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Fevipirant, a DP₂ Receptor Antagonist, Inhibits Eosinophil Migration Towards Mast Cells

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To the Editor:

Prostaglandin D₂ Receptor 2 (DP₂; previously CRTh2), a receptor for prostaglandin
D₂ (PGD₂), is expressed on adaptive and innate immune cells [1]. PGD₂ is produced mainly

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by activated mast cells (MCs) and serves as a chemoattractant for eosinophils and other immune cells. It is found at increased levels in asthmatic lungs, with further increases occurring according to disease severity [1] and therefore the DP₂ receptor pathway plays a major role in the pathophysiology of asthma. Fevipiprant is a potent selective antagonist of the DP₂ receptor and blocks agonist-induced shape change in human eosinophils and type-2 cytokine secretion (i.e. IL-4, IL-5 and IL-13) from human CD4⁺ Th2 cells *in vitro* [2]. Clinically, fevipiprant has demonstrated efficacy and reduced eosinophilic airway inflammation in patients with persistent asthma and raised sputum eosinophil counts [3] and is currently in phase 3 development for asthma.

Two key effector cells in asthma are MC and eosinophils. We have extensively described their pro-inflammatory cross-talk, characterized as the Allergic Effector Unit (AEU), that comprises both soluble and physical cellular interactions [4, 5]. Here, we investigate the effects of fevipiprant on the different AEU proinflammatory parameters such as eosinophil migration, eosinophil survival and MC degranulation.

We first evaluated the secretion of PGD₂ by activated human cord-blood MC (CBMC). After 30 min of anti-IgE activation, mono-cultured CBMC secreted 43.4 ± 7.6 ng/ml PGD₂ compared to 5.2 ± 3.2 ng/ml secreted by non-activated (NA) CBMC (**Fig. 1a**). Similarly, co-culture of NA human peripheral blood (PB) eosinophils with anti-IgE activated CBMC for 90 min secreted 58 ± 4.1 ng/ml PGD₂ compared to 2.6 ± 1.3 ng/ml secreted from co-culture with NA CBMC (**Fig. 1b**). Monoculture of NA eosinophils secreted only 0.58 ± 0.06 ng/ml PGD₂. Although eosinophils were shown to secrete low levels of PGD₂ in response to variety of stimuli [6-7], our results indicate that the main contributors of PGD₂ in these co-culture settings are CBMC, but not eosinophils.

Next, we examined the expression levels of DP₂ on eosinophils and CBMC. As shown in **Fig. 1c** surface immuno-staining of DP₂ receptors demonstrated expression on eosinophils (**Fig.1c, left panel**), but not on NA or anti-IgE activated CBMC (**Fig.1c, right panel**). Our results are in line with previous studies showing DP₂ surface expression on human eosinophils [8] but not on human MC [9]. Therefore, in our MC-eosinophil co-culture experiments, eosinophil effects would be anticipated to be directly blocked by fevipiprant and not indirectly via fevipiprant-effects on MC. First, DP₂ functionality on eosinophils was examined by assessing eosinophil transwell migration toward PGD₂. As expected, eosinophils displayed significant chemotactic activity towards PGD₂, reaching migration of 25% of total cells after 90 min (**Supplemental Fig. 1a**). In a second step we examined the ability of fevipiprant to inhibit this activity. As shown in Fig 1D. IgE-activated CBMC induced a significant 1.67-fold increase in the percentage of eosinophil migration compared to NA CBMCs (from 17% to 28%) (**Fig 1d.**). Preincubation of CBMC with different concentrations of fevipiprant (10⁻⁹-10⁻⁵ M) significantly inhibited eosinophil migration towards IgE-activated CBMC in a dose-dependent manner, with a 2.3-fold reduction (from 28% to 12%) at the highest drug concentration. In addition, fevipiprant decreased, by 2.1-fold, the percentage of eosinophil migration toward NA CBMC (from 17% to 8%).

Fevipiprant had no effect on DP₂ surface expression on eosinophils in the absence of PGD₂ (**Supplemental Fig. 2a**). In addition, fevipiprant had no effect on the surface expression of CCR3, another major eosinophil chemokine receptor (**Supplemental Fig. 2b**), nor on its function as found in CCL11 (eotaxin)-mediated transwell migration assay (**Supplemental Fig. 2c**).

The proinflammatory cross-talk between eosinophils and MCs includes induction of MC degranulation by eosinophils in a contact-dependent manner [4]. Therefore, we examined the effect of fevipiprant on eosinophil-mediated CBMC degranulation in the

absence or presence of MC activation stimuli with anti-IgE. The extent of MC degranulation was measured by the surface expression of the established MC degranulation marker lysosomal-associated membrane protein-1 (LAMP-1) [10], and by measuring the levels of secreted granule enzyme tryptase by an activity assay, in the co-culture supernatants. IgE-activated CBMCs showed an increase in LAMP-1 expression (**Supplemental Fig. 3a**) and in tryptase in the supernatants (**Fig. 2**). Co-culture of anti- IgE activated CBMC with eosinophils further increased tryptase secretion (**Fig. 2**), as previously shown [6-7]. However, eosinophil-mediated CBMC degranulation was not affected by the highest concentration of fevipirant (10 μ M) as measured by tryptase release in supernatants (**Fig. 2**) or LAMP-1 expression (**Supplemental Fig. 3a**). These results suggest that the DP₂ pathway is not involved in eosinophil-mediated MC degranulation.

Another aspect of the proinflammatory AEU is the induction of eosinophil survival by MCs (8) but we were able to demonstrate that co-culturing of eosinophils and CBMC for 3 days in the presence of different concentrations of fevipirant had no impact on eosinophil survival (**Supplemental Fig. 4**). These results are in line with recently published study showing DP₁, another PGD₂ receptor that is expressed on eosinophils, but not DP₂ is required for PGD₂-induced eosinophil survival [11].

Taken together our observations suggest that fevipirant impacts the proinflammatory activity of the AEU as a selective blocker of DP₂ pathway-dependent eosinophil migration toward MC, an early and crucial stage of allergic inflammation and a Th2-mediated exacerbation of asthma. These results are well aligned with recent clinical data [3] showing reduction in sputum and tissue eosinophil levels in uncontrolled asthma patients treated with fevipirant. Interestingly, further AEU proinflammatory activities, such as MC-dependent eosinophil survival induction or eosinophil-dependent MC-degranulation, are not affected by fevipirant, probably because these activities are DP₂-independent.

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Author contributions

RS designed the research, performed experiments, analyzed data and wrote the manuscript; DM provided human cord blood; DS, VE, JD and FLS designed the research, provided overall supervision and edited the manuscript.

Conflict-of-interest disclosure: The authors F Levi-Schaffer, R Shamri and D Mankuta declare no competing financial interests. VJ Erpenbeck and DA Sandham are employees of Novartis; G Dubois is a former Novartis employee.

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Figure legends:

Figure 1: Fevipiprant inhibits eosinophil migration toward activated mast cells. (A,B) PGD₂ in supernatants of non-activated (NA) or anti- IgE activated CBMCs monoculture **(A)** or co-culture with eosinophils **(B)** as described in (supp. M&M). **(C)** Surface DP₂ expression (grey shade) on human eosinophils (left panel) compared to isotype control (dashed line), but not on NA CBMC (grey-shade-right panel), or anti-IgE activated CBMC (thin black line) compared to isotype control (dotted line), as described in supp. M&M. Data are a

representative of three independent experiments. **(D)**. Migration of non-treated (NT) or fevipirant (FEVI)-treated eosinophils toward NA or anti-IgE activated CBMC, as described in supp. M&M. Asterisks represent P values < 0.05 (*), compared to NT- eosinophil migration toward anti-IgE activated hCBMC (second bar). Data of A, B are mean $\pm$ SD of three (A, B), or 3-7 (D) independent experiments.

Figure 2: Eosinophil-mediated MC degranulation is not affected by fevipirant. tryptase in supernatants of NA or anti- IgE activated CBMCs monoculture or co-cultured with NT or fevipirant (FEVI)-treated eosinophils as described in supp. M&M. Data are mean $\pm$ SD of three independent experiments. Asterisks represent P values < 0.01 (**) or 0.05 (*), compared to NT and NA hCBMC (third bar).

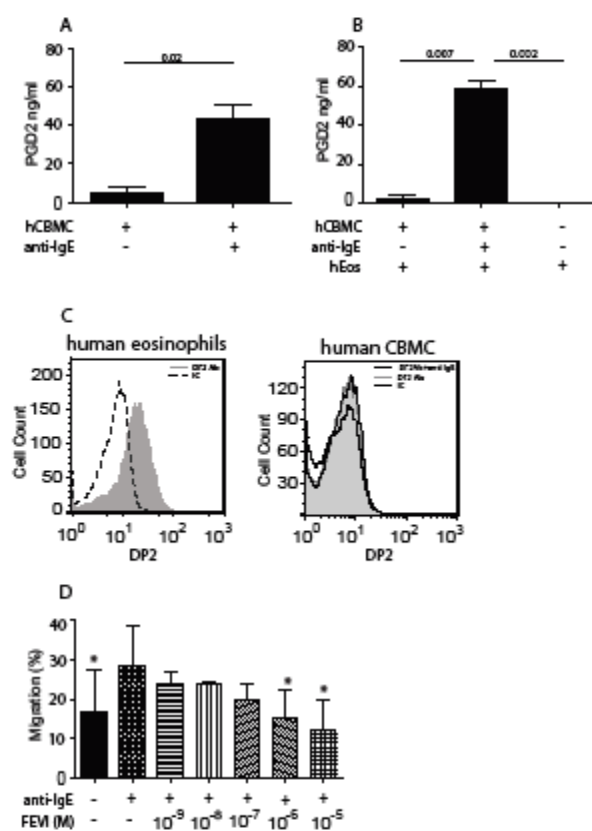
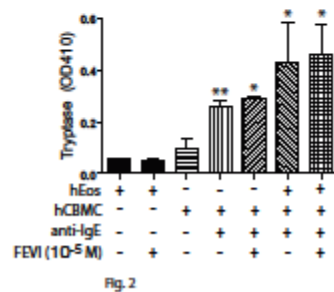
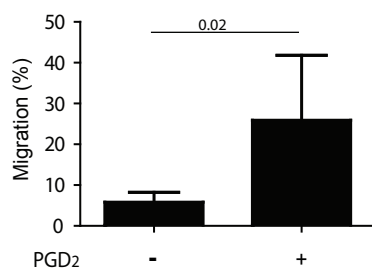
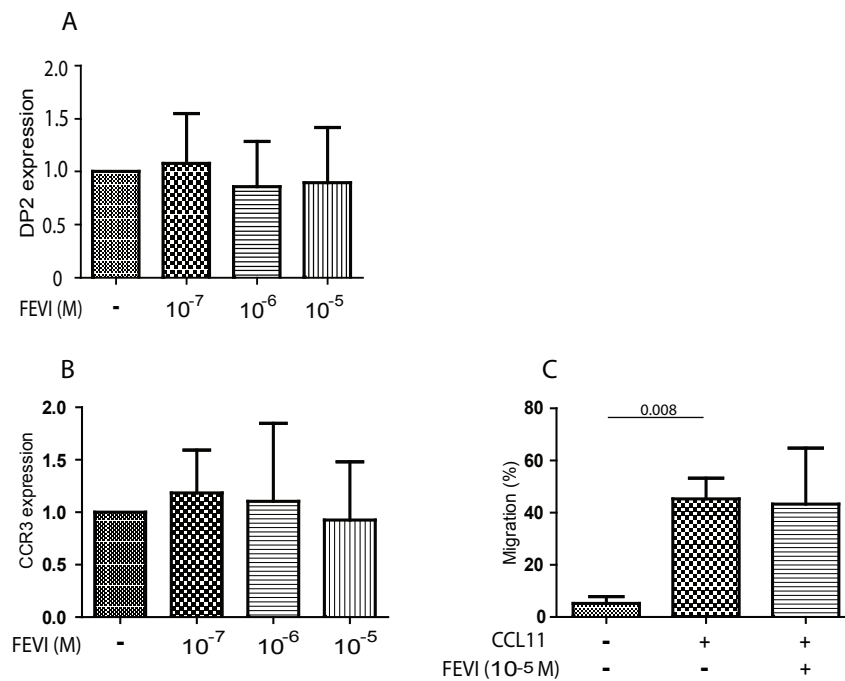


Fig. 1

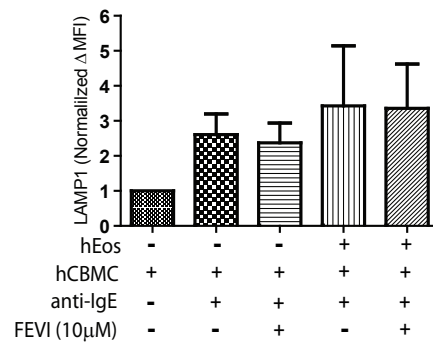




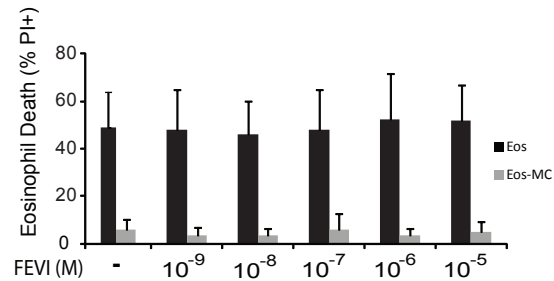
Supplemental Figure 1: Eosinophils migrate toward PGD₂. Migration of eosinophil toward PGD₂ , as described in supp. M&M. Data are mean $\pm$ SD of four independent experiments.



Supplemental Figure 2: Fevipiprant had no effect on DP₂ or CCR3 expression or CCR3-mediated eosinophil migration. (A) Surface DP₂ expression on NT or treated- eosinophils with different concentrations of fevipiprant (FEVI), as described in supp. M&M. Data of A, B are mean ± SD of three (A, B), four (E), or 3-7 (D) independent experiments. **(B).** Surface CCR3 expression on human eosinophils, NT or treated with different concentrations of fevipiprant (FEVI), as described in supp. M&M. **(C)** Migration assay of NT or fevipiprant (FEVI)-treated eosinophils toward CCL11 (eotaxin), as described in supp. M&M. Data in A and B are mean ±SD of three independent experiments.



Supplemental Figure 3: Fevipiprant had no effect on LAMP-1 expression. Surface LAMP-1 expression on NA or anti- IgE activated CBMC monoculture or co-culture with NT- or fevipiprant (FEVI)-treated eosinophils as described in supp. M&M. Data are mean $\pm$ SD of three independent experiments.



Supplemental Figure 4: MC-induced eosinophil survival are not affected by fevipiprant. Survival of NT or fevipiprant (FEVI)-treated eosinophil monoculture (black bars) or co-cultured with CBMC (grey bars) for 72 hrs, as described in supp. M&M. Data are mean $\pm$ SD of three independent experiments.

Supplemental Material and Methods:

Isolation of human eosinophils and human cord-blood derived mast cells:

Peripheral blood eosinophils were purified from healthy or asymptomatic mildly allergic or asthmatic subjects not on medication before or during sampling. Eosinophils were purified by negative selection, as described in Gangwar RS et al., 2014. *Methods Mol Biol.* 1178:231-249. Human cord blood derived mast cells (CBMC) were cultured from cord blood of healthy women, as described in Gangwar RS et al., 2014. *Methods Mol Biol.* 1178:231-249.

All human blood samples were collected following ethical approval of the Hadassah Medical Center-the Hebrew University Human Experimentation Helsinki Committee (ref #0410-14 HMO, #7-14.01.05), and written informed consent was obtained according to its guidelines. Purity >95% were determined by microscopic analyses after Kimura staining for Eosinophils and Toluidine blue for CBMC. Viability >98% was determined by microscopic analyses of trypan blue exclusion.

Cell activation and inhibition:

CBMC were pre-sensitized with human myeloma IgE antibody (0.3 µg/ml; Calbiochem, EMD Millipore, Billerica, MA) in sensitization medium (MEMα 10% heat-inactivated fetal-calf serum; Biological Industries, Beit Haemek, Israel), 100 ng/ml human SCF, 10 ng/ml human IL-4 (Peprotech, Rocky Hill, NJ) for 72 hours. Then cells were washed with medium (300g, 5 min at RT) and stimulated with anti-IgE antibody (5 µg/ml; Dakocytomation, # A0094) for 30 min prior to co-culture with eosinophils or used in the migration experiments in a humidified incubator (37°C, 5% CO₂).

DP₂ inhibition on eosinophils was done by preincubation of eosinophils with different concentrations (10^{-9} - 10^{-5} M in DMSO) of fevipiprant (Novartis Pharma AG, Basel, Switzerland) or the DMSO vehicle, for 30 min in a humidified incubator (37°C, 5% CO₂) prior to co-culture or migration experiments.

Flow cytometry:

For chemokine receptor staining, CBMC were presensitized with human IgE followed by anti-IgE activation as described above, while eosinophils were preinhibited with fevipiprant for 30 min as described above. Cells (1×10^5 /sample) were washed (300g, 5 min at 4°C) with FACS buffer (0.1% BSA/PBS), blocked with serum and incubated for 30 min at 4°C with 0.1 µg of anti DP₂ antibody (CRTH2 (DP₂)- A647 (Biolegend, San Diego, CA, USA) or rat anti-human CCR3- PE (R&D Systems, Minneapolis, MN, USA) or equivalent isotype controls for 30 min at 4°C. Cells were then washed and samples were acquired using the FACSCalibur flow cytometer (BD Bioscience, San-Jose, CA, USA) and analyzed by Flow Jo (Tree Star, Ashland, OR, USA) software. CCR3 and DP₂ expression levels were calculated as ΔMFI (mean fluorescence intensity) by subtracting the geometric mean fluorescence of stained samples from that of the non-stained (isotype control) samples. ΔMFI fold increases is the ratio of ΔMFI of stimulated cells out of non-stimulated and/or non-inhibited samples.

Migration assay:

Cells were washed (300g, 5 min at 4°C) and resuspended (CBMC, 0.3×10^6 / ml, eosinophils 2×10^6 / ml) in chemotaxis medium (RPMI 1640, supplemented with 1% heat-inactivated fetal calf serum, 10%, penicillin 100 U/ml, streptomycin; all from Biological

Industries, Beit Haemek, Israel), cultured in a humidified incubator (37°C, 5% CO₂), in the presence of 100 ng/ml human SCF to maintain CBMC viability.

Eosinophils were pretreated with different concentrations of fevipiprant, or vehicle in chemotaxis medium as described above, and then were added (2×10^5 cells in 0.1 ml) in duplicates to the upper chambers of 24-well transwell plates (5 micron pore size, Corning, Tewksbury, MA). The lower chamber was prefilled with 0.6 ml of chemotaxis medium supplemented with 100 ng/ml CCL11 (Peprotech) or 10^{-6} M PGD₂ (Sigma-Aldrich-Merck, Knilworth, NJ, USA) or non-activated (NA) or anti-IgE activated CBMC (2×10^5 per well) as described above. Eosinophils were allowed to migrate for 90 min in a humidified incubator (37°C, 5% CO₂). Transmigrated cells from the lower chamber including adherent cells were collected with 5 mM EDTA chemotaxis medium. Cells were stained with the MC marker, PE-conjugated anti-human cKit antibody (Biolegend) to better distinguish between eosinophils and CBMC. Percentages of migrated eosinophils were calculated from total eosinophils number originally loaded in the upper chamber.

Cell viability assay:

CBMC and eosinophils were resuspended (1×10^6 / ml) in MEM alpha medium supplemented with: heat-inactivated fetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 µg/ml), ribonucleosides (10 µg/ml) (all from Biological Industries, Beit Haemek, Israel) and fresh human SCF (100 ng/ml) and placed in a 96-well plate in 1:1 ratio (1×10^5 for each cell type) for 72 hrs in a humidified incubator (37°C, 5% CO₂). Monoculture of eosinophils or CBMC consisted of 2×10^5 cells/ 0.2 ml, in order to maintain equivalent volume and cell

concentration as in co-culture samples. At the end-point, samples were washed (300g, 5 min at 4°C) with FACS buffer and stained for the MC marker, mouse anti human cKit (eBioscience, affymetrix, Santa-Clara, CA, USA), for 30 min at 4°C, followed by a staining with Alexa 647^(C) - conjugated goat anti mouse secondary antibody (Jackson ImmunoResearch **Laboratories, Inc.**, West Grove, PA, USA) for 30 min at 4°C, and Propidium Iodide (PI) staining (Sigma-Aldrich-Merck). Data were acquired and analyzed as described above. Results are presented as percentage of dead eosinophils (ckit⁻/PI⁺) from total eosinophils in the sample.

ELISA for PGD₂:

PGD₂ was measured in supernatants of CBMC/eosinophils in mono/co-culture using the PGD₂ ELISA kit (Cayman, Ann Arbor, MI, USA) according to the manufacture instructions. The lower detection limit for this cytokine was 19 pg/ml.

Degranulation assay:

CBMC or eosinophils were washed and resuspended in degranulation medium (RPMI 1640 media (Biological Industries, Beit Haemek, Israel), supplemented with 0.1% gelatin, 100 ng/ml hSCF, 1.8 mM Ca²⁺ and 0.9 mM Mg²⁺).

Eosinophils were pretreated with fevipirant, or vehicle in degranulation medium as described above and co-cultured for 1.5 hr in 1:1 ratio with CBMC (1x 10⁵ /0.1 ml for each cell type) in 96-well plate (Nunc, Thermo Scientific, Denmark). Samples of monoculture of eosinophils or CBMC consisted of 2x10⁵ cells/ 0.2 ml. At the end-point, samples were centrifuged (300g, 5 min at 4°C) and supernatants were transferred to new tubes and kept at -

80°C for Tryptase activity assay. The cells were analyzed for CBMC activation by staining (30 min at 4°C) with APC-conjugated mouse anti human LAMP-1 antibody (11) together with PE-conjugated anti-human cKit antibody (Biolegend),. Samples were acquired and analyzed as described above. Data are presented as Δ MFI fold increase of stimulated CBMC samples (with or without eosinophils, treated or not with Fevipiprant) out of non-stimulated monoculture CBMC samples.

Tryptase assay:

Tryptase in supernatants was determined by an activity assay as previously described (Bachelet I et al. Journal of Immunology. 2005). Briefly, 48 μ l of the experimental sample was added to 2 μ l of the specific substrate solution of *N-p*-tosyl-gly-pro-lys-*p*-nitroanilide (Sigma Aldrich-Merck; 25 mM), mixed in a flat 96-well plate and incubated at 37°C until chromogenesis. OD was measured at 410 nm.

Statistical analysis

Levels of significance between groups were analyzed by two-tailed paired Student's t tests. P values < 0.05 were considered statistically significant.