 min, mobile phase A was further decreased to 10% and held for 1 min. Within 1 min, the initial conditions were restored and the column was re-equilibrated for 6 min. 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 Sciex, Darmstadt, Germany) were used. The following precursor-to-product ion transitions used for quantification: m/z 351.2 → m/z 315.0 for PGE₂, m/z 351.2 → m/z 233.3 for PGD₂, m/z 353.2 → m/z 193.0 for PGF_{2α}, m/z 369.2 → m/z 163.0 for 6-keto PGF_{1α}, m/z 369.2 → m/z 195.0 for TXB₂, m/z 279.1 → m/z 178.9 for 12-HHT, m/z 353.2 → m/z 309.2 for 11β-PGF_{2α}, m/z 351.2 → m/z 333.2 for 13,14dh-15-keto-PGD₂, m/z 333.2 → m/z 233.3 for PGJ₂ and m/z 333.2 → m/z 233.3 for delta12-PGJ₂. The peak area of each analyte was corrected by the peak area of the corresponding internal standard. Calibration curves were constructed 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 – 10 ng/mL plasma for PGE₂, PGD₂, TXB₂, 13,14dh-15keto-PGD₂, PGJ₂, delta12-PGJ₂; 0.04 – 20 ng/mL plasma for PGF_{2α}; 0.08 – 40 ng/mL plasma for 6-keto PGF_{1α}; 0.1 – 10 ng/mL plasma for 11β-PGF_{2α} and 0.2 – 10 ng/mL plasma for 12-HHT.

2.9. 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, *PTGS2* (for COX-2), *HPGDS*, *HPGD*, *PTGDR* (for DP1), *GPR44* (for CRTH2) and 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).

2.10. RNA sequencing analysis

RNA sequencing analysis was performed by Åsa Björklund (Science for Life Laboratory, Uppsala, Sweden). 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. ²⁵ 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.

2.11. Statistical analyses

In all experiments, ‘n’ represents 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.

3. Results

3.1. PGE₂ suppresses human group 2 innate lymphoid cell function

Data presented in this chapter have in part been published in:

Jovana Maric, Avinash Ravindran, Luca Mazzurana, Åsa K. Björklund, Aline Van Acker, Anna Rao, Danielle Friberg, Sven-Erik Dahlén, Akos Heinemann, Viktoria Konya and Jenny Mjösberg. *PGE₂ suppresses human group 2 innate lymphoid cell function. J Allergy Clin Immunol*, 2017. Accepted September 11, 2017¹.

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

We sorted ILC2 from human tonsil mononuclear cells as viable Lin⁻CD127⁺CD161⁺CRTH2⁺ lymphocytes (Figure 5A). Since the yield of tonsil ILC2 is relatively low, i.e. between 1000-6000 cells, freshly sorted ILC2 were expanded *in vitro* with irradiated feeder cells for two weeks in the presence of high level of IL-2 (100 U/mL), PHA (1 µg/mL) and addition of IL-4 (25 ng/mL) which maintains ILC2 phenotype. After expansion, ILC2 retained their phenotype as they expressed CD161 and CRTH2 but not CD3 and CD56, same as freshly isolated cells (Figure 5B, C).

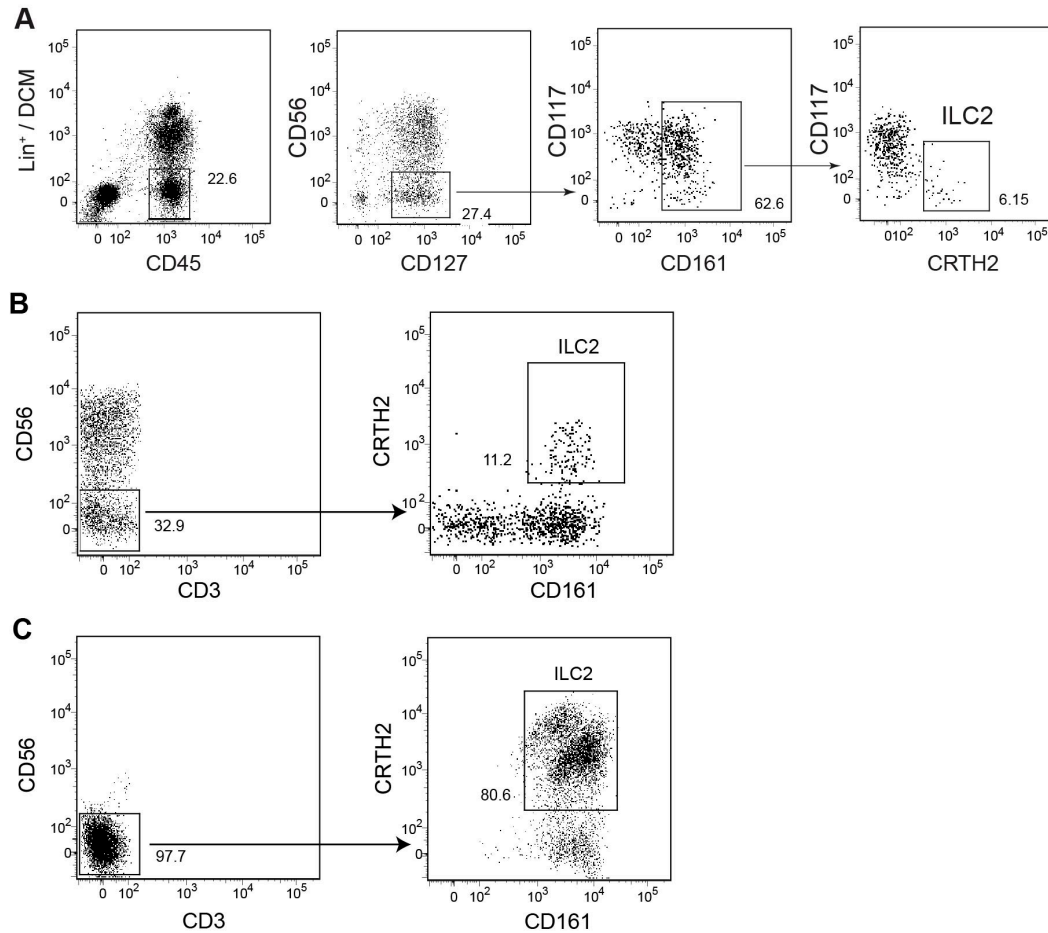


Figure 5. Gating strategy of ILC2.

A. Gating strategy for sort-purifying human tonsil ILC2. **B.** Gating of ILC2 from lineage-depleted alive freshly isolated human tonsil mononuclear cells. **C.** Sorted and expanded ILC2 retain their phenotype after two weeks of culture expressing CD161 and CRTH2 and lacking expression of CD3 and CD56 as analyzed by flow cytometry. Figure is adapted from ¹, with a right granted from Elsevier, as for all subsequent figures in this chapter. Sorting of ILCs was performed by Viktoria Konya, Luca Mazzurana, Aline Van Acker and Anna Rao; Expansion of some ILC2 cultures was done with help of Avinash Ravindran.

To examine the effects of PGE_2 , tonsil ILC2 were incubated with cytokines known to activate human ILC2: IL-33, TSLP and IL-25 (labeled as IL; each 50 ng/mL), plus IL-2 (10 U/mL), in the presence or absence of PGE_2 for 24 hours. Non-stimulated (ns) ILC2 were treated only with IL-2 (10 U/mL). The stimulatory cytokines activated ILC2 to secrete high levels of IL-5 and IL-13, as assessed by ELISA (Figure 6A-D). Addition of PGE_2 caused concentration-dependent inhibition of cytokine secretion (Figure 6A, B). In

all further experiments, PGE₂ was used in a concentration 30 nM, which was a little higher than IC₅₀ and caused approximately 70% suppression of cytokine secretion. Of note, combination of all three used cytokines: IL-33, IL-25 and TSLP induced much higher IL-5 and IL-13 release by ILC2 than each of the cytokines alone (Figure 6E, F). These findings suggest that the stimulatory cytokines have synergistic effects and all are necessary for complete ILC2 response. IL-33 alone could induce prominent IL-5 and IL-13 production, while IL-25 and TSLP caused only moderate ILC2 activation. PGE₂ inhibited the response to a combination of all the cytokines, as well as to each of them separately (Figure 6E, F).

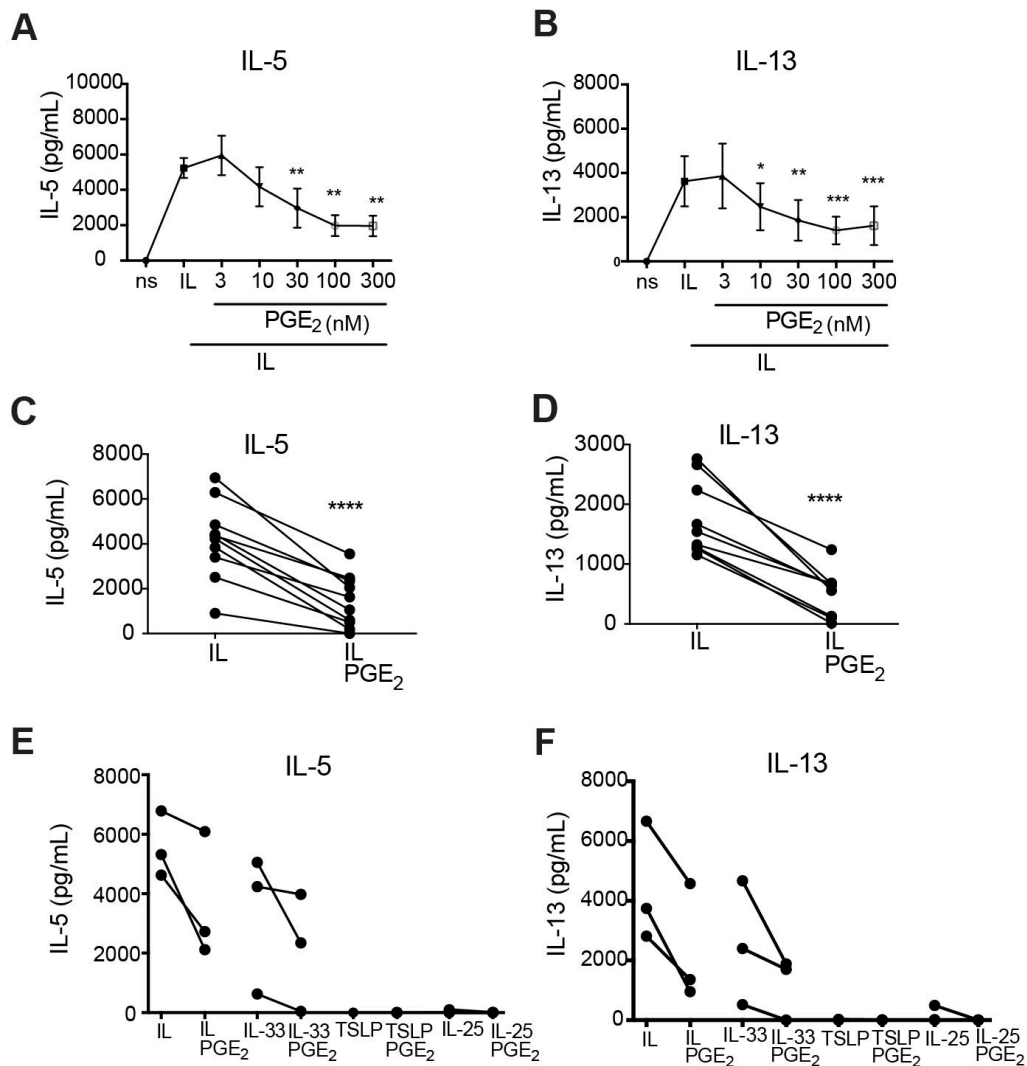


Figure 6. PGE₂ suppresses IL-5 and IL-13 release from human tonsil ILC2.

A. 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. A and B. 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 $\pm$ SEM

(* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 4$). **C** and **D**. graphs show individual concentrations of released IL-5 (**C**) and IL-13 (**D**) for effects of 30 nM PGE₂ (**** $p < 0.0001$, $n = 9-10$). ILC2 were stimulated with a cocktail of cytokines or individual cytokines in addition to IL-2 (**E**, **F**). Graphs show individual IL-5 (**E**) and IL-13 (**F**) concentrations, $n = 3$. Figure is adapted from ¹.

Next, the aim was to confirm the effects of PGE₂ on a per-cell basis. Hence, intracellular expression of IL-5 and IL-13 in ILC2 was analyzed. Same pattern of results was obtained as PGE₂ significantly abolished the increased percentage of IL-5- and IL-13- producing ILC2 induced by stimulation with cytokines (Figure 7A-C).

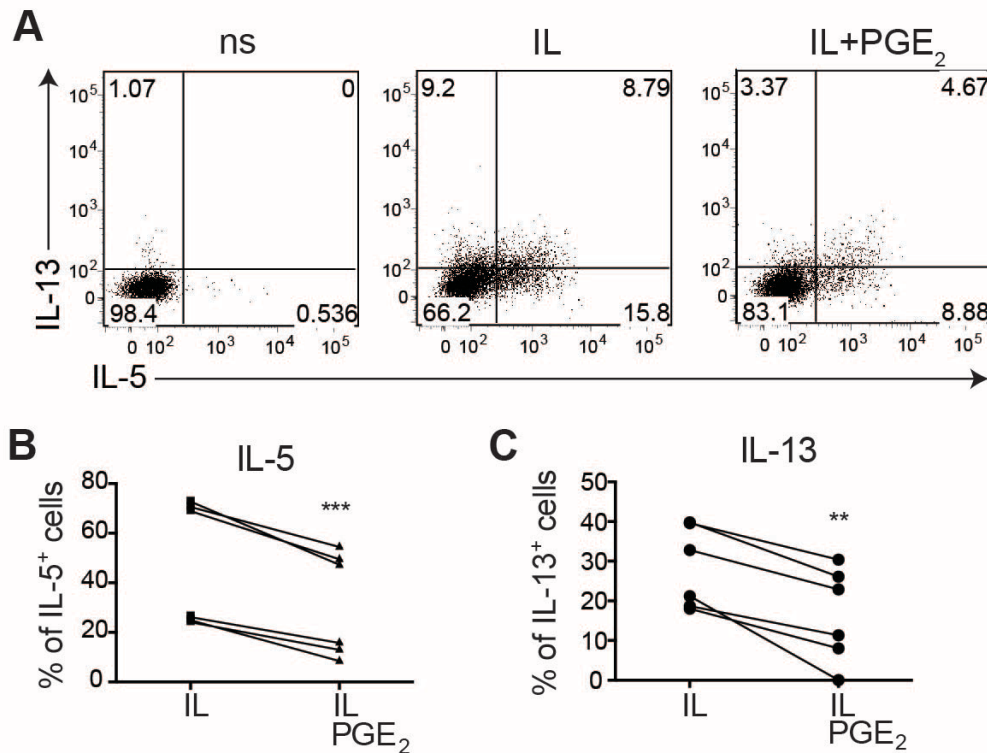


Figure 7. PGE₂ reduces intracellular production of IL-5 and IL-13 in stimulated ILC2.

A. Intracellular cytokine expression of IL-5 and IL-13 was determined by flow cytometry. **B** and **C.** The graphs present individual percentages of IL-5- (**B**) and IL-13- (**C**) producing cells (** $p < 0.01$, *** $p < 0.001$, $n = 6$). Figure is adapted from ¹.

To examine whether alteration of ILC2 count is a possible reason for the suppressive effect of PGE₂ on the cytokine secretion, cell viability and proliferation after 24 hours of culture were assessed. Importantly, there were no differences in these parameters in the presence

of PGE₂ treatment (Figure 8A, B), which gives us information about effects on ILC2 on a per-cell basis.

Altogether, these results clearly show that PGE₂ reduces synthesis and secretion of IL-5 and IL-13 by cytokine-stimulated ILC2. These data have been reported in similar fashion in ¹.

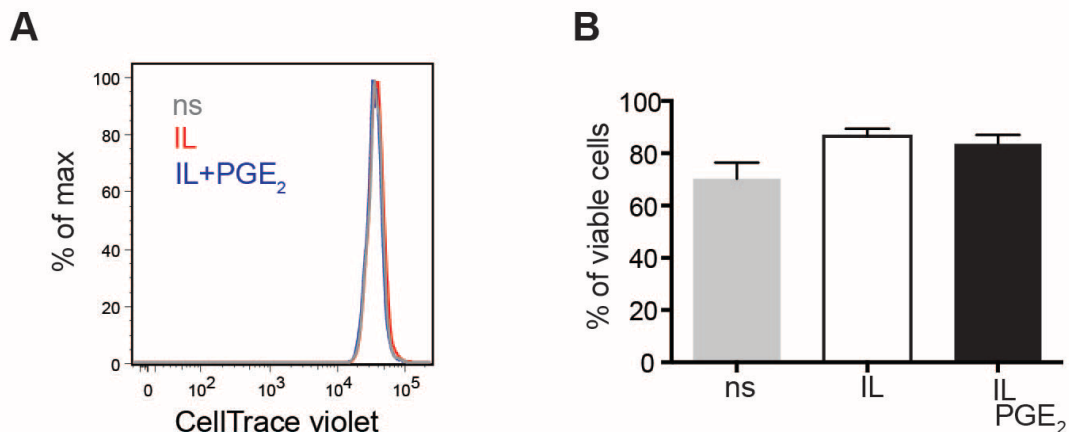


Figure 8. PGE₂ does not affect ILC2 viability and proliferation after 24 hours.

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 is adapted from ¹.

3.1.2. PGE₂ prevents GATA3 and CD25 upregulation

As previously described, the transcription factor GATA-3 is required for ILC2 function, regulating the production of cytokines by these cells. Thus, it was important to examine intracellular protein expression of GATA3 in ILC2 upon the treatments. As previously shown, we now confirmed that non-stimulated ILC2 already express high level of GATA3, which is more increased upon cytokine stimulation for 24 hours (Figure 9A-C). Presence of PGE₂ caused significant reduction of the GATA-3 expression, by preventing GATA3 upregulation in a subpopulation of ILC2 (Figure 9A-C).

IL-2Rα (CD25) is an additional marker of ILC2 and IL-2 is crucial in co-stimulating ILC2 activation ⁴⁴. Therefore, next aim was to determine the impact of PGE₂ treatment on CD25 cell surface expression on ILC2. As expected, cytokine stimulation of ILC2 induced

increase in CD25 expression after 24 hours compared to non-stimulated control. Strikingly, co-incubation of ILC2 with PGE₂ significantly decreased upregulation of CD25 expression (Figure 9D-F).

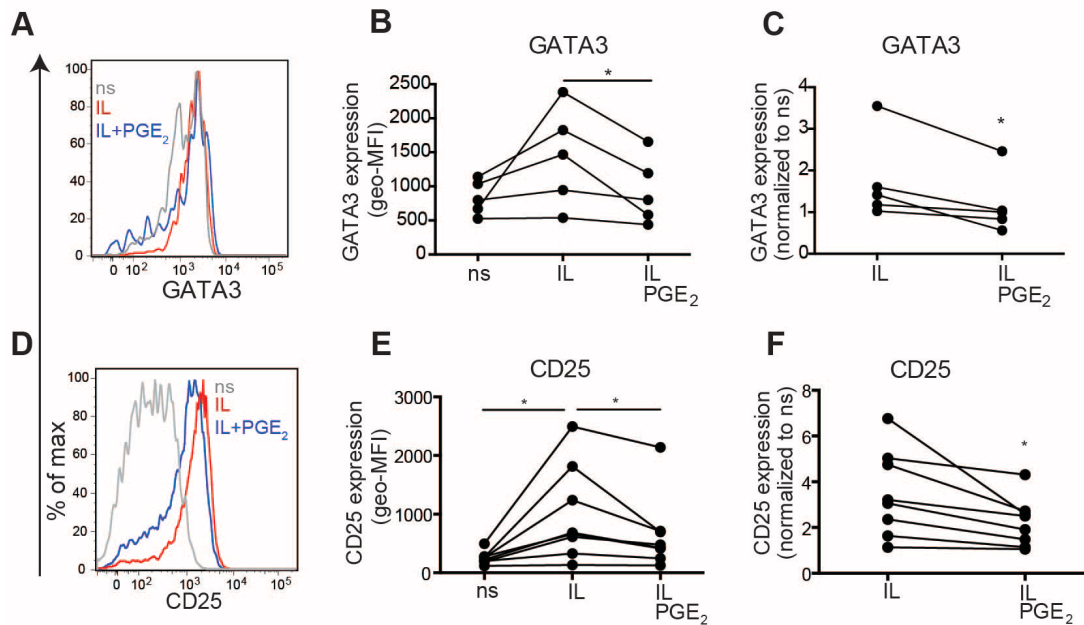


Figure 9. PGE₂ reduces GATA3 and CD25 upregulation.

ILC2 were stimulated as indicated for 24 hours. Expression of GATA3 (A) and CD25 (D) was analyzed by flow cytometry. Graphs show individual geometric mean fluorescence intensity of GATA3 (B) and CD25 (E) (*p<0.05, n=5-8) and geometric mean fluorescence intensity normalized to non-stimulated cells of GATA3 (C) and CD25 (F) expression (*p<0.05, n=5-8). Figure is adapted from ¹.

Taken together, PGE₂ reduced upregulation of GATA3 and CD25 expression in stimulated ILC2, which suggest that these steps are part of the underlying mechanisms behind the observed inhibitory effects of PGE₂ on IL-5 and IL-13 production. These finding have been previously discussed ¹.

3.1.3. PGE₂ additionally suppresses ILC2 proliferation after longer culture

Next objective was to examine whether the effects of PGE₂ are long lasting. Thus, ILC2 were incubated with the treatments described above for 72 hours. Cytokine-stimulated ILC2 produced higher amounts of IL-5 and IL-13 when compared to 24 hours, while the

suppressive effects of PGE₂ remained significant (Figure 10A, B). Similar pattern regarding GATA3 expression was observed, as PGE₂ significantly decreased cytokine-triggered enhanced GATA3 expression (Figure 10C-E).

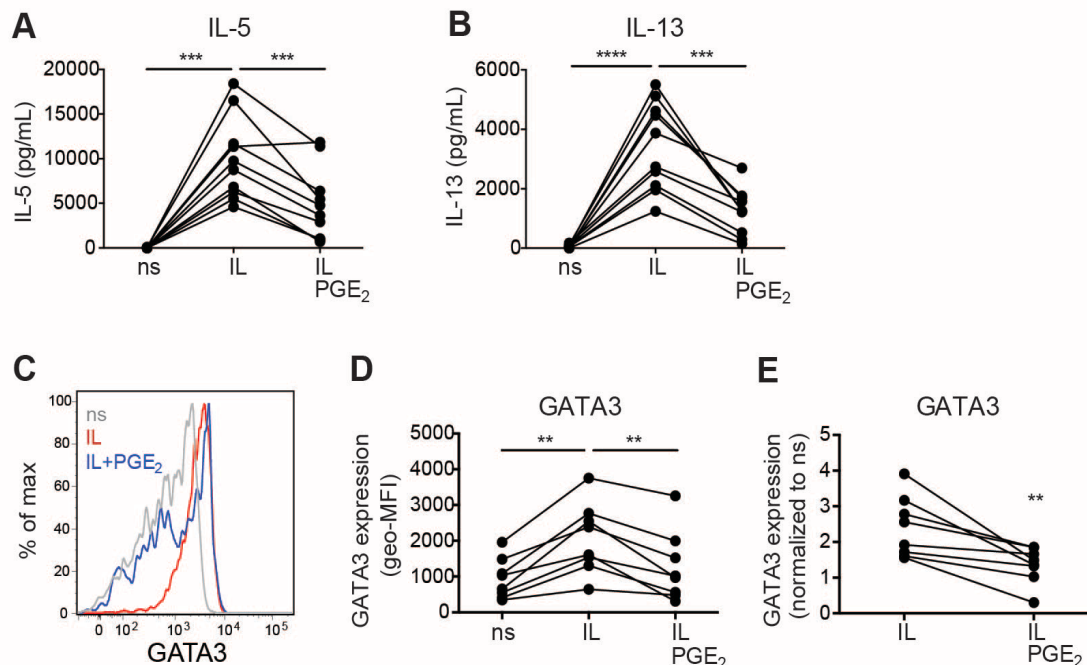


Figure 10. PGE₂ prevents GATA3 upregulation after 72 hours.

ILC2 were stimulated as indicated for 72 hours. Concentrations of IL-5 (A) and IL-13 (B) in ILC2 supernatants were determined by ELISA; individual concentrations of the cytokines in the supernatants are shown (*** p <0.001, **** p <0.0001, n =10). 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 is reproduced from ¹.

Furthermore, determining CD25 expression after 72 hours revealed even more pronounced suppressive effects of PGE₂ on CD25 upregulation when compared to 24 hours of culture (Figure 11A-C). Considering that IL-2 initiates cell proliferation, the effects of PGE₂ on ILC2 proliferation were assessed using Cell trace violet dye. The cells were loaded with the proliferation dye before adding the treatments. As the cells divided, the dye was diluted, resulting in low MFI, as detected by flow cytometry. IL-stimulation triggered ILC2 proliferation, as the cells went through a second, third and some even a fourth division (Figure 11D-F). Proliferation is likely an additional reason for increased level of IL-5 and IL-13 release after 72 hours of culture. As expected, PGE₂ blocked cytokine-

induced ILC2 proliferation, since most of the cells remained in cell division phase 0 and 1, similar to non-stimulated cells (Figure 11D, E). These data were similarly described in ¹.

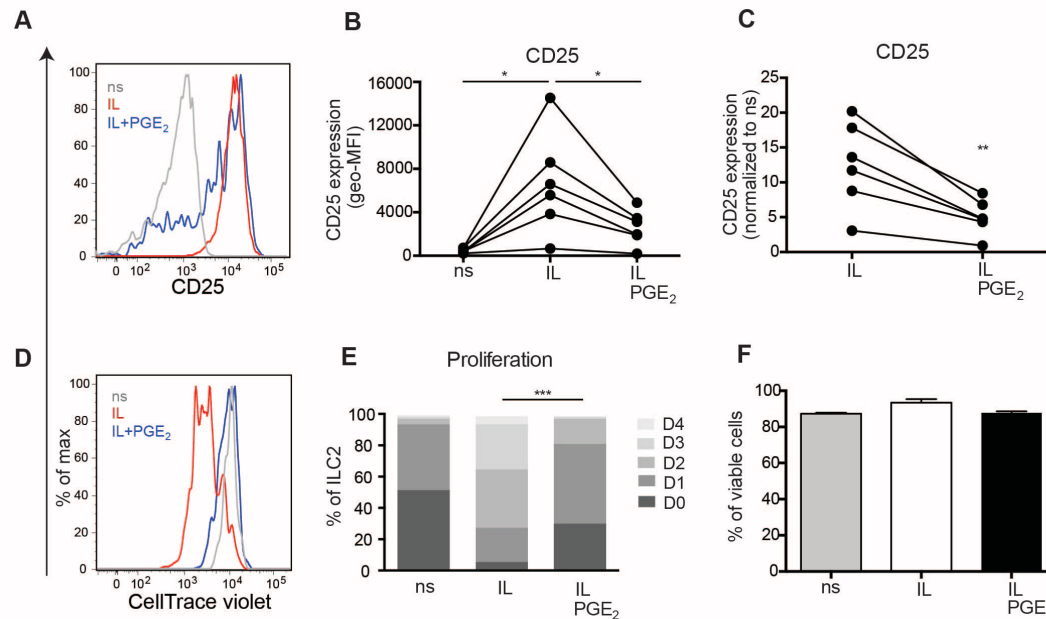


Figure 11. PGE₂ prevents cytokine-induced CD25 upregulation and proliferation of ILC2.

ILC2 were stimulated as indicated for 72 hours. **A.** Expression of CD25 was analyzed by flow cytometry, representative histograms are shown. Graphs show individual geometric mean fluorescence intensity of CD25 expression (**B**) and geometric mean fluorescence intensity normalized to non-stimulated cells of CD25 expression (**C**) (* $p < 0.05$, ** $p < 0.01$, $n = 6$) **D.** Proliferation of CellTrace violet dye-loaded ILC2 after 72 hours incubation was analyzed by flow cytometry. **E.** Data are shown as percentage of ILC2 in different division phases (D0-D4) (** $p < 0.001$, $n = 4$). **F.** 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 is adapted from ¹.

These data indicate that PGE₂, besides acting on the per cell basis by reducing CD25 and GATA-3 expression, also affects production of type 2 cytokines after longer culture by suppressing ILC2 proliferation.

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

Previous experiments were performed with expanded cells due to the low number of freshly sorted ILC2. However, next objective was to show that obtained results are not a consequence of potential changes during the cell expansion and confirm the inhibitory effect of PGE₂ on freshly sorted human ILC2. To address this issue, sorted ILC2 from buffy coats (Figure 12A, B and Figure 13A-C) and tonsils (Figure 12C, D) were used. As freshly isolated ILC2 secrete less cytokines as compared to expanded cells on a per cell basis⁴¹, ILC2 were incubated for 5 days. Cytokine stimulation induced secretion of IL-5 and IL-13 from both freshly isolated blood ILC2 (Figure 12A, B) and tonsil ILC2 (Figure 12C, D). Importantly, release of IL-5 and IL-13 from stimulated freshly sorted ILC2 was reduced by PGE₂ (Figure 12A-D).

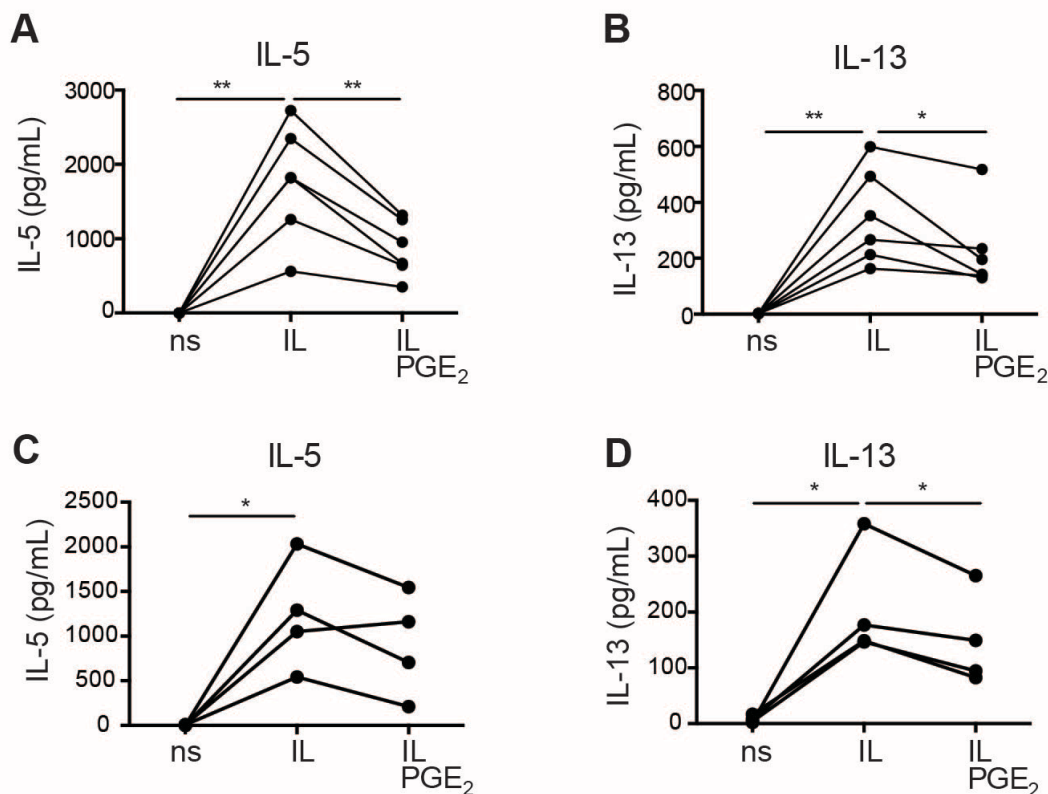


Figure 12. PGE₂ reduces IL-5 and IL-13 secretion from fresh blood and tonsil ILC2.

Freshly sorted human blood (A, B) and tonsil (C, D) ILC2 were incubated as indicated for 5 days. Concentrations of released IL-5 from blood ILC2 (A) and tonsil ILC2 (C) and IL-13 from blood ILC2 (B) and tonsil ILC2 (D) were measured by ELISA; graphs show

individual concentrations of the cytokines in the supernatants (* $p < 0.05$, ** $p < 0.01$; $n = 6$ for blood ILC2 and $n = 4$ for tonsil ILC2). Figure is adapted from ¹.

Next, CD25 expression in freshly sorted blood ILC2 was assessed. Cytokine stimulation strikingly induced upregulation of CD25 expression, which was prevented by PGE₂ co-incubation (Figure 13A, B). To analyze the effects of the treatments on ILC2 proliferation, microscopic images of fresh blood ILC2 were captured after 5 days of culture. These images showed that cytokine stimulation triggered proliferation and growth of ILC2 (Figure 13C), whereas these effects were less pronounced in the presence of PGE₂ (Figure 13C). These data have been reported in ¹.

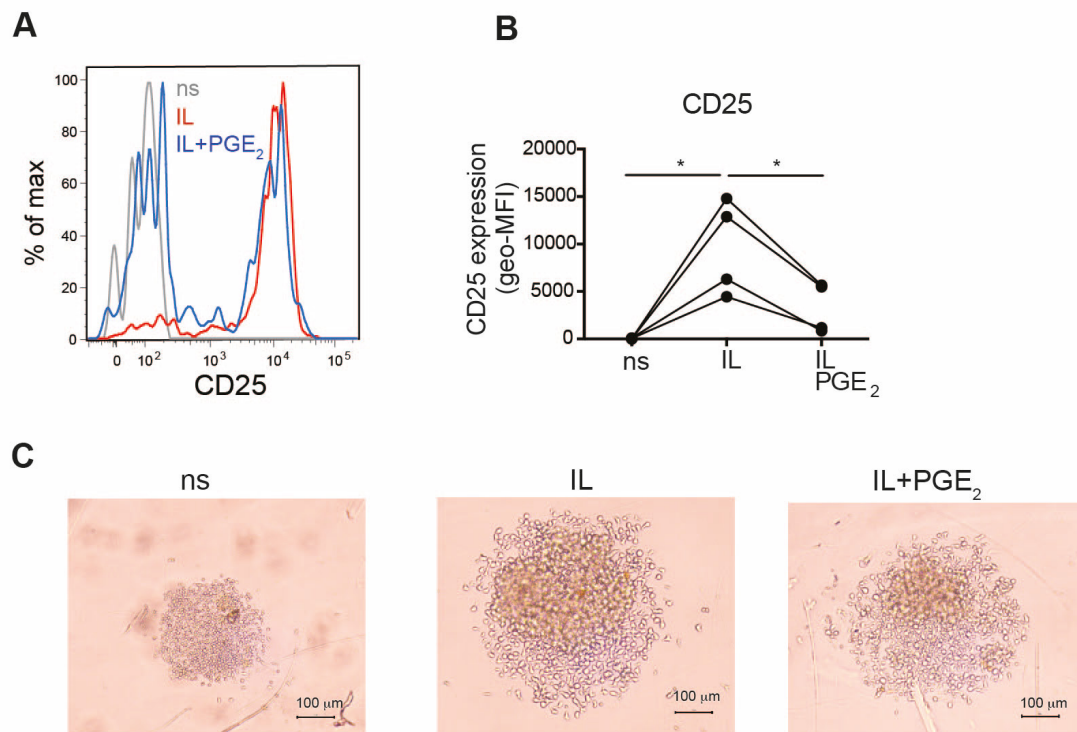


Figure 13. PGE₂ prevents CD25 upregulation in freshly isolated blood ILC2.

A. Expression of CD25 in freshly sorted blood ILC2 was analyzed by flow cytometry, representative histograms are shown. **B.** Graph shows individual geometric mean fluorescence intensity of CD25 expression in blood ILC2 after 5 days of culture (* $p < 0.05$, $n = 4$). **C.** Images of freshly sorted blood ILC2 after 5 days of treatments; 10 x magnification, scale bar = 100 μm. Figure is adapted from ¹.

These results show that activation of freshly isolated ILC2 is also reduced by PGE₂ treatment and confirm the inhibitory effects of PGE₂ observed in expanded ILC2.

Additionally, it is shown that suppressive effects of PGE₂ on ILC2 function are not limited to tonsil tissue, as they are also demonstrated in blood.

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

Effects of PGE₂ are mediated through four different G protein coupled receptors: EP1-EP4. To identify which EP receptors are responsible for the PGE₂-induced suppression of ILC2 function, expression of all four EP receptors in human tonsil ILC2 was determined. Previously published single-cell RNA sequencing study performed in freshly isolated tonsil ILCs²⁵ shows that ILC2 express *PTGER2* and *PTGER4*, transcripts of EP2 and EP4 receptors. In contrast, transcripts of EP1 and EP3 receptors are absent in ILC2 (Figure 14). Analysis of single cell RNA sequencing was performed by Åsa Björklund.

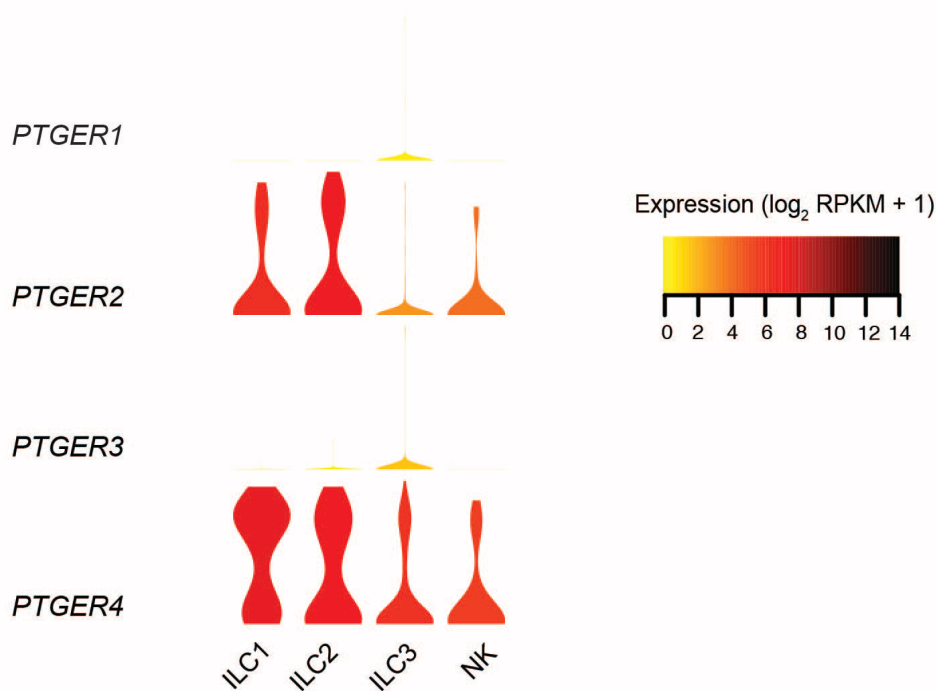


Figure 14. Human freshly sorted tonsil ILC2 express EP2 and EP4 receptors.

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 (RPKM). Bioinformatic analysis was provided by Åsa Björklund. Figure is adapted from

1.

To analyze whether the mRNA expression pattern of EP receptors is retained in expanded ILC2, RT-qPCR was performed after 24 hours of culture. Similar results were obtained, as only mRNA for EP2 and EP4 receptors could be detected in non-stimulated expanded tonsil ILC2 (Figure 15A). Of note, expression of EP2 and EP4 was not affected by cytokine stimulation showing that responsiveness to PGE₂ was not changed upon the treatments (Figure 15B).

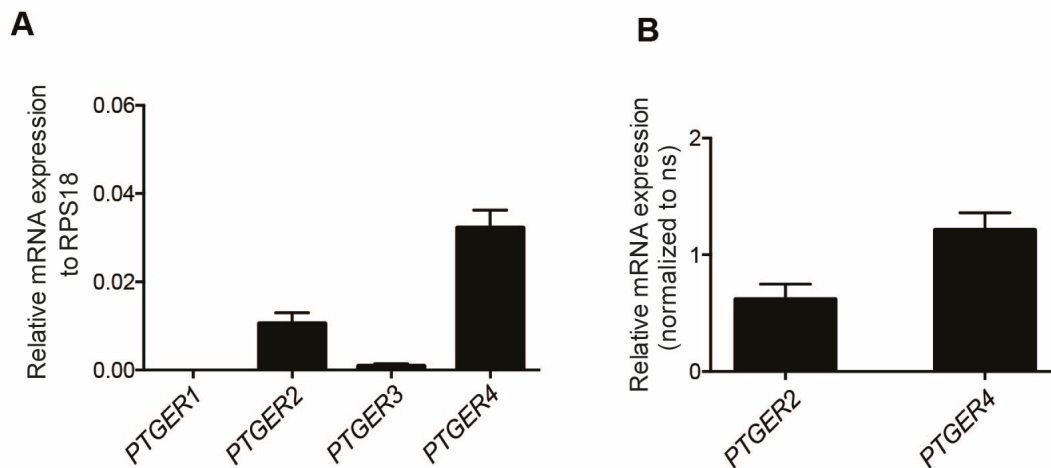


Figure 15. Expanded human tonsil ILC2 express EP2 and EP4 receptors.

A. mRNA expression of EP receptors (PTGER1-4) in sorted and expanded tonsil ILC2 was determined by RT-qPCR (n=5). **B.** mRNA expression of EP2 and EP4 receptors after 24 hours stimulation normalized to non-stimulated cells. Graphs show mean expression +SEM (n=5). Figure is adapted from ¹.

Having verified the expression of EP2 and EP4 receptors in tonsil ILC2, the next goal was to investigate whether activation of these two receptors is responsible for the suppressive effects of PGE₂. Therefore, ILC2 were pre-incubated with selective EP2 (PF-04418948) and EP4 (ONO AE3-208) receptor antagonists for 20 minutes before the PGE₂ and cytokine treatments (Figure 16). Levels of IL-5 and IL-13 in ILC2 supernatants were determined after 24 hours of culture and showed that individual EP2 or EP4 antagonism partially prevented the inhibitory effects of PGE₂. Only combined EP2 and EP4 receptors blockade significantly reversed the PGE₂-induced reduction of IL-5 and IL-13 secretion (Figure 16A, B). These observations were confirmed on the per-cell basis, as demonstrated that EP2 and EP4 antagonist used together were necessary to abolish the PGE₂-caused reduction of IL-5 and IL-13 production in ILC2 (Figure 16C-E).

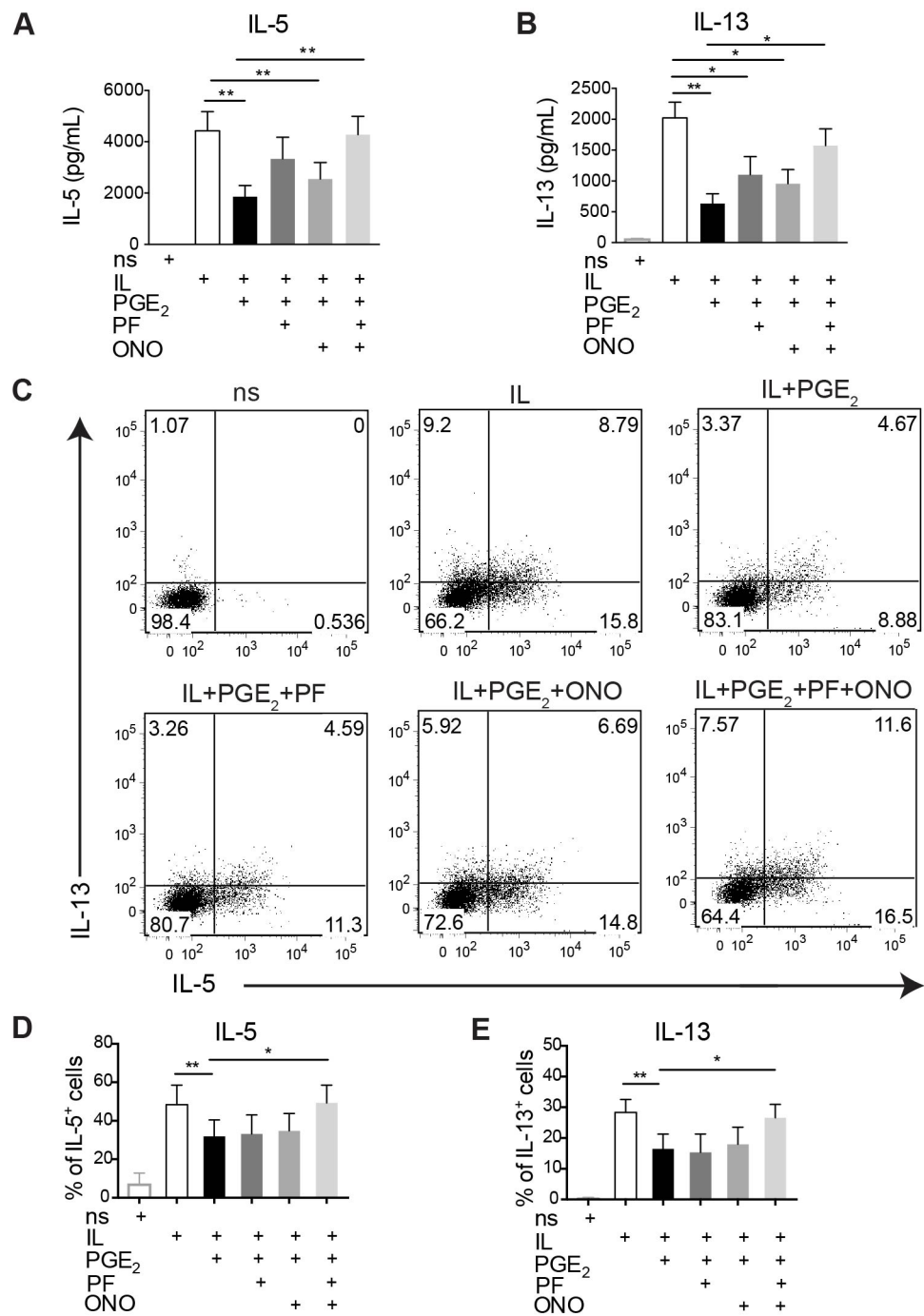


Figure 16. PGE₂ reduces IL-5 and IL-13 production in ILC2 through activation of EP2 and EP4 receptors.

Sorted and expanded tonsil ILC2 were stimulated as indicated for 24 hours. 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** 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 $\pm$ SEM of IL-5- (**D**) and IL-13- (**E**) producing ILC2 (* $p < 0.05$, ** $p < 0.01$, $n = 6$). Figure adapted from¹.

EP2 and EP4 receptors antagonists were used at 1 μ M concentration, but single antagonist treatment did not reverse the suppressive PGE₂ effects on ILC2 activation. This raised the question whether other concentrations would provide more efficient inhibition. Therefore, ILC2 were pre-incubated with EP2 and EP4 antagonists in the concentration range of 0.1-3 μ M. Detection of IL-5 and IL-13 amount in ILC2 supernatants revealed that antagonists at concentrations higher than 1 μ M did not have a more pronounced inhibitory effects on PGE₂ suppression of ILC2 cytokine secretion (Figure 17).

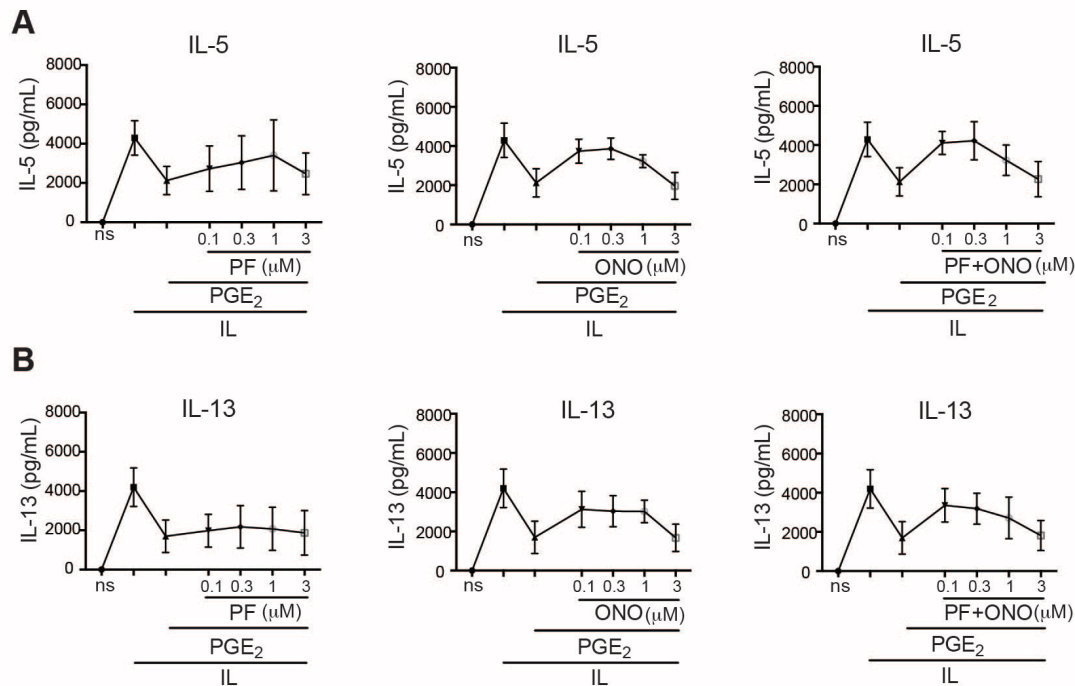


Figure 17. Dual engagement of EP2 and EP4 receptors is essential for the PGE₂-induced reduction of ILC2 cytokine secretion.

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 at 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 ($n = 4$). Figure is adapted from¹.

In addition, to confirm engagement of both EP2 and EP4 receptors, ILC2 were incubated with specific agonists of EP2 (butaprost) and EP4 (L-902, 688) receptors in the concentration range of 10-1000 nM. Engaging of EP4 receptor showed more inhibitory potential on ILC2 cytokine secretion than that of EP2 receptor. However, effects of individual agonists did not reach statistical significance. Data show that, at the concentration of 100 nM, joint agonism of EP2 and EP4 receptors could mimic the inhibitory effects of PGE₂ on ILC2 activation (Figure 18).

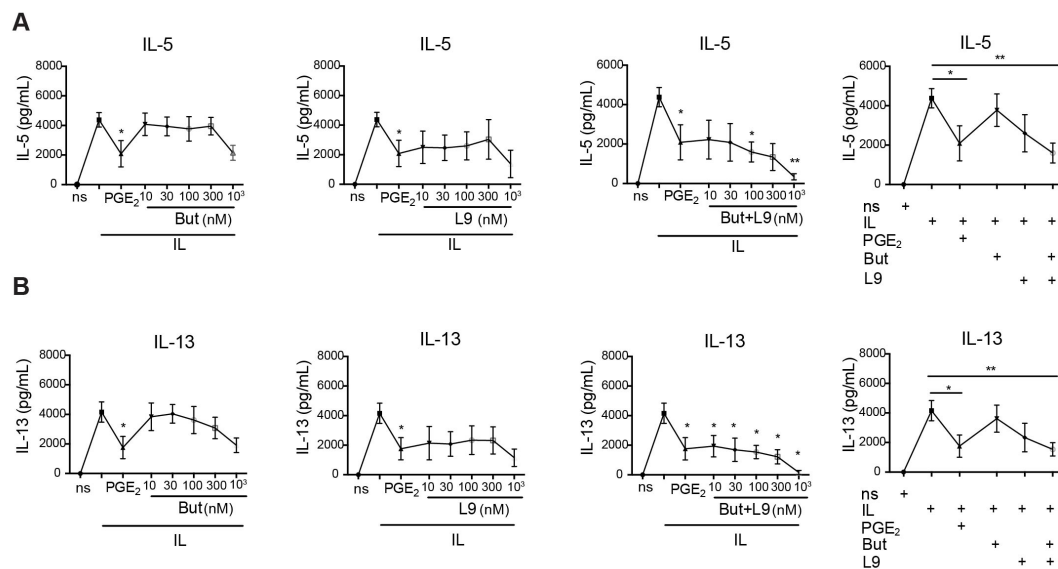


Figure 18. Simultaneous EP2 and EP4 receptors activation is crucial for the PGE₂-caused reduction of ILC2 cytokine secretion.

Sorted and expanded tonsil ILC2 were treated with EP2 receptor agonist, butaprost (But) and EP4 receptor agonist, L-902, 688 (L9), separately or together at 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 (A) and IL-13 (B) 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). Statistical differences for concentrations of IL-5 (A, far right panel) and IL-13 (B, far right panel) are shown after treatments with But and L9 both used at 100 nM concentration and PGE₂ in 30 nM (* p <0.05, ** p <0.01, n =4). Figure is adapted from ¹.

Since PGE₂ showed pronounced inhibitory effects on ILC2 proliferation after 72 hours in culture, role of EP2 and EP4 receptors in this process was investigated. Indeed, simultaneous antagonism of EP2 and EP4 receptors reversed PGE₂-induced suppression of ILC2 proliferation, as it enabled ILC2 to go to the second and third division phases (Figure

19A, B). Dual EP2 and EP4 blockade also reversed the PGE₂-induced reduction of ILC2 cytokine secretion, further confirming the role of both receptors in mediating the long-term inhibitory effects of PGE₂ in ILC2 function (Figure 19C, D).

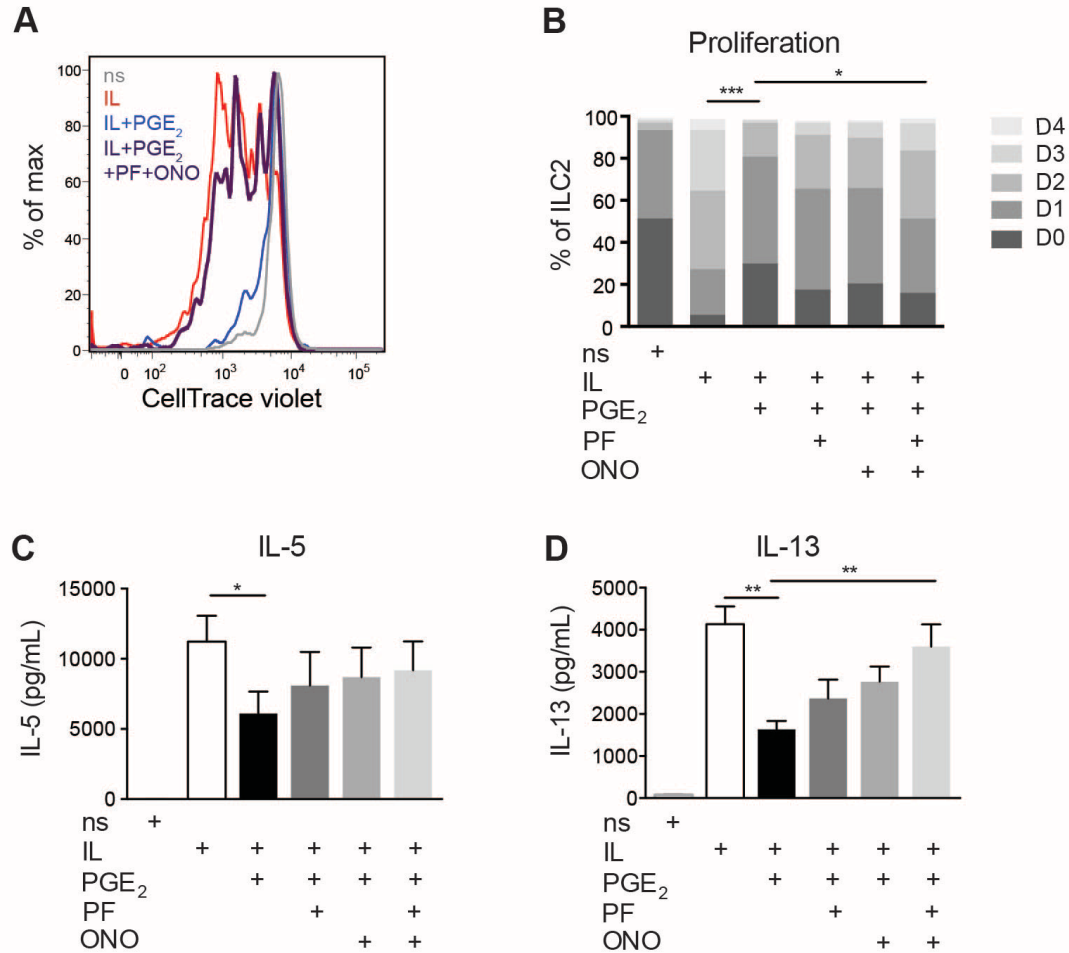


Figure 19. Activation of EP2 and EP4 receptors inhibits ILC2 cytokine release via preventing ILC2 proliferation.

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 $\pm$ SEM (* p <0.05, ** p <0.01, n =7). Figure is adapted from ¹.

Simultaneous antagonism of EP2 and EP4 receptors also reversed the PGE₂-induced reduction of GATA3 expression after both 24 hours and 72 hours of culture (Figure 20). Altogether, these data demonstrate that PGE₂ suppressive effects on IL-5 and IL-13 production and proliferation occur via engagement of both EP2 and EP4 receptors. These findings have been published in ¹.

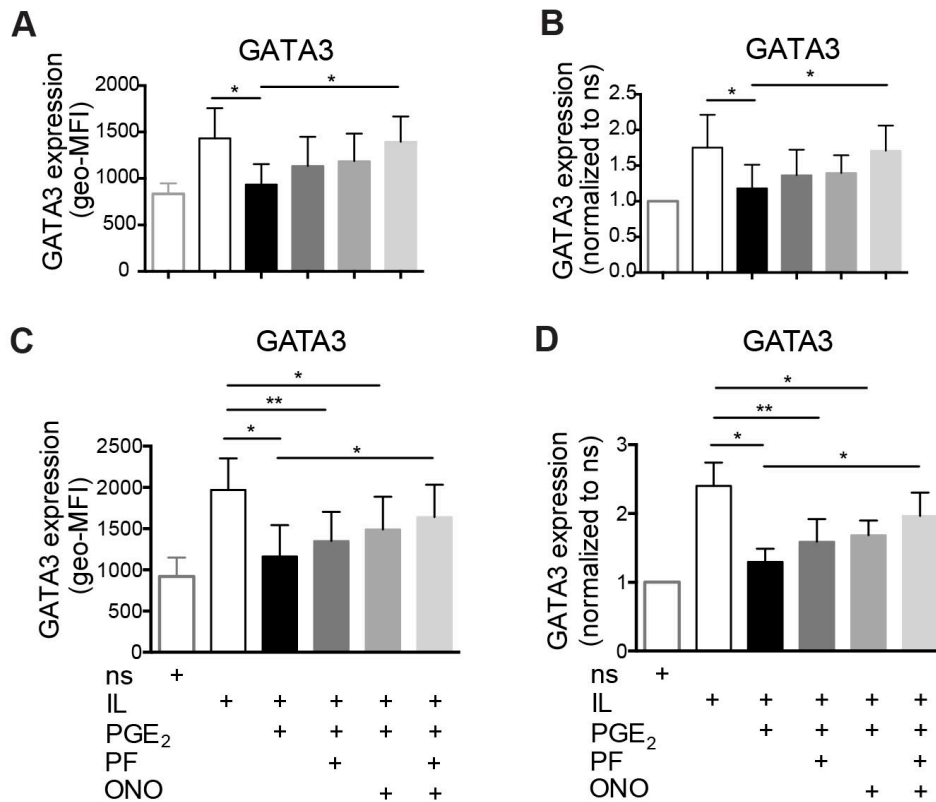


Figure 20. GATA3 is downregulated by the activation of EP2 and EP4 receptors in ILC2.

Expression of GATA3 was analyzed by flow cytometry. Graphs show geometric mean fluorescence intensity +SEM after 24 hours (A) and 72 hours (C) and geometric mean fluorescence intensity normalized to non-stimulated cells +SEM after 24 hours (B) and 72 hours (D), of GATA3 expression (* $p < 0.05$, ** $p < 0.01$, $n = 5-7$). Figure adapted from ¹.

3.2. Cytokine-induced endogenous PGD₂ production is essential for human ILC2 activation

Data presented in this chapter are content of a manuscript under review:

Jovana Maric, Avinash Ravindran, Luca Mazzurana, Aline Van Acker, Anna Rao, Maria Ekoff, Dominique Thomas, Alexander Fauland, Gunnar Nilsson, Craig E. Wheelock, Sven-Erik Dahlén, Nerea Ferreirós, Gerd Geisslinger, Danielle Friberg, Akos Heinemann, Viktoria Konya, and Jenny Mjösberg:

Cytokine-induced endogenous production of PGD₂ is essential for human ILC2 activation.

3.2.1. Production of IL-5 and IL-13 by ILC2 is dependent on endogenously released prostaglandins

Since it was recently shown in Dr. Mjösberg's group that freshly isolated human ILC2 express HPGDS, our hypothesis was that ILC2 are able to produce prostaglandins, which could be important for ILC2 regulation. Sorted and expanded tonsil ILC2 were, as mentioned earlier, incubated with cytokines known to activate human ILC2: IL-33, TSLP and IL-25 (IL; each 50 ng/mL), plus IL-2 (10 U/mL). To examine the impact of endogenous prostaglandins in ILC2 stimulation, ILC2 were pre-treated with the COX-1/2 inhibitor flurbiprofen 40 min before the cytokine stimulation. Blockade of COX-1/2 enzymes almost completely prevented IL-5 and IL-13 release of stimulated ILC2 (Figure 21A, B). Suppression of type 2 cytokine production on a per cell basis was confirmed by analyzing intracellular cytokine expression, which showed that presence of flurbiprofen blocked synthesis of IL-5 and IL-13 in stimulated ILC2 (Figure 21C-E). These data suggest that prostaglandins produced by cytokine-stimulated ILC2 are essential for ILC2 activation.

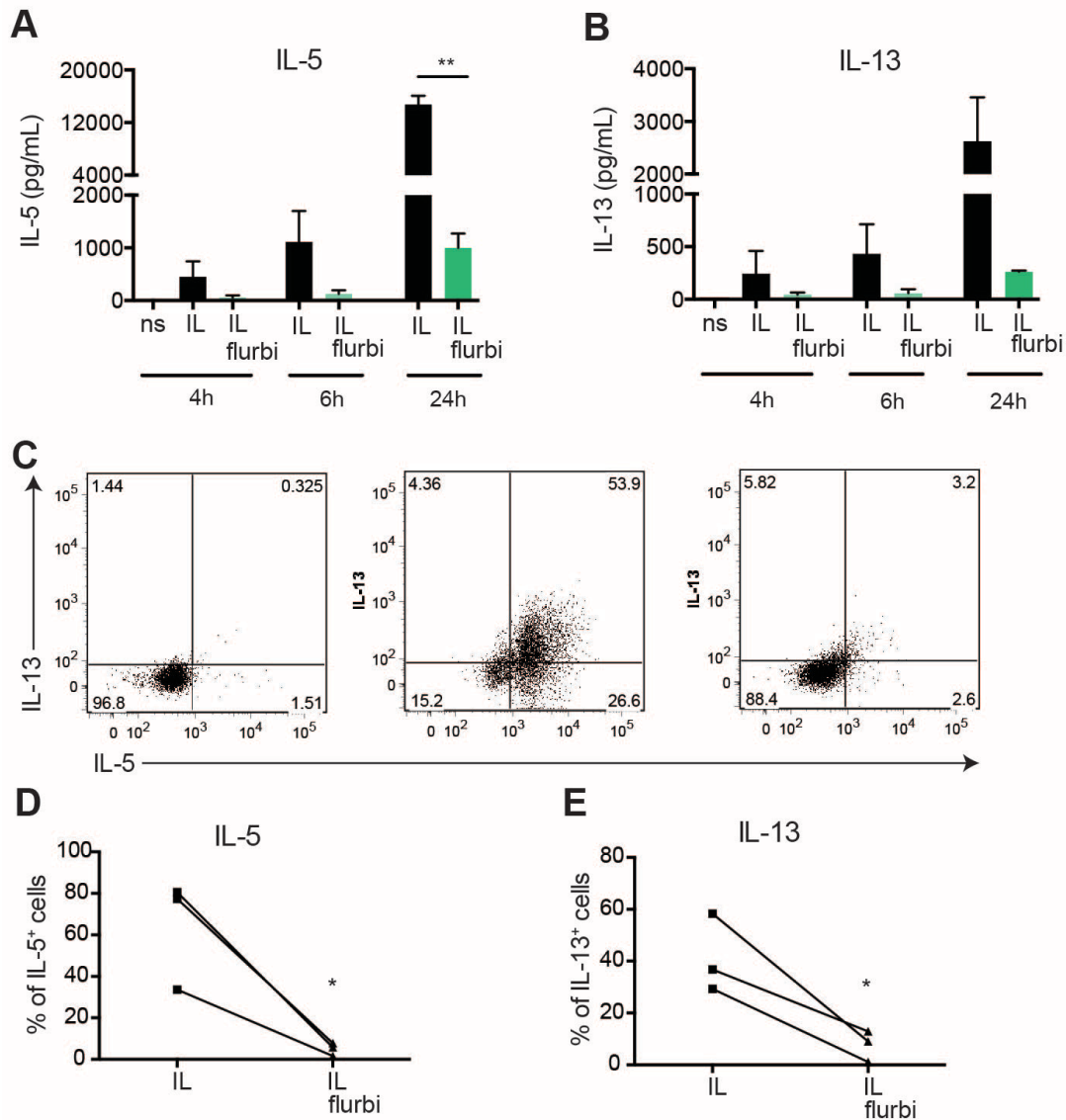


Figure 21. The production of IL-5 and IL-13 by ILC2 is dependent on endogenously produced prostaglandins.

A and B. Sorted and expanded ILC2 in the presence of IL-2 (10 U/mL) were stimulated with IL-33, IL-25 and TSLP (50 ng/mL each), denoted as IL, or left non-stimulated (ns) for 4 hours, 6 hours and 24 hours. Flurbiprofen (1 μ M) was used as 40 min pre-treatment. Concentrations of IL-5 (**A**) and IL-13 (**B**) in ILC2 supernatants were detected by ELISA, graphs show means +SEM (** p <0.01, n =3). **C.** Intracellular expression of IL-5 and IL-13 in ILC2 was assessed after 24 hours by flow cytometry, Graphs show individual percentages of IL-5- (**D**) and IL-13- (**E**) producing ILC2 (* p <0.05, n =3).

3.2.2. The PGD₂ pathway is turned on in stimulated human ILC2

As incubation with flurbiprofen dramatically reduced ILC2 activation showing the importance of endogenous prostaglandin production, we next aimed to determine which prostaglandins can be released by stimulated ILC2. It is known that exogenous PGD₂ acts as a stimulator of ILC2 via activating the CRTH2 receptor⁷⁰. However, it has not yet been shown whether ILC2 might produce PGD₂ on their own. Therefore, supernatants of expanded tonsil ILC2 were collected after 24 hours of stimulation and released lipid mediators were detected using a highly sensitive mass spectrometry approach. This analysis showed that non-stimulated ILC2 do not constitutively secrete any lipids. Cytokine stimulation initiated PGD₂ biosynthesis, as PGD₂ and its metabolites PGJ₂ and delta-12-PGJ₂ (Δ^{12} -PGJ₂) (Figure 22A, C, D) could be detected in the supernatants. PGD₂ metabolites, as 15-deoxy-delta-12, 14-PGJ₂ (15d-PGJ₂) (Figure 22E) and in smaller amount 13,14 dihydro-15-keto PGD₂ (DK-PGD₂) were also present in ILC2 supernatants (Figure 22B). Co-incubation with flurbiprofen completely prevented release of these lipid mediators, confirming specific inhibition.

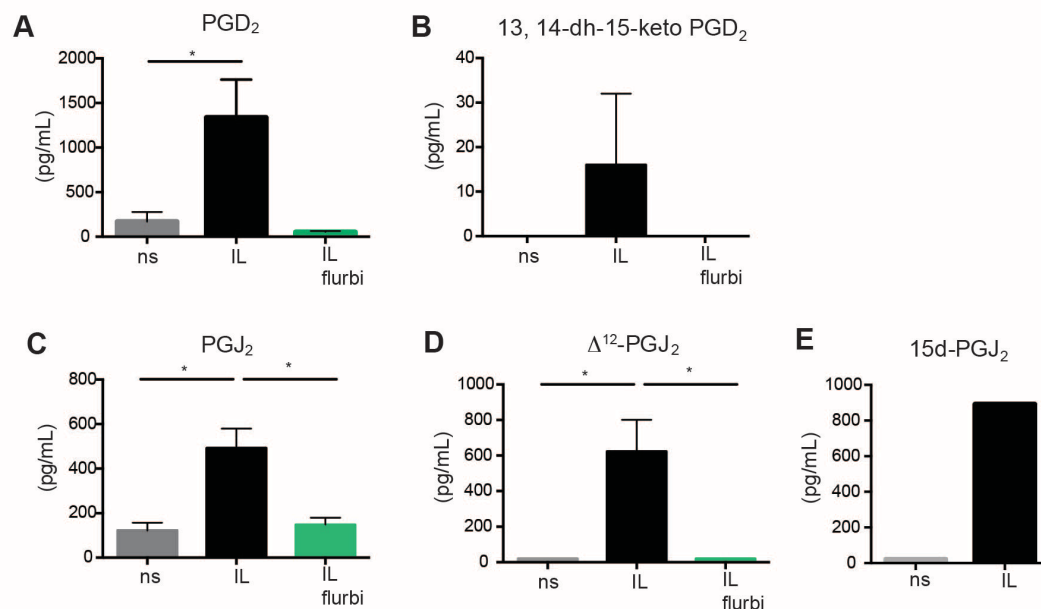


Figure 22. Human tonsil ILC2 produce PGD₂ and its metabolites upon cytokine stimulation.

Sorted and expanded tonsil ILC2 were stimulated as indicated for 24 hours with or without flurbiprofen (1 μ M, 40 min pre-treatment). Mediators of the PGD₂ pathway were detected in ILC2 supernatants by using LC-MS/MS. Graphs show mean concentration +SEM of

PGD_2 (* $p < 0.05$, $n = 4-5$), 13, 14-dihydro-15-keto PGD_2 ($n = 4$), PGJ_2 (* $p < 0.05$, $n = 7$), Δ^{12} - PGJ_2 (* $p < 0.05$, $n = 7$) and 15d- PGJ_2 ($n = 1$). LC-MS/MS analyses were performed by Alexander Fauland, Craig E. Wheelock, Nerea Ferreirós and Dominique Thomas.

Besides, we detected TXB_2 in ILC2 supernatants, which was also completely blocked by using the COX-1/2 inhibitor (Figure 23). Of note, other eicosanoids, such as PGE_2 , PGI_2 were not detected in the supernatants of stimulated ILC2.

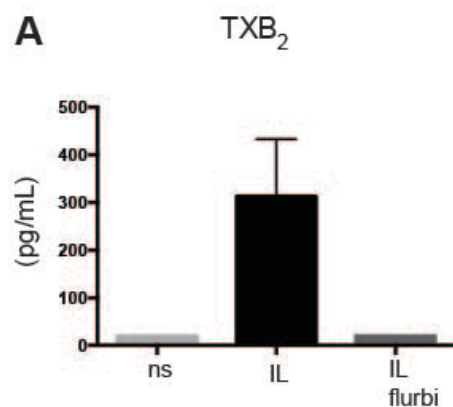


Figure 23. Stimulated ILC2 produce TXB_2 .

Sorted and expanded tonsil ILC2 were stimulated for 24 hours. TXB_2 was detected in ILC2 supernatants by using LC-MS/MS. Graph shows mean concentration +SEM ($n = 4$). LC-MS/MS analyses were performed by Alexander Fauland, Craig E. Wheelock, Nerea Ferreirós and Dominique Thomas

Since mast cells are known to be one of the major sources of PGD_2 ¹⁹¹, lipid mediators released by cord blood derived mast cells were determined to assess the relative contribution of ILC2 to generation of PGD_2 . Upon IgE-receptor activation, mast cells secreted high levels of PGD_2 (Figure 24). Compared to mast cells, ILC2 released less PGD_2 indicating importance of autocrine role of PGD_2 in ILC2 self-stimulation rather than contributing to the increase of the systemic level of PGD_2 .

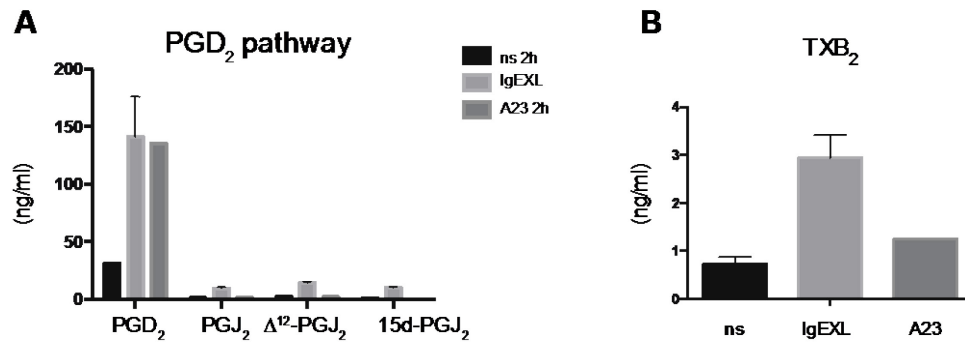


Figure 24. Metabolites of the PGD₂ pathway and TXB₂ pathway are released by activated human mast cells.

Cord blood-derived mast cells were stimulated with IgEXL for 6 hours or A23 for 2 hours. Mediators of the PGD₂ pathways and TXB₂ were detected in mast cell supernatants by using LC-MS/MS. Graphs show mean concentrations +SEM (n=1-3). Mast cell culture and treatments were done by Maria Ekoff; LC-MS/MS analysis was performed by Alexander Fauland and Craig E. Wheelock.

Furthermore, after PGD₂ and its metabolites were detected in ILC2 supernatants, next aim was to analyze gene expression of the molecules involved in PGD₂ synthesis and metabolism: COX-2, HPGDS and 15-hydroxyprostaglandin dehydrogenase (HPGD). Non-stimulated ILC2 did not express *COX-1* and *COX-2* mRNA, which gives an explanation for the absence of prostanoid production from resting ILC2. Activating cytokines highly upregulated *COX-2* expression in ILC2 after 6 hours (Figure 25A, B) and sustain increased expression levels even after 24 hours (Figure 25E). *COX-1* expression was not induced upon cytokine stimulation. Surprisingly, using of the COX-1/2 inhibitor flurbiprofen reduced upregulation of *COX-2* mRNA, indicating a possible regulatory role of COX-2 inhibition in the transcription of COX-2. (Figure 25B, E). Under basal conditions, ILC2 expressed *HPGDS* transcripts (Figure 25A, D), while lacking expression of *LPGDS* (data not shown). Additionally, here I found that non-stimulated expanded ILC2 express *HPGD* (Figure 25A, D). These findings are in agreement with previous observations about ILC2 expressing HPGDS and HPGD, detected by single cell RNA sequencing of freshly isolated human ILC2²⁵. HPGD is the enzyme responsible for conversion of PGD₂ to 13,14 dihydro-15-keto PGD₂¹⁹², and small amounts of this metabolite (Figure 25B), a natural selective agonist of the CRTH2 receptor¹⁶⁶, were detected in the supernatants of stimulated ILC2. Cytokine stimulation slightly downregulated *HPGDS* expression (Figure

25C). However, expression of HPGD was not affected by treatment with cytokines (Figure 25C, F).

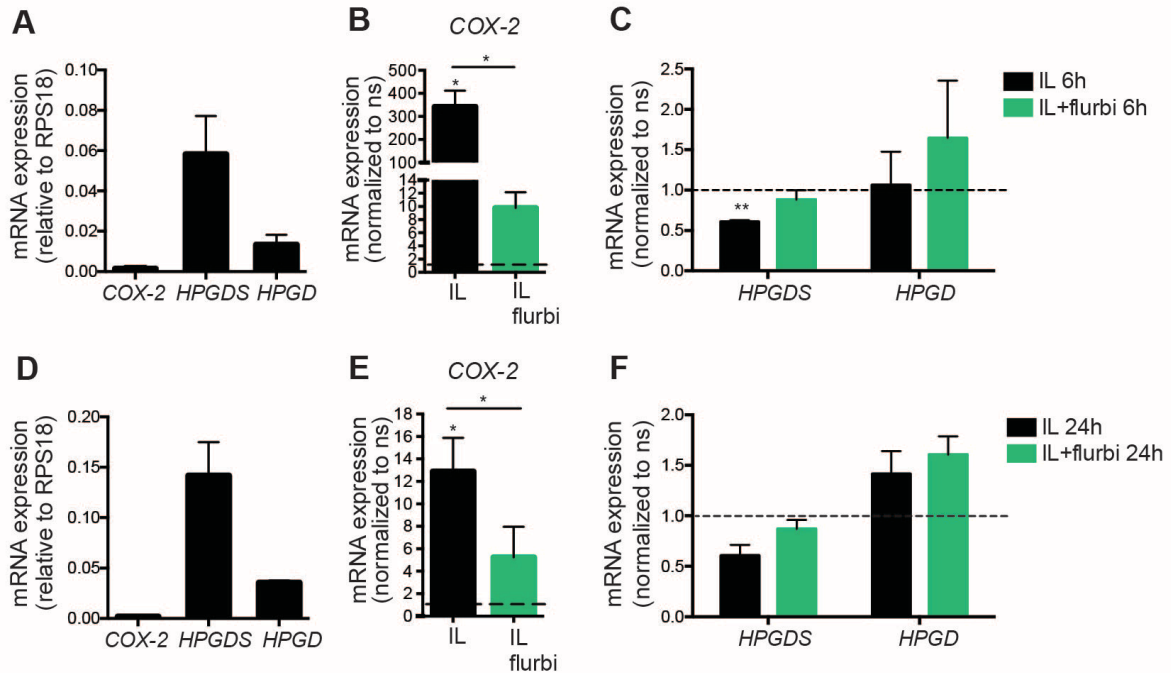


Figure 25. ILC2 express enzymes necessary for PGD_2 production and metabolism.

Sorted and expanded tonsil ILC2 were stimulated as indicated for 6 hours (A-C) and 24 hours (D-F), with or without flurbiprofen (1 μ M, 40 min pre-treatment). Basal mRNA expression of COX2, HPGDS, and HPGD after 6 hours culture (A) and 24 hours (D) were analyzed by qPCR. Data are shown as mean +SEM (n=4). Changes of gene expression were normalized to non-stimulated cells after 6 hours stimulation (B, C) (*p<0.05 between IL treatment and ns and between IL+flurbi and IL for COX-2 and **p<0.01 between IL treatment and ns for HPGDS, n=3-4) and after 24 hours (E, F) (*p<0.05 between IL treatment and ns and between IL+flurbi and IL, n=4) are shown as mean +SEM.

Taken together, ILC2 express the enzymes required for PGD_2 synthesis: HPGDS and COX-2, the latter being upregulated after treatment with stimulatory cytokines. Accordingly, stimulated ILC2 activate the PGD_2 pathway, as PGD_2 and its metabolites were found. Moreover, ILC2 expressed HPGD, and its product, 13,14 dehydro-15-keto PGD_2 , was detected in ILC2 supernatants.

3.2.3. Endogenously produced PGD₂ is crucial for the activation of ILC2 upon cytokine stimulation

Since PGD₂ and its metabolites were detected in supernatants of stimulated ILC2, further objective was to confirm if the observed suppressive effects of flurbiprofen were due to the lack of PGD₂ production. Therefore, ILC2 were pre-treated with a selective CRTH2 antagonist, CAY10471, 20 minutes before the addition of stimulatory cytokines. Obtained data revealed that, similarly to the treatment with flurbiprofen, the CRTH2 antagonist completely inhibited IL-5 and IL-13 release of cytokine-stimulated ILC2 (Figure 26A, B). Accordingly, CRTH2 receptor blockade prevented the synthesis of IL-5 and IL-13 by stimulated ILC2 (Figure 26C-E), as determined by intracellular cytokine staining. These data clearly show that endogenous PGD₂ produced by ILC2 upon cytokine stimulation is necessary for proper activation of ILC2.

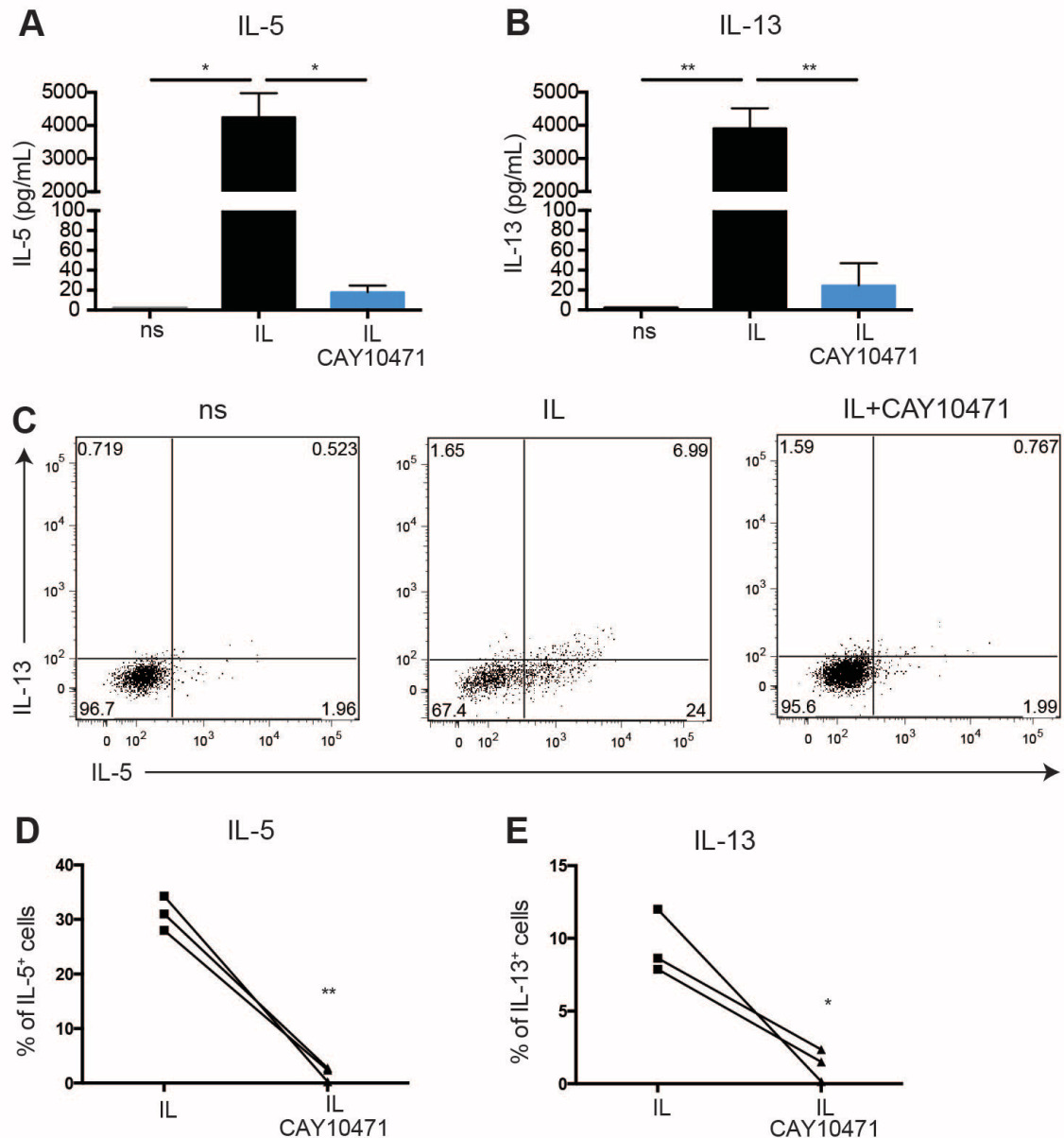


Figure 26. Endogenously produced PGD_2 is essential for IL-5 and IL-13 production by ILC2.

A and B. Sorted and expanded ILC2 were treated as indicated in the presence or absence of CRTH2 selective antagonist, CAY10471 (1 μ M, 20 min pre-treatment) for 24h. Concentrations of IL-5 (**A**) and IL-13 (**B**) in ILC2 supernatants were detected by ELISA, graphs show mean +SEM (* p <0.05, ** p <0.01, n =4). **C.** Intracellular expression of IL-5 and IL-13 in ILC2 was assessed after 24h by flow cytometry; graphs show individual percentages of IL-5- (**D**) and IL-13- (**E**) producing ILC2 (* p <0.05, ** p <0.0, n =3).

It was recently shown that exogenous PGD_2 directly stimulates ILC2 acting via CRTH2 receptor and this leads to downregulation of CRTH2 mRNA and protein expression ⁷⁰.

Thus, we further investigated the role of endogenously produced prostaglandins in regulating the expression of CRTH2 in ILC2. We observed that *CRTH2* mRNA expression in ILC2 was significantly decreased by stimulatory cytokines, which was partially reversed upon inhibition of COX1/2 activity with flurbiprofen (Figure 27A, B).

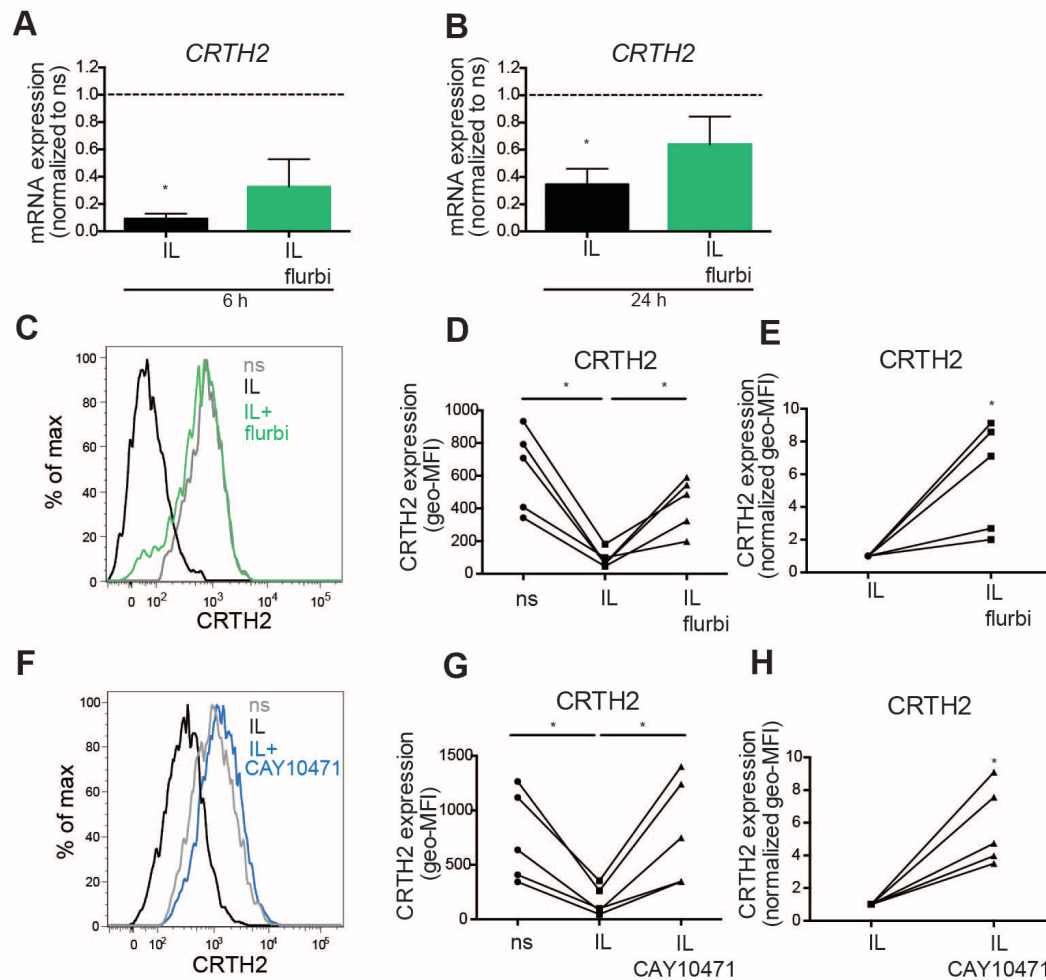


Figure 27. The cytokine-induced downregulation of *CRTH2* is prevented by inhibition of endogenous prostaglandin production or by blocking the *CRTH2* receptor.

Tonsil ILC2 were stimulated as indicated for 6 or 24 hours, with pre-treatment of flurbiprofen (1 μ M, 40 min) (A-E) or CAY10471 (1 μ M, 20 min) (F-H). Changes of mRNA *CRTH2* expression normalized to non-stimulated cells after 6 hour stimulation (A) (* p <0.05, n =3) and after 24 hours (B) (* p <0.05, n =4) are shown as mean +SEM. C and F. *CRTH2* protein expression was analyzed by flow cytometry. Graphs show individual geometric mean fluorescence intensity (D, G) and individual geometric mean fluorescence intensity normalized to stimulated cells of *CRTH2* expression after 24 hours (E, H) (* p <0.05, n =5). Part of these data were provided by Avinash Ravindran.

Furthermore, cytokine stimulation also reduced CRTH2 protein expression on ILC2 cell surface (Figure 27). Pre-treatment of ILC2 with either flurbiprofen (Figure 27C-E) or a CRTH2 antagonist (Figure 27F-H) reversed protein expression of CRTH2 on the cell surface to the level as in non-stimulated ILC2. These findings indicate that, most likely, endogenously released PGD₂ is responsible for cytokine-induced reduction of CRTH2 expression and additionally confirm the involvement of endogenous PGD₂ in activation of stimulated ILC2.

Our RT-qPCR data show that while non-stimulated ILC2 express high levels of *CRTH2* transcripts, only low expression of DP1 receptor is detected (Figure 28A, B). DP1 expression was not modulated by cytokine stimulation for 6 hours, and was slightly downregulated after 24 hours of stimulation (Figure 28C, D).

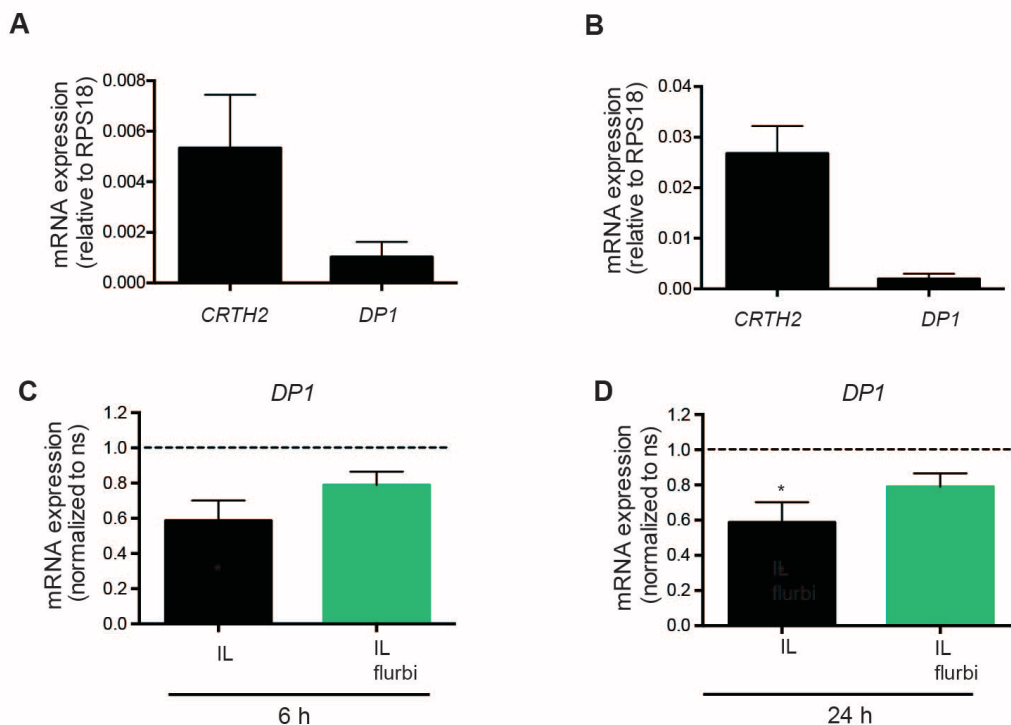


Figure 28. ILC2 express lower mRNA levels of DP1 than CRTH2 receptor.

Sorted and expanded tonsil ILC2 were stimulated as indicated for 6 hours (A, C) and 24 hours (B, D), with or without flurbiprofen (1 μ M, 40 min pre-treatment). Basal mRNA expression of CRTH2 and DP1 after 6 hours culture (A) and 24 hours (B) were analyzed by qPCR. Data are shown as mean +SEM (n=2-4). Changes of DP1 mRNA expression normalized to non-stimulated cells after 6 hours of stimulation (C) (n=2) and after 24 hours (D) (* p <0.05, n=4) are shown as mean +SEM.

3.2.4. The upregulation of CD25 cell surface expression is dependent on the endogenously produced PGD₂ in cytokine-stimulated ILC2

As IL-2 is an important co-stimulator of ILC2⁴⁴ and CD25 (IL-2 R α) is one of the defining markers of these cells, the aim was to determine how CD25 expression is affected by the endogenous PGD₂ in ILC2. As shown already, stimulatory cytokines upregulated CD25 expression in ILC2 (Figure 29). Co-incubation of ILC2 with either flurbiprofen (Figure 29A-C) or a CRTH2 antagonist (Figure 29D-F) largely prevented CD25 upregulation. Taken together, endogenously produced PGD₂, acting through CRTH2 receptors, plays a key role in the CD25 upregulation upon cytokine-stimulation of ILC2.

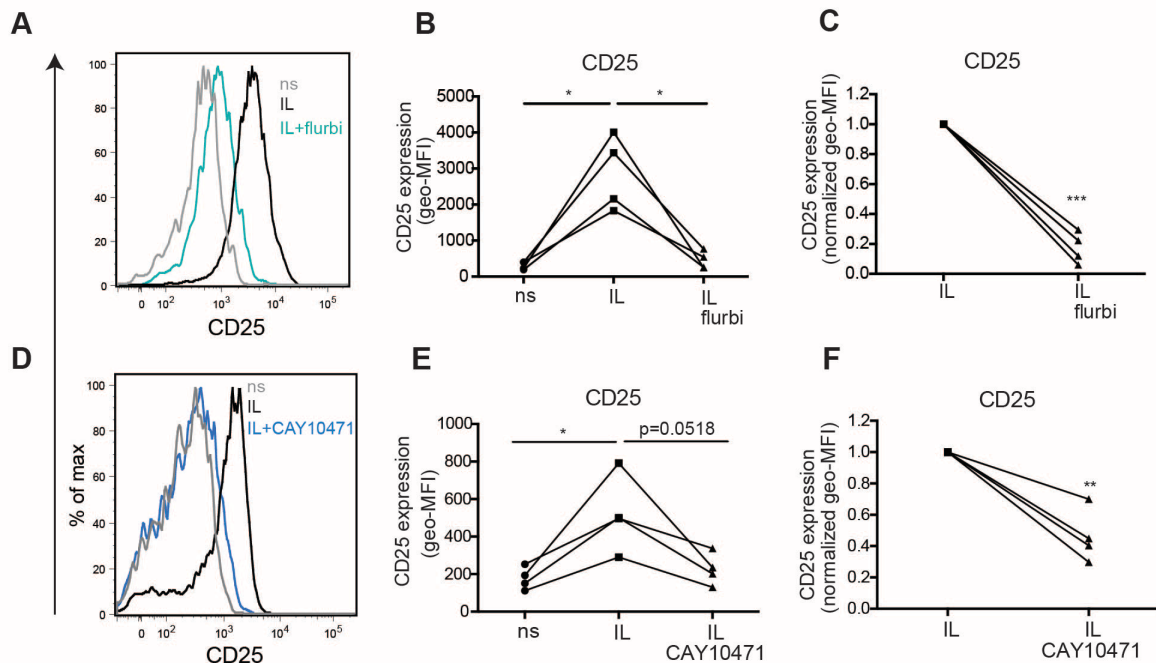


Figure 29. Endogenously produced PGD₂ is essential for CD25 upregulation in stimulated ILC2.

Tonsil ILC2 were stimulated as indicated for 24 hours, subsequent to pre-treatment with flurbiprofen (1 μ M, 40 min) (A-C) or CAY10471 (1 μ M, 20 min) (D-F). 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, E) (* p <0.05, n =4) and geometric mean fluorescence intensity normalized to stimulated cells of CD25 expression after 24 hours (C, F) (** p <0.01, *** p <0.001, n =4).

3.2.5. Endogenously produced PGD₂ contributes to stimulation of fresh blood and tonsil ILC2

My next objective was to confirm findings observed in expanded tonsil ILC2 by using freshly sorted ILC2. Beside investigating fresh tonsil ILC2, peripheral blood ILC2 were also studied (Figure 30). Freshly isolated ILC2 from tonsils (Figure 30A, B) and blood (Figure 30C, D) generated IL-5 and IL-13 after 5 days of stimulation. As in expanded ILC2, treatment with flurbiprofen (Figure 30A, B) or the CRTH2 antagonist (Figure 30C, D) significantly reduced IL-5 and IL-13 release by freshly isolated ILC2.

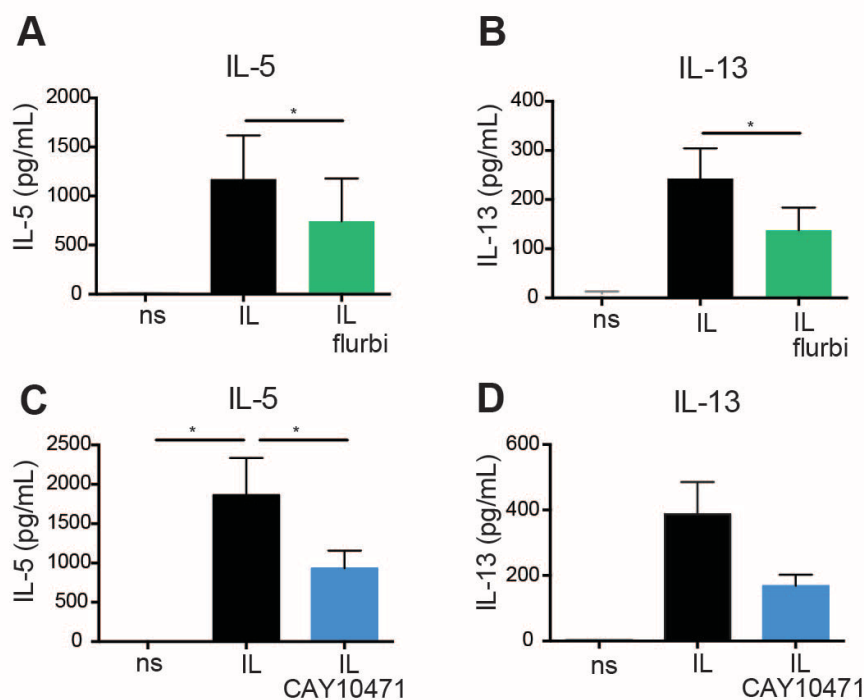


Figure 30. Endogenously produced PGD₂ contributes to IL-5 and IL-13 secretion from freshly sorted tonsil and blood ILC2 upon cytokine stimulation.

Freshly sorted human tonsil (A, B) and blood (C-D) ILC2 were incubated as indicated for 5 days, in the presence or absence of flurbiprofen (1 μ M) (A, B) or CAY10471 (1 μ M) (C, D). Concentrations of released IL-5 from tonsil ILC2 (A) and blood ILC2 (C) and IL-13 from tonsil ILC2 (B) and blood ILC2 (D) were measured by ELISA; graphs show means +SEM (* p <0.05; n =4 for blood ILC2 and n =3 for tonsil ILC2).

Changes of CD25 expression in freshly isolated ILC2 were also determined. The CRTH2 antagonist prevented cytokine-induced upregulation of CD25 expression in freshly isolated blood ILC2 (Figure 31A, B). Similarly to the findings in expanded ILC2, the CRTH2

antagonist reversed cytokine-induced downregulation of CRTH2 protein expression in freshly isolated ILC2 (Figure 31C-E). Furthermore, microscopic images of freshly isolated blood ILC2 were captured after 5 days of incubation. These images revealed that blockage of the CRTH2 receptor caused less pronounced cell growth comparing to the treatment with only stimulatory cytokines (Figure 31F). Together, these data confirm that endogenously produced PGD₂ is also important in complete activation of freshly isolated blood and tonsil ILC2.

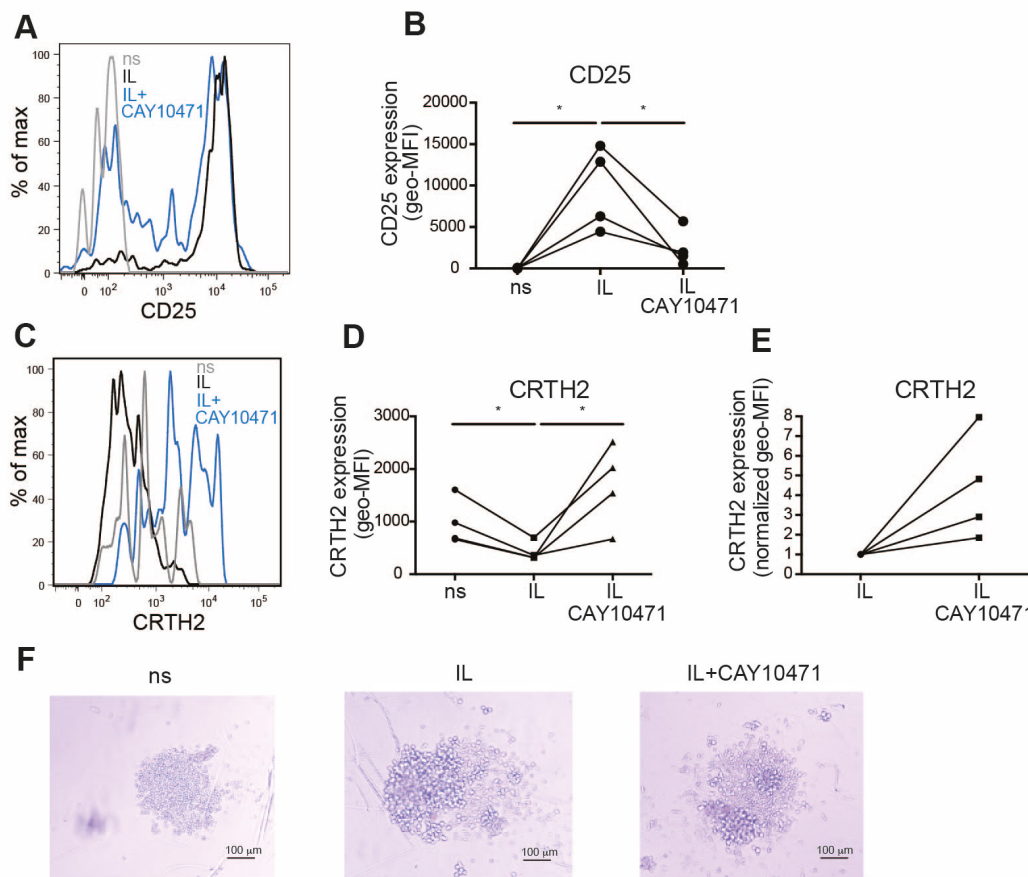


Figure 31. Endogenous PGD₂ contributes to the activation of freshly sorted blood ILC2.

A and C. Expression of CD25 and CRTH2 in freshly sorted blood ILC2 was analyzed by flow cytometry; representative histograms are shown. Graphs show individual geometric mean fluorescence intensity of CD25 expression in blood ILC2 after 5 days of culture (**B**) (*p<0.05, n=4) and of CRTH2 expression (**D**) (*p<0.05, n=4) and individual geometric mean fluorescence intensity of CRTH2 expression normalized to stimulated cells (**E**) (n=4). **F.** Images of freshly sorted blood ILC2 after 5 days of treatments; 10 x magnification, scale bar = 100 μm.

4. Discussion

Asthma is a complex disorder characterized by bronchial obstruction and airway inflammation and affects 5-16% of human population ^{76, 193}. Airway epithelium, as a barrier between the environment and our organism, plays a critical role in the mechanisms leading to asthma ¹⁹⁴. Although there have lately been advantages in understanding causes and pathogenesis of asthma, there are still no efficient strategies for the prevention of this disease ^{76, 195}. ILC2 are a group of recently discovered leukocytes which translate signals from damaged epithelial cells to enhanced immunological reactions. ILC2 are essential in initiation, maintenance and amplification of type 2 immune responses ^{6, 93}. Different studies with mouse models clarified important roles of ILC2 activation in asthma and allergic immune responses ^{14, 72}, even in the absence of T or B cells. In agreement with these observations, involvement of ILC2 in asthma and allergic inflammation was confirmed in humans, as increased numbers of ILC2 were detected in samples of patients with allergic asthma ^{44, 78}, lung fibrosis ⁹⁰, chronic rhinosinusitis ⁹⁶ and atopic dermatitis ¹⁰⁰. Beside allergic asthma, ILC2 play significant roles in non-allergic asthma and steroid-resistant severe airway inflammation ⁸⁶. ILC2 require several signals for optimal activation. STAT- activators TSLP and IL-2, as well as NF-κB stimulators IL-33 and IL-25 synergistically stimulate human ILC2 and lead to type 2 cytokine production. Of note, ILC2 can also be stimulated by lipid mediators such as PGD₂, LTD₄ and LTE₄ ^{59, 70, 196}. Therefore, the aim of my thesis was to further investigate the roles of prostanoids in ILC2 function. Despite growing knowledge on various stimulators of ILC2, very little is known about factors which may inhibit ILC2 function. Thorough understanding of ILC2 regulation is required for development of novel therapeutic approaches.

4.1. PGE₂ suppresses human group 2 innate lymphoid cell function

In the first part of the thesis I showed that PGE₂ negatively regulates the function of human tonsil and blood ILC2 by suppressing IL-5 and IL-13 expression and release, as well as blocking the proliferation of ILC2. Furthermore, we revealed the mechanistic events of the action of PGE₂ on ILC2, which we found to involve prevention of the upregulation of CD25 and GATA3 expression in stimulated ILC2. Moreover, it was demonstrated that

PGE₂ exerts suppressive effects on ILC2 function via binding to both EP2 and EP4 receptors.

PGE₂ is one the most abundantly released lipid mediator in the human lung, where it is produced by alveolar epithelial and endothelial cells, fibroblasts, as well as different immune cells such as macrophages and dendritic cells¹⁹⁷. Protective roles of PGE₂ in respiratory tract have been demonstrated¹³⁹.

Importantly, clinical studies reported prevention of bronchoconstriction in healthy and asthmatic subjects by PGE₂ inhalation^{154-156, 198}. Furthermore, PGE₂ is known to reduce human Th2 cell cytokine production,¹⁴⁷ eosinophil trafficking^{149, 199} and activation of mast cells¹⁴⁸. In several mouse models of asthma and allergy, PGE₂ has been shown to have beneficial effects by inhibiting Th2 cells and eosinophil function, as well as by preventing airway inflammation^{149, 151, 153}.

As PGE₂ is present in the respiratory system, endogenous PGE₂ is likely to prevent or control dysregulated inflammatory events. There are opposing reports considering PGE₂ production during inflammation caused by different stimuli. Various inflammatory signals such as LPS, IL-1 β , TNF- α , or trypsin cause COX-2 upregulation and thereby trigger production of PGE₂^{138, 150}. In addition, blockade of COX-1/2 activity worsened the symptoms in OVA mouse models of asthma²⁰⁰ indicating protective role of PGE₂. Interestingly, cytokine released during type 2 inflammation, IL-13, showed differential influence on PGE₂ biosynthetic pathway: one report demonstrated that IL-13 inhibited PGE₂ production in human bronchial primary epithelial cells by suppressing COX-2 and PGE₂ synthesis and upregulating HPD²⁰¹. Furthermore, lower levels of PGE₂ were found in the sputum of asthmatics compared to patients with eosinophilic bronchitis²⁰². These findings may provide an explanation for the lack of protective effects of PGE₂ in some of the asthmatic patients²⁰². In contrast, other studies showed that IL-13 increased PGE₂ production from dendritic cells and macrophages by stimulating the liberation of arachidonic acid^{203, 204}. In addition, airway smooth muscle cells from asthmatics produced more PGE₂ upon stimulation with trypsin compared to non-asthmatics²⁰⁵. Elevated levels of PGE₂ have been detected in another study with asthmatic patients¹⁵⁹. It seems that PGE₂ production depends on the stimulus and the cellular context. Importantly, in our work, PGE₂ was not detected in the supernatants of stimulated ILC2, showing that there was not impact of endogenous PGE₂ in these *in vitro* experiments.

ILC2 are crucial players in allergic responses, thus data of this thesis extend previously described observations, demonstrating that PGE₂ directly inhibits ILC2 activation. These findings contribute to a better understanding of immunoregulatory processes in type 2 inflammation.

PGE₂ exerts its effect binding to four different receptors: EP1-EP4. Human tonsil ILC2 express only EP2 and EP4 receptors among the PGE₂ receptors, as detected by single-cell RNA sequencing in freshly isolated ILC2 and by RT-qPCR in expanded ILC2. Selective antagonism of either EP2 or EP4 receptors caused only partial reduction of the suppressive effects of PGE₂. Of interest, simultaneous blockade of EP2 and EP4 receptors was necessary to completely reverse PGE₂-caused inhibition of ILC2 activation. To strengthen these observations, ILC2 were also treated with EP2 and EP4 agonists prior to the cytokine stimulation. In accordance with the data obtained by using EP antagonists, combined EP2 and EP4 receptor activation by selective agonists was required to completely suppress ILC2 cytokine release, comparable to the effects of PGE₂. EP4 receptor activation seemed to have a stronger suppressive effect on ILC2 stimulation as compared to EP2. A likely reason for this observation could be higher expression level of EP4 than EP2 receptor in both fresh and expanded ILC2. Interestingly, PGE₂ was also shown to affect ILC3 potentiating IL-22 production, and reducing LPS-induced systemic inflammation as well as contributing to intestinal barrier maintenance through EP4 receptor activation ²⁰⁶. However, in our work, only dual engagement of EP2 and EP4 receptors could provide full inhibition of ILC2 function. In line with these findings, anti-inflammatory effects of EP2 and EP4 receptors were previously described ^{151, 152, 207, 208}. PGE₂ inhibited antigen-specific responses of human peripheral blood Th2 cells through activation of both EP2 and EP4 receptors ¹⁴⁷. Similarly, EP2 and EP4 receptors mediated suppression of human eosinophils and alveolar macrophages, regarding their typical functions such as migration, degranulation, ROS production and cytokine release ^{149, 209, 210}. Similar to our observation with ILC2, it was found that both EP2 and EP4 receptors are required for inhibition of mast cell activation ²¹¹. In contrast, bronchoprotection caused by PGE₂-induced mast cell inhibition ²¹² and reduction of cytokine production of human nasal polyp cells ²⁰⁸ were exerted by the EP2 receptor. In addition, the PGE₂-EP2 signaling was impaired in patients with aspirin-exacerbated respiratory disease contributing to disease pathogenesis ^{213, 214}. Studies in mouse models confirmed beneficial effects of EP2 and EP4 receptors. EP2 *knockout* mice exhibited higher levels of type 2 cytokines in BAL in a model of OVA-

induced allergic asthma ¹⁵¹. A study with EP4 *knockout* mice showed increased numbers of eosinophils and neutrophils in BAL after OVA and LPS treatment, respectively ¹⁵³. Activation of EP3 receptor caused cough, which supports the use of specific EP2 and EP4 agonists in the treatment of asthma and allergic diseases, instead of PGE₂. Interestingly, a study performed with isolated bronchi demonstrated that although EP2 receptor was responsible for bronchorelaxation in several animal models, only EP4 receptor mediated PGE₂-induced relaxation of human bronchi ²¹⁵. Bronchodilation induced by EP2 and EP4 receptors activation would provide an additional protective effect of these agonists, along with inhibition of several other immune cells involved in type 2 immune responses. Beside described beneficial effects, activation of EP2 and EP4 receptor can cause vasodilation and consequently decrease of blood pressure ²¹⁶⁻²¹⁸. Therefore, local airway administration of EP2 and/or EP4 agonists would be preferable to minimize systemic side effects.

Both EP2 and EP4 receptors are Gs protein-coupled receptors and trigger cAMP/PKA pathways. However, some of the effects of EP4 receptor activation are not mediated through cAMP signaling. EP4 activation can stimulate the EP4-associated protein (EPRAP), which stabilizes the NF-κB inhibitor leading to reduction of human macrophage activity ²¹⁹. Furthermore, EP4 engagement suppressed human eosinophil migration by triggering PI 3-kinase and PKC pathways ²¹⁰. Additionally, the phosphoinositide-dependent protein kinase 1 (PDK1) mediated EP4 receptor-induced suppression of eosinophil function ²²⁰. These mechanisms may provide an explanation for the requirement of both EP2 and EP4 receptors in inhibiting ILC2 function.

Additionally, we show that combination of IL-33, IL-25 and TSLP in addition to IL-2 was necessary for complete ILC2 stimulation. This finding is supported by previous studies ^{13, 44}. Importantly, PGE₂ attenuated activating effects of each cytokine separately, as well as their effects when they were added simultaneously to stimulate ILC2. In further experiments, I addressed the underlying mechanisms of the PGE₂-induced suppression of ILC2 function upon activation with all stimulatory cytokines at the same time.

Importantly, we confirmed the suppressive effects of PGE₂ also in ILC2 that were freshly isolated both from tonsils and blood. ILC2 are tissue resident and capable of fast immune response. However, migration of ILC2 from peripheral blood to the tissue is also important for accumulation of ILC2 and their enhanced activation ^{79, 100}. Studies with human samples showed increased responses of peripheral blood ILC2 from asthmatic patients indicating

activation of these cells⁴⁴. Thus, it is important that the suppressive effects of PGE₂ are additionally confirmed in blood ILC2.

Furthermore, I investigated if PGE₂ affects signaling pathways involved in ILC2 activation. GATA3 is constitutively expressed in ILC2, as well as in Th2 cells, as these cell types share transcriptional program^{15, 221}. GATA3 is important for development of ILC2, maintenance and activation. Expression of GATA3 is regulated by NF-κB, STAT5 and STAT6 signaling^{13, 222}. Moreover, upregulation of GATA3 was shown to be essential for TSLP and IL-33 induced production of IL-5 and IL-13 in human ILC2¹³. Here we have shown that PGE₂ prevents GATA3 upregulation induced by activating cytokines. To the best of our knowledge, this is the first instance showing that PGE₂ reduces GATA3 expression in immune cells. Since NF-κB is involved in GATA3 upregulation²²² and PGE₂ has been shown to inhibit NF-κB activation, this signaling could provide the explanation for the observed effects of PGE₂. Additionally, of particular interest is the finding that PGE₂ blocks cytokine-induced ILC2 proliferation. As ILC2 can be tissue resident, besides their recruitment from the blood, their proliferation is important for the amplification of local immune response, as it was shown *in vitro* and *in vivo*²²³ in allergic diseases. Proliferation of ILC2 can be triggered by IL-2, IL-33, TSLP, IL-25 or IL-9,⁵³ and it was shown that combination of several stimuli had more pronounced effects than individual cytokines⁵³, likely via synergism of NF-κB and STAT5 signaling. Moreover, IL-2 is an important co-activator of human ILC2, potentiating stimulation caused by other cytokines^{44, 59}. A recent study showed that IL-2 is necessary for ILC2 activation in type 2 lung inflammation in mice⁵⁸. IL-2 signaling upregulates CD25 expression (*i.e.* the α chain of the IL-2 receptor) via positive feedback, hence CD25 expression level can serve as an indicator of ILC2 activation. PGE₂ reduced CD25 upregulation of stimulated ILC2, strongly suggesting its influence on ILC2 activation and in the longer run also on ILC2 proliferation. These findings are in accordance with previous studies which showed that CD25 expression and IL-2 signaling in human primary peripheral blood T cells²²⁴ and bovine CD4⁺ T cells²²⁵ are impaired by PGE₂ treatment. It was also demonstrated that PGE₂ upregulates expression of genes which block cell cycle and proliferation of human primary CD4⁺ T cells^{147, 226} and that lung resident mesenchymal stem cells inhibit proliferation of T cells via soluble mediator such as PGE₂²²⁷.

Taken together, these data demonstrate a novel negative regulation of human ILC2 by PGE₂ through simultaneous EP2 and EP4 receptor activation.

4.2. Cytokine-induced endogenous PGD₂ production is essential for human ILC2 activation

The key role of lipid mediators in ILC2 function is currently emerging. Exogenous PGD₂ is described to be important in activation of ILC2 through the CRTH2 receptor^{59, 70, 228}. However, it has remained still unknown whether ILC2 are able to produce prostaglandins.

In the second part of my thesis I confirmed that ILC2, constitutively express *HPGDS*, and went on to demonstrate that these cells upregulate *COX-2* upon cytokine stimulation and consequently produce PGD₂, which in turn leads to CRTH2 receptor activation. Thus these experiments unraveled that the endogenous PGD₂-CRTH2 axis is essential for cytokine-induced ILC2 activation.

To test the relevance of endogenous prostaglandins in ILC2 function, cytokine-treated ILC2 were first incubated with the COX-1/2 inhibitor flurbiprofen. Surprisingly, general blockade of prostanoid production in ILC2 had a great impact on the IL-5 and IL-13 production upon cytokine stimulation. Flurbiprofen almost completely inhibited type 2 cytokine release of ILC2 implicating a crucial role of endogenous prostanoids in ILC2 activation. Upon interrogating which prostaglandins are produced by ILC2, we detected PGD₂ and its metabolites PGJ₂, Δ^{12} -PGJ₂, 15d-PGJ₂ and DK-PGD₂. Notably, flurbiprofen completely inhibited the release of these lipid mediators.

In this study we confirmed that *in vitro*-expanded tonsil ILC2 express *HPGDS*, as it was previously shown in freshly isolated ILC2²⁵. However, non-stimulated ILC2 do not produce PGD₂ which is most probably due to the lack of COX-1 and COX-2 expression. In contrast, mast cells express COX-1 and release PGD₂ even under resting conditions, while COX-2 can be induced by different stimuli, for instance upon immunological activation with IgE crosslinking¹⁶³. Here we demonstrated that stimulation of ILC2 with activating cytokines: IL-33, TSLP, IL-25 plus IL-2 induces strong upregulation of *COX-2* mRNA expression. COX-2 subsequently leads to formation of PGH₂, the substrate for the already

present HPGDS enzyme to produce PGD₂. The above mentioned combination of ILC2 activating cytokines was applied, as it enables full stimulation of human ILC2^{13, 41}. It was shown that IL-33, member of the IL-1 cytokine family, induces PGD₂ synthesis in mast cells²²⁹ and a recent study demonstrated that TSLP synergistically with IL-33 contributes to PGD₂ production by human mast cells²³⁰. It has been shown that blocking IL-33 decreased COX-2 expression in renal parenchyma of mice, indicating a role of IL-33 in COX-2 upregulation. The amount of PGD₂ released by ILC2 is lower than what we observed for IgE-stimulated mast cells, suggesting the importance of ILC2-derived PGD₂ in internal ILC2 signaling. Similar to ILC2, Th2 cells, dendritic cells and eosinophils can also upregulate COX-2 upon various stimuli and hence, are capable of PGD₂ production which may be important for autocrine or paracrine cell activation²³¹⁻²³³. Interestingly, the COX-1/2 inhibitor flurbiprofen also decreased the upregulation of COX-2 at the mRNA level in stimulated ILC2. Flurbiprofen is known to block COX-1/2 activity, but its effect on COX-2 expression remains unclear. Some studies showed that flurbiprofen and other non-steroidal anti-inflammatory drugs (NSAIDs) do not influence expression of COX-2^{234, 235}. In contrast, another report demonstrated that flurbiprofen in a concentration-dependent manner reduces zymosan-induced COX-2 upregulation in inflamed rat paw, and inhibits LPS-induced NF-κB activation in RAW 264.7 macrophages²³⁶. Furthermore, flurbiprofen decreased IL-1β-induced COX-2 expression²³⁷. It was also found that NSAIDs induce COX-2 expression under basal conditions, whereas NSAIDs inhibit upregulation of COX-2 expression upon stimulation with LPS or TNF-α²³⁸. Additionally, prostaglandins can regulate COX-2 expression in a positive feedback²³⁹. With respect to PGE₂, there were different findings: while in a positive feedback loop it regulated myeloid-derived suppressor cells (MDSCs) and fibroblasts^{239, 240} by increasing COX-2 expression, endogenous PGE₂ inhibited COX-2 expression in porcine aortic smooth muscle cells²⁴¹ and spinal cord culture²⁴². In our in vitro system, we used only ILC2, which did not release PGE₂. It is possible that endogenous PGD₂, and its metabolites, acting via a positive feedback loop further contribute to COX-2 upregulation in ILC2. As a consequence of flurbiprofen-induced inhibition of PGD₂ production, upregulated COX-2 expression is largely decreased.

Noteworthy, we demonstrated here that reduction of endogenous PGD₂ is responsible for the observed effect of flurbiprofen, as a selective CRTH2 antagonist mimicked the effect

of flurbiprofen and almost completely inhibited the cytokine-induced ILC2 activation. This finding reveals a novel interesting mechanism of ILC2 activation, where the response of ILC2 to stimulating cytokines depends on PGD₂ production and its action in an autocrine manner. Importantly, PGD₂ treatment alone exerted less prominent stimulating effects than activating cytokines⁴³. These findings indicate that cytokine signaling pathways act synergistically with endogenously produced PGD₂ to fully activate ILC2.

Interestingly, we additionally observed that CRTH2 mRNA and protein expression was reduced upon cytokine stimulation of ILC2. It was previously shown that exogenous PGD₂ downregulates CRTH2 expression in ILC2 both at mRNA and protein levels⁷⁰. Furthermore, PGD₂ caused internalization and desensitization of CRTH2 receptor in HEK293 cells and in human eosinophils^{166, 243}. Restored cell surface expression of CRTH2 on ILC2 by flurbiprofen and CRTH2 antagonist treatment strengthened the hypothesis that endogenous PGD₂ is important for ILC2 stimulation. Additionally, these findings indicate that, in contrast to COX-2 upregulation, ILC2-derived prostaglandins provide a self-limiting mechanism by reducing CRTH2 expression in activated ILC2. Furthermore, as flurbiprofen did not completely reverse reduced CRTH2 mRNA expression upon cytokine stimulation, it is likely that ILC2-stimulatory cytokines can also directly affect *CRTH2* gene expression in a PGD₂-independent manner.

Further analysing the mechanisms underlying the actions of endogenous prostaglandins, we also detected *HPGD* expression in ILC2, and the product of this enzyme, 13, 14-dihydro-15-keto PGD₂ (DK-PGD₂) was found in supernatants of simulated ILC2. DK-PGD₂ is a natural selective CRTH2 agonist which has been reported to activate human Th2 cells and eosinophils^{166, 171}. Additionally, other PGD₂ metabolites present in ILC2 supernatants such as PGJ₂, Δ^{12} -PGJ₂ and 15d-PGJ₂ can also mediate their pro-inflammatory effects through the CRTH2 receptor^{165, 172}. These metabolites, along with PGD₂ may be responsible for CRTH2 receptor-dependent ILC2 activation. As PGD₂ can rapidly be degraded, its metabolites maintain physiological effects of CRTH2 receptor stimulation. 15d-PGJ₂, besides mediating its effects through the CRTH2 receptor, also binds to the peroxisome proliferator-activated receptor- γ (PPAR- γ). Although anti-inflammatory effects of PPAR- γ activation have been reported²⁴⁴, other studies demonstrated that it triggered human eosinophil chemotaxis²⁴⁵ and promoted type 2

responses in mouse Th2 cells and ILC2²⁴⁶. Therefore, the 15d-PGJ₂-PPAR- γ axis might also be partly involved in the ILC2 activation.

I also found that ILC2 express low levels of DP1 receptor. Since CRTH2 antagonist almost completely inhibited ILC2 activation, it is most likely that PGD₂ mediates its effects only through CRTH2 receptor. In line with this conclusion, it was previously shown that DP1 receptor activation did not have a significant effect on ILC2 function⁴³.

In addition to metabolites of the PGD₂ pathway, low levels of TXB₂ were detected. Although there is no evidence yet for TP receptor expression on ILC2, thromboxane metabolites still could play some role in ILC2 activation, as a major thromboxane metabolite, 11-dehydro-TXB₂ has been shown to be a CRTH2 agonist²⁴⁷.

As in other immune cells¹⁴¹, here I demonstrated different roles of prostaglandins in ILC2 function. PGD₂, acting via CRTH2 receptor has pro-inflammatory effects, thereby opposing EP2- and EP4- mediated PGE₂ suppression of inflammation. As inhibition of COX-2 inhibits synthesis of all prostanoids, and PGE₂ has beneficial effects, selective CRTH2 antagonism would be a better approach in blocking effects of PGD₂. There are several phase II clinical trials showing that some of the CRTH2 antagonists provide moderate improvement of lung function in asthmatic patients, particularly those with eosinophilic inflammation in their lungs. Our findings highlight the importance of CRTH2 activation for stimulation of ILC2 and additionally encourage further research regarding potential beneficial effects of CRTH2 antagonists.

Importantly, I also confirmed the role of endogenous PGD₂ in freshly isolated ILC2 from blood and tonsil tissue. In these experiments I cultured ILC2 for 5 days, as it was previously observed that this longer incubation time is required for activation of primary ILC2 as compared to *in vitro*-expanded cells. CRTH2 antagonism and COX-1/2 inhibition suppressed freshly isolated ILC2 function but these effects were less pronounced than those observed in *in vitro*-expanded ILC2. It is possible that flurbiprofen and the CRTH2 antagonist did not fully maintain their effects added two times during the 5 days of culturing, due to metabolic instability. Another explanation may see freshly expanded cells depending less on endogenous PGD₂ production than expanded cells, and that the stimulatory cytokines trigger other molecular pathways that activate ILC2, acting synergistically with endogenous PGD₂. Synergistic effects of innate cytokines and PGD₂ have already been documented in freshly sorted blood human ILC2⁴³. Nevertheless, I

could confirm that also freshly isolated ILC2 use endogenous PGD₂ as a mechanism for cytokine-induced activation.

Further I examined if PGD₂ affects some of the mechanisms of ILC2 stimulation. IL-2 is an important co-stimulator of ILC2 and CD25 can serve as marker of cell activation^{44, 59}. Suppression of cytokine-induced CD25 upregulation with CRTH2 antagonist and flurbiprofen indicates that regulation of CD25 expression is one of the downstream mechanisms of activated PGD₂-CRTH2 axis.

Together, these data demonstrate an interesting mechanism of cytokine-induced activation of ILC2 by endogenous production of PGD₂.

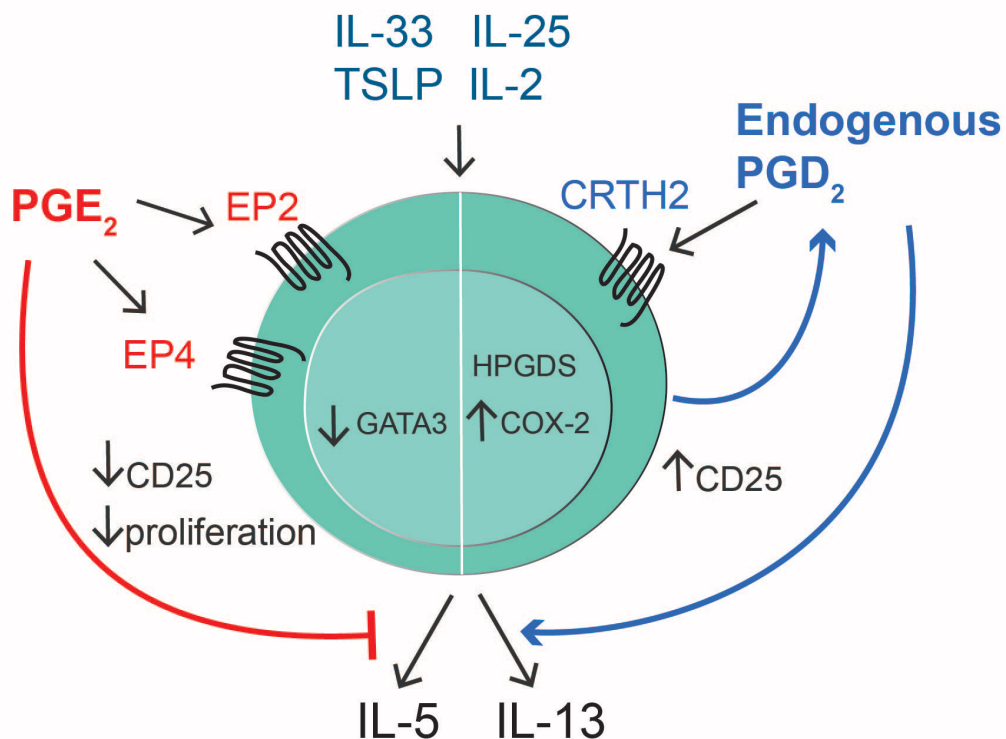


Figure 32. Regulation of ILC2 function by PGE₂ and PGD₂.

Upon different stimuli, epithelial cells produce innate cytokines: IL-25, IL-33 and TSLP. These cytokines plus IL-2 stimulate ILC2 to produce IL-5 and IL-13. On the left hand side, lipid mediator PGE₂ suppresses IL-5 and IL-13 production, likely via preventing CD25 and GATA3 upregulation and blocking ILC2 proliferation. Inhibitory effects of PGE₂ are

mediated by EP2 and EP4 receptors. On the right hand side, ILC2 express HPGDS and upregulate COX-2 upon stimulation with activating cytokines. These events lead to PGD₂ production. Endogenous PGD₂, acting via CRTH2 receptor, is crucial for IL-5 and IL-13 production of ILC2 induced by activating cytokines.

5. Conclusion

Findings of the first part of this thesis reveal that PGE₂ impairs activation of fresh and expanded human blood and tonsil ILC2, by suppressing production and release of the type 2 cytokines IL-5 and IL-13. Additionally I show that PGE₂ interferes with pathways involved in type 2 cytokine production, downregulates CD25 and GATA3 expression and consequently inhibits ILC2 proliferation. Furthermore, I demonstrate that both EP2 and EP4 receptors mediate the PGE₂-induced impairment of ILC2 activation.

Second part of the thesis revealed a novel mechanism of ILC2 activation, where cytokine-induced ILC2 stimulation largely depends on endogenous PGD₂ production. It is demonstrated that human ILC2 express HPGDS, upregulate COX-2 upon stimulation with activating cytokines and consequently produce PGD₂. Particularly, we observed that endogenously produced PGD₂, and its metabolites, acting in an autocrine manner via the CRTH2 receptor, are crucial for production of IL-5 and IL-13 in innate cytokine-stimulated ILC2. Hence, these findings support a potential benefit of CRTH2 antagonists in the treatment of allergic diseases.

Taken together, results of this thesis provide a better understanding of ILC2 regulation by lipid mediators and reveal novel mechanisms in the regulation of ILC2 function that may serve as therapeutic approaches in controlling allergic inflammation and asthma.

6. References

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