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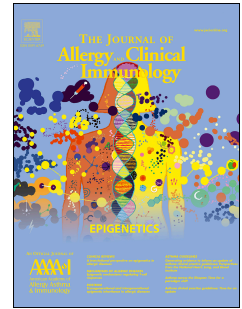
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Fevipirant, a selective prostaglandin-D₂ receptor-2 antagonist, inhibits human group-2 innate lymphoid cell aggregation and function

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Capsule summary: Fevipirant is a potent inhibitor of PGD₂-mediated ILC2 activation. The findings support further development as a therapeutic intervention for asthma.

Keywords: PGD₂ ILC2 asthma allergy

To the Editor:

Lipid mediators are increasingly recognised as activators of inflammatory immune responses. Prostaglandin-D2 (PGD₂) has been strongly implicated in a number of allergic diseases. PGD₂, derived from arachidonic acid metabolism, is released predominantly by activated mast cells. Increased production of and sensitivity to PGD₂ is associated with poor asthma control. Levels of airway PGD₂ and enzymes involved in PGD₂ synthesis are upregulated in the lungs of severe asthmatics (1, 2). Furthermore, PGD₂ receptor-bearing cell populations are expanded within the asthmatic lung.

The effects of PGD₂ are mediated in concert by PGD₂ receptor-1 (DP₁) and DP₂ (CRTH2). DP₁ is broadly expressed, whereas DP₂ is present exclusively on immune cells including eosinophils, basophils, Th2 and Tc2 cells and group-2 innate lymphoid cells (ILC2). Notably, human ILC2 are often characterised by high constitutive expression of DP₂, and PGD₂ is an activating and chemotactic factor for ILC2. Following activation by PGD₂, ILC2 produce large amounts of type-2 cytokines, predominantly IL-13 (3). ILC2 are present at low frequency in the blood but are enriched in number and function at barrier tissues. Increased levels of ILC2 have been found in the blood and sputum of severe therapy-resistant asthmatics (4). Elevated levels of ILC2 are hypothesized to drive the chronic eosinophilia associated with severe asthma. Consistent with this, ablation of ILC2 in murine allergic lung inflammation models, induced by aeroallergens papain or house dust mite, resulted in diminished Th2 responses, reduced eosinophil, neutrophil, and dendritic cell infiltration, and decreased lung chemokine levels and serum IgE. These effects were due to the loss of ILC2-derived IL-13 (5).

Fevipirant is a potent and selective DP₂ antagonist, which reduces eosinophilic airway inflammation and improves lung function and asthma-related quality of life in persistent eosinophilic asthma patients (6). Fevipirant is in clinical development for treatment of asthma (6-8).

To characterize the ILC2 intrinsic inhibitory effects of fevipirant on DP₂ signalling, ILC2 were isolated from blood of healthy participants and expanded *in vitro* by mixed lymphocyte reaction (Fig. E1). ILC2 migration towards PGD₂ (200nM) was measured using IncuCyte live-cell microscopy. Fevipirant (0.01nM-1µM) inhibited PGD₂-induced migration with an average IC₅₀ of 6.3nM (Fig. 1A and 1B).

PGD₂ is a survival factor for Th2 cells. Apoptosis was induced by serum/nutrient deprivation. The addition of PGD₂ (1 µM) promoted ILC2 survival, which was reversed by addition of fevipirant (1 µM; Fig. 1C).

To assess the potential role for fevipirant in regulating ILC2 effector function, PGD₂-induced type-2 cytokine production was measured using qPCR and ELISA. ILC2 were stimulated with PGD₂ (200nM) for 4 hours, supernatant was harvested and assayed for protein and the

cells were lysed for mRNA analysis. Prior incubation of ILC2 with fevipiprant (0.01nM-1 μ M) significantly reduced IL-4, IL-5 and IL-13 production in a dose-dependent manner at both the mRNA level, with average IC₅₀ of 0.2, 1.14, 0.71nM respectively, (Figs. 1D and E2A) and protein level with average IC₅₀ = 0.88, 1.56, 1.96nM respectively (Fig. 1E and E2B). Further cytokine production was assessed by qPCR and multiplex bead array. We observed that ILC2 production of IL-8, GM-CSF and CSF-1 was similarly induced by PGD₂ and inhibited by fevipiprant (Figs. 1F-G and E2C-D). These data highlight the capacity of ILC2 to act outside of the classical type-2 cytokine production pathway. To assess the potency of fevipiprant on asthma-associated ILC2, we derived ILC2 from mild-moderate and severe asthmatic patient blood and analysed cytokine production by ELISA (Fig. 1H). There were small but significant differences in the IC₅₀ from blood ILC2 from health controls vs mild asthmatics (IL-13 p=0.001 and IL-5 p<0.0001) and healthy controls vs severe asthmatics (IL-13 and IL-5, p<0.0001) but whether these represent functional differences for tissue ILC2 in vivo is not known. There were no significant differences in the IC₅₀ for IL-8 or GM-CSF production. Interestingly, we observed that ILC2 aggregated rapidly, within 1 hour of PGD₂ stimulation, forming colony-like clusters (Fig. 2A). This phenotype was ablated in a dose-dependent manner with increasing concentrations of fevipiprant (Fig. 2B). The degree of aggregation was quantified as integrated density from light microscopy images. The effect of fevipiprant on aggregation was not significantly affected by ILC2-donor disease status (Fig. 2C). To determine the mechanism by which this phenotype was regulated, adhesion molecule expression was measured by qPCR. PGD₂ induced upregulation of ICAM-1 and PECAM-1, and was blocked by fevipiprant, with an average IC₅₀ of 0.56nM and 0.82nM respectively (Fig. 2D and 2E). Indeed, antibody-mediated neutralization of ICAM-1 and PECAM-1 in ILC2 cultures significantly reduced the degree of ILC2 aggregation following PGD₂ stimulation (Fig. 2F). To investigate the functional impact of rapid ILC2 aggregation following PGD₂ signalling, we assayed the supernatant of ICAM-1 and PECAM-1 neutralised cultures. We assessed type-2 cytokine production by ELISA (Fig. 2G), and found that inhibition of aggregation alone significantly reduced the PGD₂-induced IL-5 and IL-13 production potential of ILC2. In this study, inhibition of PGD₂-induced activation of ILC2 with fevipiprant significantly reduced ILC2 type-2 cytokine production and also GM-CSF, CSF-1 and IL-8 expression and release. These data highlight the role of ILC2 as potent early responders during allergy, capable of orchestrating infiltration and activation of a number of innate and adaptive immune populations with consequent target potential. Importantly all IC₅₀ values we report are well below the derived C_{trough} concentrations of 200nM at the 225mg bd dose from in vivo data (6).

We showed that in addition to regulating cytokine production, fevipiprant could neutralize the protective effect of PGD₂ on ILC2 survival. Blockade of PGD₂ signalling may result in reduction of the ILC2 population *in vivo*, which could, according to murine experimental allergic lung inflammation studies, contribute to alleviation of asthma symptoms (5).

Upon PGD₂-stimulation we found that ILC2 formed colony-like clusters, and observed for the first time that ILC2 cytokine production was partly dependent on cellular aggregation and ICAM-1 and PECAM-1 expression. Complete cluster-abrogation was not observed in all cultures by ICAM-1 and PECAM-1 neutralization, and so additional adhesion molecules and chemotactic factors are likely to contribute. IL-5 and IL-13, but not IL-4 production, was reduced when ILC2 aggregation was diminished. This could be due to differential cytokine production kinetics, with IL-4 induction occurring before the effects of aggregation are relevant. Although ILC2 represent a rare lymphocyte population, here we suggest that clustering could aid and amplify local ILC2 responses, enhancing effector function at the site of wounding or allergen detection. Interestingly, atopic dermatitis lesions show ILC2 enrichment with close contacts with known DP2⁺ populations including other ILC2, T cells and basophils (9). Indeed, although unlikely to recapitulate the cluster phenotype observed in ILC2 monoculture exactly, aggregation-dependent cytokine production could be relevant at barrier tissues such as the lung, skin and gut where ILC2 are enriched in number and function. Fevipiprant effectively blocks aggregation and cytokine production induced by PGD₂ and could be in part interfering with a positive feedback loop of aggregation and cytokine production.

Phase II studies showed fevipiprant to be effective in uncontrolled eosinophilic asthmatics, patients with severe airflow limitation, and low-dose steroid resistant allergic asthmatics (6-8). An advantage of targeting inflammation upstream of ILC2, rather than anti-type-2 cytokine therapies, is intervention earlier in the pathway and reduction in redundancy between type-2 effector cytokines. Furthermore, blockade of PGD₂ signalling will additionally target non-ILC2 DP₂⁺ populations, such as Th2, Tc2, basophils and eosinophils, all of which have been implicated in asthma immunopathology.

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Conflicts of Interest

The work was funded through a research grant to GO and LX from Novartis, administered through the University of Oxford. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health. GO has received research grants, consultancies or served on Advisory Boards for UCB, Celgene, AnaptysBio, Sanofi/Genzyme, La Roche Posay, Orbit Discovery, Grunenthal, Evelo, Eli Lilly, Leo. VJE is a former and DAS is a current employee of Novartis. In the last 5 years IDP has received speaker's honoraria for speaking at sponsored meetings from Astra Zeneca, Boehringer Ingelheim, Aerocrine, Almirall, Novartis, Teva, Chiesi, Sanofi/Regeneron and GSK and payments for organising educational events from AZ, GSK, Sanofi/Regeneron and Teva. He has received honoraria for attending advisory panels with Genentech, Sanofi/Regeneron, Astra Zeneca, Boehringer Ingelheim, GSK, Novartis, Teva, Merck, Circassia, Chiesi and Knopp and payments to support FDA approval meetings from GSK. He has received sponsorship to attend international scientific meetings from Boehringer Ingelheim, GSK, Astra Zeneca, Teva and Chiesi. He has received a grant from Chiesi to support a phase 2 clinical trial in Oxford. In 2014-5 he was an expert witness for a patent dispute involving Astra Zeneca and Teva. The remaining authors have nothing to disclose.

Figure Legends:

Figure.1: Effect of Feviprant on ILC2 migration, survival and cytokine production.

ILC2 migration to PGD₂ (200nM) in presence of 1μM (A) or concentration range (B) Feviprant determined with IncuCyte, and ILC2 survival measured by flow cytometry (C). ILC2 were incubated with combinations of PGD₂ (200nM) and Feviprant (0.01nM-1μM) and measurements taken of type-2 cytokine gene expression (D) and protein production (E); and GMCSF, CSF-1 and IL-8 gene expression (F), and protein production (G). Asthmatic donor (mild-moderate n=3 and severe n=3 donors) ILC2 were incubated with combinations of PGD₂ (200nM) and Feviprant (0.01nM-1μM) and cytokine production was measured (H). n=3-6 donors and data representative of at least 3 independent experiments per donor. Mean +/- SEM

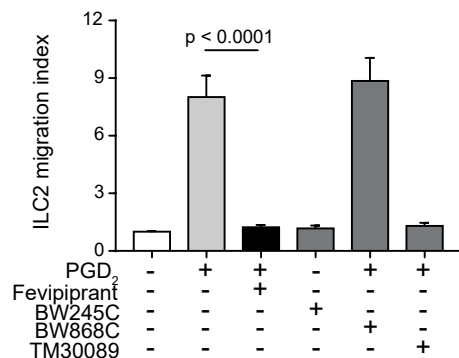
Figure.2: Effect of Feviprant on ILC2 aggregation and cytokine production. ILC2

aggregation induced by PGD₂ (200nM) in presence of Feviprant (0.01nM-1μM) measured by microscopy from healthy (A, B) and asthmatic donors (C). Adhesion molecule gene expression, *ICAM1* (D) and *PECAM1* (E) measured by qPCR. ICAM-1 and PECAM-1

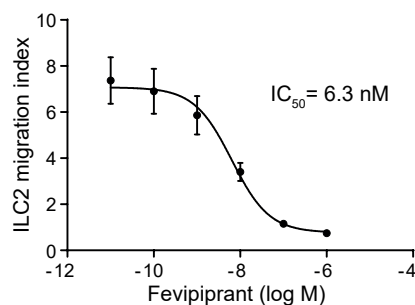
blockade effect on PGD₂-induced ILC2 aggregation (**F**). Effect of Fevipirant (1 μ M) and anti-ICAM-1 (20 μ g/ml) and anti-PECAM-1 (40 μ g/ml) on ILC2 cytokine production following PGD₂ stimulation. n=3-6 donors and data representative of at least 3 independent experiments.

Mean +/- SEM (**G**).

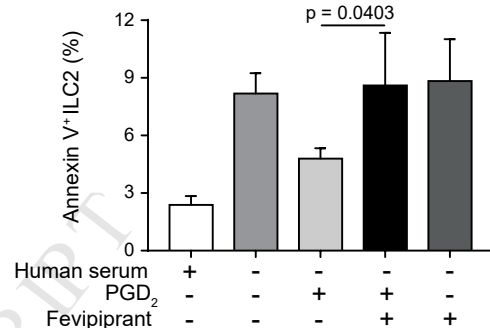
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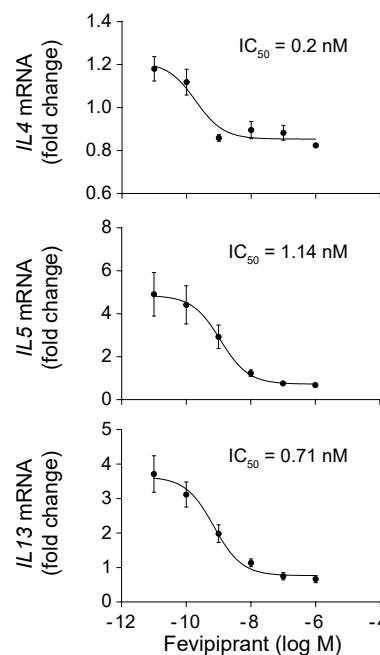
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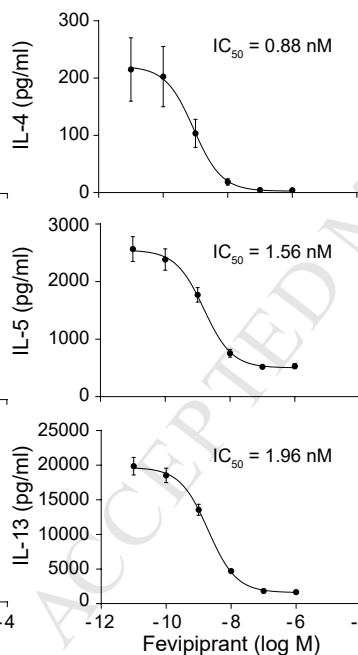
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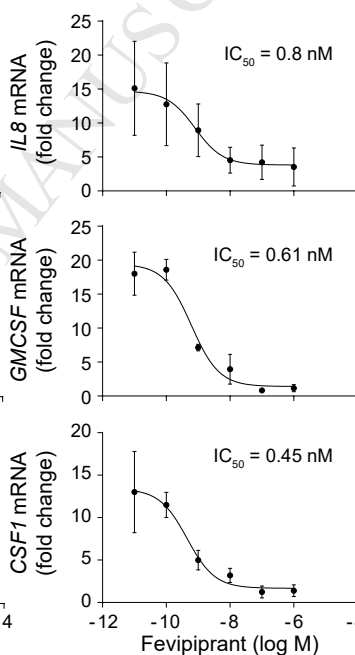
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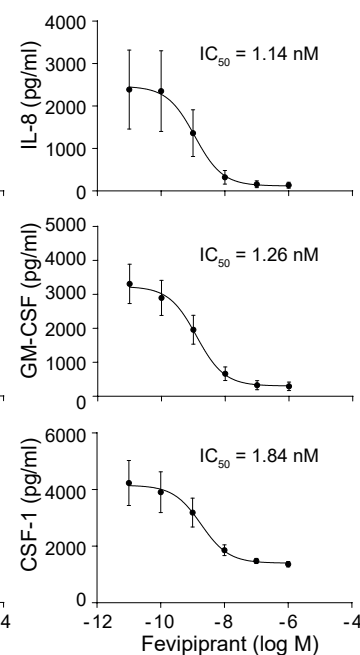
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G



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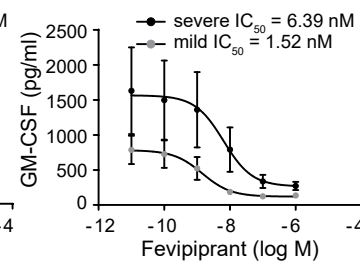
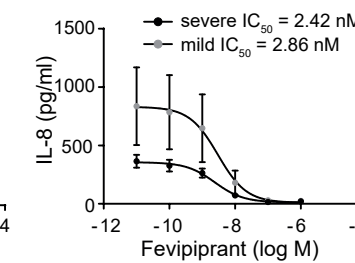
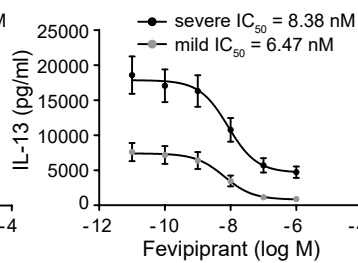
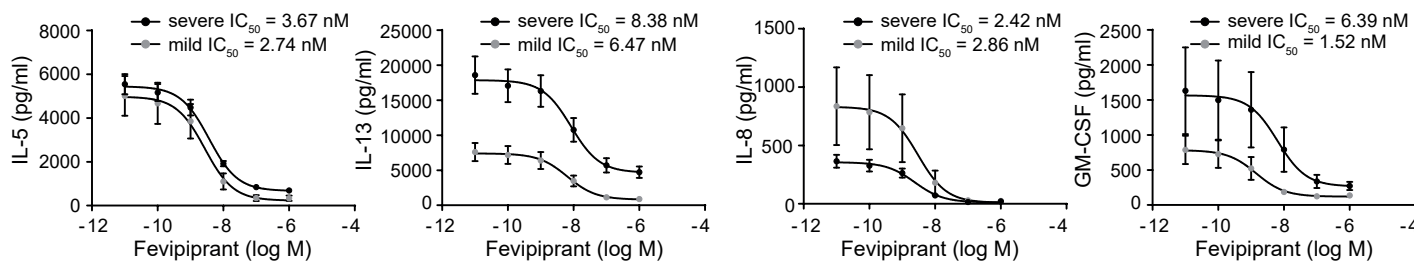
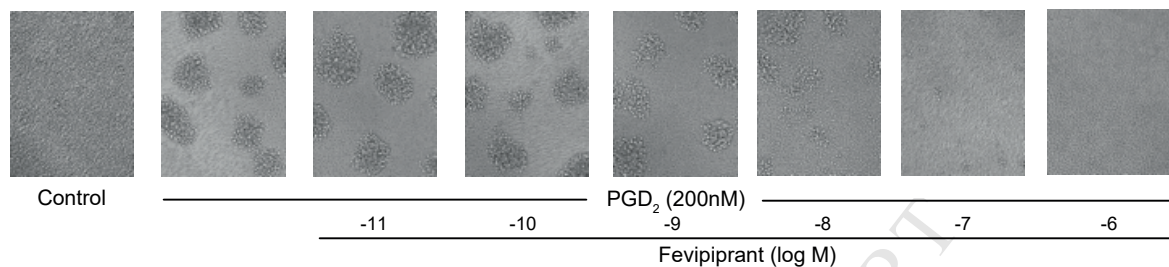


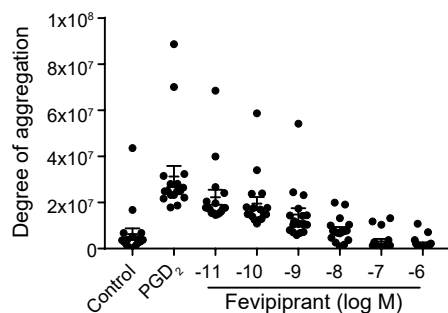
Figure 2

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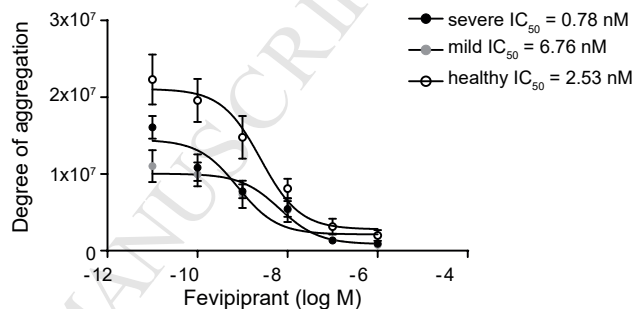
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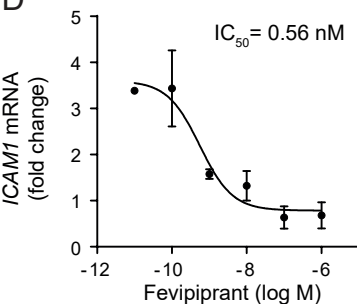
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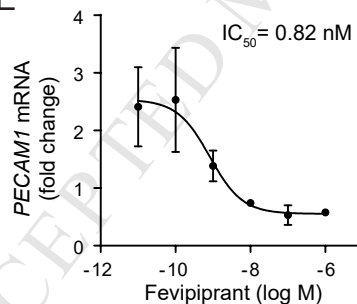
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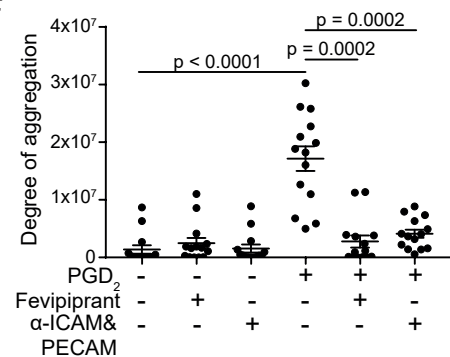
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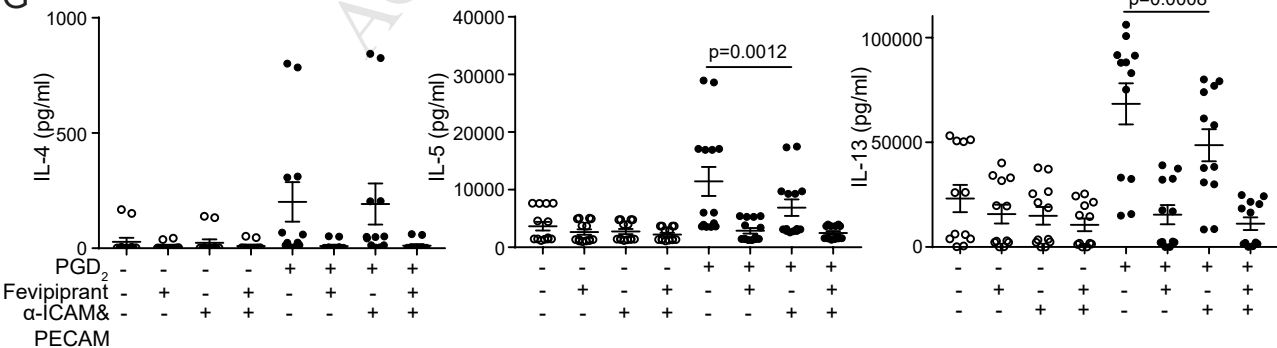
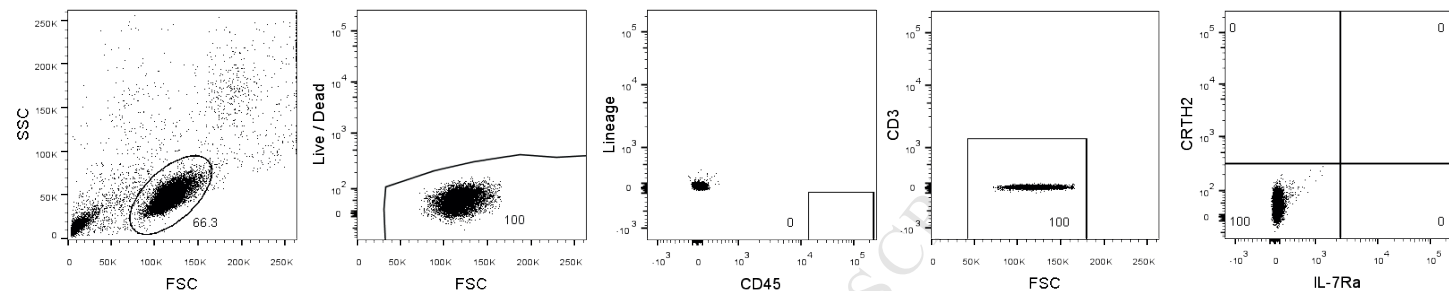
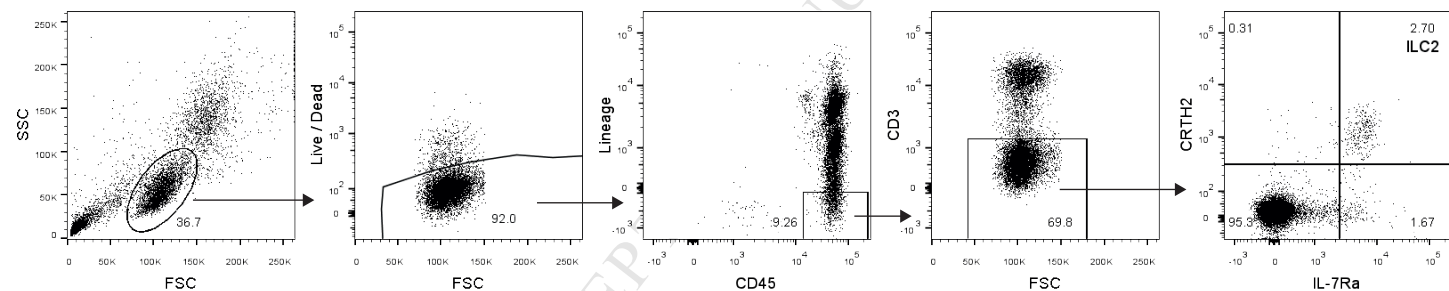


Figure E1

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B



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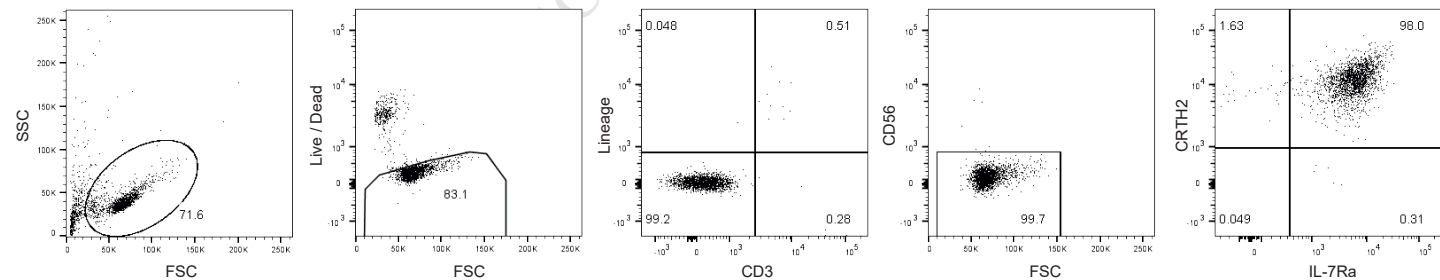
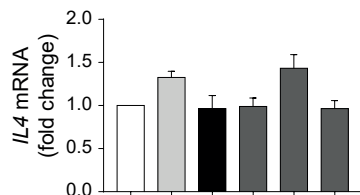
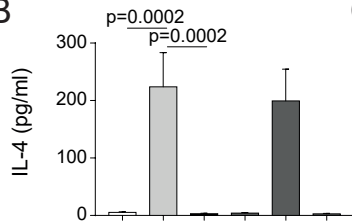


Figure E2

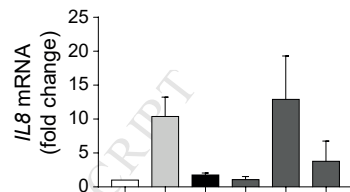
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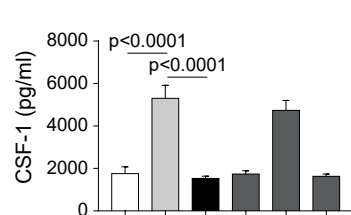
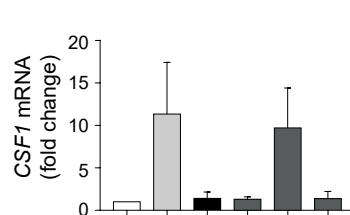
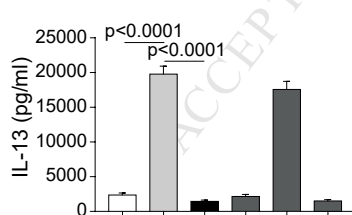
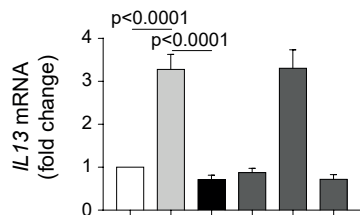
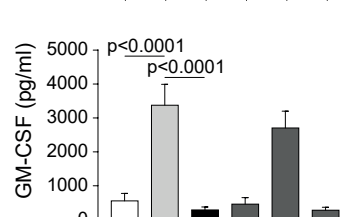
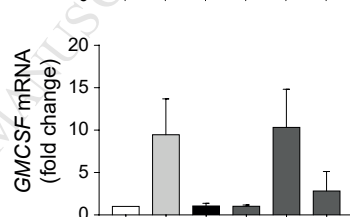
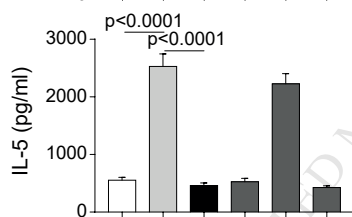
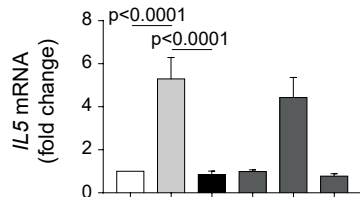
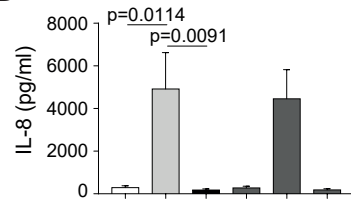
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C



D



PGD ₂	+	+	+	-	+	+
Fevipirant	-	-	+	-	-	-
BW245C	-	-	-	+	-	-
BW868C	-	-	-	-	+	-
TM30089	-	-	-	-	-	+

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Online repository materials**Supplementary Figure Legends**

Figure E1. **Gating strategy for analysis of ILC2.** **A.** Isotype controls for ILC2 flow cytometry antibody stain. **B.** ILC2 are CD45⁺/CD3⁻/Lineage⁻/DP₂⁺/IL7R α ⁺. Full gating strategy for isolation of ILC2. **C.** Flow cytometry antibody stain of ILC2 6 weeks post culture.

Figure E2. **Specificity of Fevipiprant inhibition of DP₂-mediated ILC2 cytokine production.** ILC2 incubated with PGD₂ (200nM) with and without fevipiprant or controls (1 μ M) and measurements taken of type-2 cytokine gene expression (**A**) and protein production (**B**); and GMCSF, CSF-1 and IL-8 gene expression (**C**), and protein production (**D**). n=3-6 donors and data representative of at least 3 independent experiments.

Table E1. qPCR primers and probes

Gene	Primer	Probe No.
<i>CSF1</i>	5'-GCAAGAACTGCAACAACAGC-3' 5'-ATCAGGCTTGGTCACCACAT-3'	19
<i>ICAM1</i>	5'-CCTTCCTCACCGTGTACTGG-3' 5'-AGCGTAGGGTAAGGTTCTTGC-3'	71
<i>IL-4</i>	5'-CACCGAGTTGACCGTAACAG-3' 5'-GCCCTGCAGAAGGTTTCC-3'	16
<i>IL-5</i>	5'-GGTTTGTTCAGCCAAAGAT-3' 5'-TCTTGGCCCTCATTCTCACT-3'	25
<i>IL-8</i>	5'-AGACAGCAGAGCACACAAGC-3' 5'-ATGGTTCCTTCCGGTGGT-3'	72
<i>IL-13</i>	5'-AGCCCTCAGGGAGCTCAT-3' 5'-CTCCATACCATGCTGCCATT-3'	17
<i>GAPDH</i>	5'-AGCCACATCGCTCAGACAC-3' 5'-GCCCAATACGACCAAATCC-3'	60
<i>GMCSF</i>	5'-TCTCAGAAATGTTTGACCTCCA-3' 5'-GCCCTTGAGCTTGGTGAG-3'	1
<i>PECAM1</i>	5'-GCAACACAGTCCAGATAGTCGT-3' 5'-GACCTCAAACCTGGGCATCAT-3'	14

E3. Materials and methods

ILC2 preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy adult donors under local ethics approval (National Research Ethics Service Committee South Central, Oxford C, 09/ H0606/71) or asthmatic patients under local ethics approval (National Research Ethics Service, Leicestershire, Northamptonshire & Rutland C, 08/H0406/189). Patients satisfied the diagnostic criteria for asthma with severity phenotypes defined by the American Thoracic Society/European Respiratory Society guidelines (1). ILC2 were isolated and cultured as previously described (2). Briefly, the lineage (CD3, CD4, CD8, CD14, CD19, CD56, CD11c, CD11b, CD123, and FcεRI)⁻, CD45⁺, IL-7Rα⁺, CRTH2⁺ ILC2 population was sorted (Fig. E1A-B), using a BD FACS Aria III cell sorter, into 96-well plates at 100 cells per well and resuspended in mixed lymphocyte reaction (MLR) of gamma-irradiated PBMCs from three healthy volunteers (2x10⁶ cells / ml) coupled with 100 IU / ml IL-2 and PHA. After 4 – 6 weeks the proliferating cells were tested by flow cytometry staining to ensure a pure population (>97%) of lineage⁻CRTH2⁺IL-7Rα⁺ ILC2 was obtained, taking particular care to exclude T cells (CD3⁺) and NK cells (CD56⁺) as likely potential contaminating cells (Fig. E1C). ILC2 were cultured in ILC2 media: RPMI supplemented with 2 mM L-glutamine, 100 IU / mL penicillin, and 100 µg / mL streptomycin, Sodium Pyruvate, non-essential amino acids, βME, HEPES, plus 10% heat inactivated human serum.

For FACS surface staining the cells were labelled with the following anti-human antibodies purchased from BioLegend unless stated otherwise: CD3 (OKT3), CD19 (SJ25C1, BD Biosciences), CD123 (FAB301C, R&D Systems), CD11b (DCIS1/18), CD11c (BU15, Abcam), CD8 (RPA-T8), FcεRI (CRA-1), CD14 (MwP9, BD Biosciences), CD4 (OKT4), CD45 (2D1), CD56 (HCD56), CRTH2 (BM16, Miltenyi Biotec), IL-7Ra (A019D5), Live/Dead violet (Invitrogen).

Chemotaxis assay

The effect of Fevipirant on chemotaxis of ILC2 to PGD₂ was assessed using the IncuCyte live cell analysis system. Cell migration was induced with PGD₂ and the dose dependent effect of Fevipirant was determined using a trans-well assay. 150,000 ILC2 were placed into the upper chamber of a 96 well transwell plate (Corning 3388) and were incubated with Fevipirant (1µM, 0.1µM, 0.01µM, 0.001µM, 0.0001µM or 0.00001µM) or controls (1µM) for 1 hour. The upper chamber of the trans-well plate was then placed into the lower chamber containing either media or PGD₂ (200nM). The plate was then incubated at 37°C for 1 hour

for migration to take place across the 5.0 μm pore polycarbonate membrane. Chemotaxis was calculated as a function of % confluency measured using an IncuCyte. A titration of cell number was performed in the lower chamber of the trans-well plate to provide a standard curve for determination of cell number from % confluency. The IncuCyte was set to measure 4 images per well using the 10 x objective.

To confirm the specificity of the PGD_2 effects seen for DP_2 , the following controls (1 μM) were employed: BW245C, a DP_1 agonist, BW868C, a DP_1 antagonist and TM30089 a positive control DP_2 antagonist.

Apoptosis assay

The effect of Fevipirant on PGD_2 -induced inhibition of ILC2 apoptosis was assessed by flow cytometry. ILC2 were incubated in ILC2 medium or medium lacking human serum, and stimulated with 1 μM PGD_2 , in the presence or absence of 1 μM Fevipirant, as indicated in the results, overnight (16 h). The cells were harvested and stained with Annexin-V-AF647 (BioLegend) and SYTOX Green Stain (Invitrogen) in Annexin V binding buffer (BioLegend) following the manufacturer's instructions. Results were acquired with a LSRII flow cytometer (BD Biosciences). Apoptotic cells were defined as Annexin V positive and SYTOX Green negative.

Measurement of ILC2 aggregation

The effect of Fevipirant on PGD_2 -induced aggregation of ILC2 was assessed by light microscopy. ILC2 were incubated in ILC2 medium in the presence or absence of 1 μM Fevipirant or controls and/or anti-ICAM-1 (20 $\mu\text{g/ml}$) and anti-PECAM-1 (40 $\mu\text{g/ml}$), as indicated in the results, for 1 hour. ILC2 were then stimulated with 1 μM PGD_2 , for 1 hour. Light microscopy images were taken using an EVOS FLoid cell imaging station and the degree of aggregation was quantified as integrated density from light microscopy images using the Trainable Weka Segmentation Fiji plugin. Cells were returned to the incubator and for 4 hour total stimulation, supernatant was then harvested and frozen for further analysis.

ELISA

The effect of Fevipirant on ILC2 type-2 cytokine production was measured by ELISA. ILC2 were incubated with Fevipirant (1 μM , 0.1 μM , 0.01 μM , 0.001 μM , 0.0001 μM or 0.00001 μM)

or controls (1 μ M) and/or anti-ICAM-1 (20 μ g/ml) and anti-PECAM-1 (40 μ g/ml) for 1 hour. And then stimulated for 4 hours with PGD₂ (200 nM) or media. The controls used for the analysis included: BW245C (DP1 receptor agonist), BW868C (DP1 antagonist) and TM30089 (CRTH2 antagonist). ILC2 were separated from the culture media by centrifugation, cell pellets frozen for mRNA analyses and the supernatant was assayed for IL-4, IL-5 and IL-13 protein by ELISA following the manufacturer's protocol (Human IL-13 ELISA Ready-SET-Go! 88-7439-88, Human IL-5 ELISA Ready-SET-Go! 88-7056-86 and Human IL-4 ELISA Ready-SET-Go! 88-7046-86).

Multi-plex bead array

The effect of Fevipirant on ILC2 cytokine production was measured by bead-based multiplex assay (Luminex). ILC2 were incubated with Fevipirant (1 μ M, 0.1 μ M, 0.01 μ M, 0.001 μ M, 0.0001 μ M or 0.00001 μ M) or controls (1 μ M)) for 1 hour. And then stimulated for 4 hours with PGD₂ (200 nM) or media. The controls used for the analysis included: BW245C (DP1 receptor agonist), BW868C (DP1 antagonist) and TM30089 (CRTH2 antagonist). ILC2 were separated from the culture media by centrifugation, the cell pellets frozen for mRNA analyses and the supernatant was assayed for IL-8, GM-CSF and CSF1 protein by human magnetic bead Luminex multi-analyte assay following the manufacturer's protocol (Bio-technique custom analyte mix). Results were obtained with a Bio-Plex 200 System (Bio-Rad).

Quantitative RT-PCR (qPCR)

Total RNA was extracted from stimulated ILC2 pellets using a RNeasy Mini kit, following pre-treatment of ILC2 with Fevipirant (1 μ M, 0.1 μ M, 0.01 μ M, 0.001 μ M, 0.0001 μ M or 0.00001 μ M) or controls (1 μ M) for 1 hour, and then stimulation with PGD₂ (200 nM) or media for 4 hours. cDNA was prepared from ILC2 mRNA using reagents (M-MLV, dNTP, Oligo(dT) and RNaseOUT) from Invitrogen. RT-PCR was conducted using Master Mix, Probes (Roche) and specific primers (Table E1) in a CFX96™ Real-Time System (Bio-Rad). GAPDH was used as the reference gene. Changes in mRNA level compared with untreated sample were calculated according to the manufacturer's instructions.

Statistics

Data are presented as means with SEM. Data were analysed using one-way ANOVA followed by Tukey's test for multiple comparisons performed using GraphPad Prism. Values of $p < 0.05$ were considered statistically significant.

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2. Xue L, Salimi M, Panse I, Mjosberg JM, McKenzie AN, Spits H, et al. Prostaglandin D2 activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on TH2 cells. *J Allergy Clin Immunol*. 2014;133(4):1184-94.