CRTH2 activation *in vivo* increases blood leukocyte counts and its blockade abrogates 13,14-dihydro-15-keto PGD_2-induced eosinophilia in rats

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CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; DK-PGD_2, 13,14-Dihydro-15-Keto-Prostaglandin D_2; DP, PGD_2 receptor; PGD_2, prostaglandin D_2; PTX, pertussis toxin; TP, TxA_2 receptor; TxA_2, thromboxane A_2;

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Abstract

We cloned, expressed, and characterized in vitro and in vivo the gene encoding the rat ortholog of CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells), a G protein-coupled receptor for prostaglandin D_2 (PGD_2). Quantitative RT-PCR analysis demonstrated highest CRTH2 expression in the lung, brain, ovary, and spleen. Pharmacologically, rat CRTH2 stably transfected in mouse pre-B lymphoma L1.2 cells behaved very similar compared to the mouse and human orthologs showing a binding affinity for PGD_2 of 11 nM, a functional calcium mobilization when exposed to agonist and similar sensitivity to agonists and antagonists.

In vivo, selective activation of CRTH2 by DK-PGD_2 injection into rats led to a dose- and time-dependent increase of the number of leukocytes in the peripheral blood. Specifically, eosinophils, lymphocytes and neutrophils were recruited with maximum effects seen 60 min after the injection of 300 μg DK-PGD_2 per rat. Pretreatment of the animals with the CRTH2/thromboxane A_2 receptor antagonist, ramatroban, completely abrogated DK-PGD_2-induced eosinophilia suggesting that CRTH2 might have a physiological and/or pathophysiological role in controlling leukocyte migration.

Chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) has been identified by a comparative differential display approach in Th1 and Th2 lymphocytes (Nagata et al., 1999a; Nagata et al., 1999b). The initial attention this receptor gained due to its restricted expression pattern on Th2 cells, basophils and eosinophils (Nagata et al., 1999a) was increased when it became clear that its ligand is the prostanoid, prostaglandin D_2 (Hirai et al., 2001) (Monneret et al., 2001). This arachidonic acid product is predominantly released by activated mast cells and its potential implication in allergic reactions has been known for many years (Lewis and Austen, 1981). Large amounts of PGD_2 are released into the airways of asthmatics immediately after challenge (Murray et al., 1985; Murray et al., 1986) (Wenzel et al., 1991) and mice overexpressing the lipocalin-type PGD synthase show stronger inflammatory reactions in the lung upon antigen challenge (Fujitani et al., 2002). In contrast, mice lacking DP, the first PGD_2 receptor identified, show a diminished response to ovalbumin challenge in animal models for asthma highlighting the importance of PGD_2 in allergic reactions (Matsuoka et al., 2000).

Despite binding the common ligand PGD_2, CRTH2 and DP differ significantly from each other by showing low sequence homology and by being coupled to different classes of G proteins. While DP couples to a stimulatory G_s protein leading to the activation of adenylyl cyclase and increasing levels of cAMP and Ca^{2+} (Hirata et al., 1994) (Boie et al., 1995), CRTH2 activation inhibits cAMP formation but induces an increase in intracellular Ca^{2+} mobilization via G_i-dependent pathways (Hirai et al., 2001) (Sawyer et

al., 2002) (Sugimoto et al., 2003). This difference in G protein coupling appears to allow CRTH2 to transmit pro-migratory signals in response to PGD_2. For instance, CRTH2-transfected Jurkat cells can migrate along a gradient of PGD_2, whereas DP-transfected Jurkat cells cannot (Hirai et al., 2001). In leukocytes of all species studied so far PGD_2 induces migration exclusively via CRTH2 (Hirai et al., 2001) (Monneret et al., 2001) (Sugimoto et al., 2003) (Gosset et al., 2003) (Hata et al., 2003).

Considering the ability of PGD_2 to stimulate the migration of inflammatory cells, it is reasonable to speculate that CRTH2 may play a role in the selective recruitment of cellular components of the allergic response into sensitized or injured tissues. Expression studies in mice, however, have shown that a variety of tissues express mRNA for CRTH2 (Abe et al., 1999; Sawyer et al., 2002) suggesting that this receptor CRTH2 may have broader functions.

In this paper, we describe the cloning and functional characterization of rat CRTH2 by comparing it to the mouse and human counterparts of the receptor. We further show the effect of the CRTH2/TxA_2 antagonist ramatroban (Sugimoto et al., 2003), a well-established medicine for the treatment of allergic rhinitis, on PGD_2-induced CRTH2 activation in vitro, and in animals exposed to the CRTH2-selective agonist, DK-PGD_2 (Giles and Leff, 1988) (Hirai et al., 2001).

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Methods

Chemicals and Reagents

Ramatroban ((+)-(3R)-3-(4-fluorobenzenesulfonamido)-1,2,3,4-tetra-hydrocarbazole-9-propionic acid) was synthesized at Bayer Yakuhin Ltd. (Shiga, Japan), ridogrel ((E)-5-[[[(3-pyridinyl)[3-(trifluoromethyl)phenyl]-methylen]amino]oxy] pentanoic acid) was prepared by Bayer AG, (Wuppertal, Germany). BWA868C was synthesized by SOGO Pharmaceutical Co. Ltd. (http://www.sogo-pharma.co.jp/index.html). PGD_2 was from Sigma-Aldrich (St. Louis. MO), and [^{3}H]PGD_2 was purchased from Amersham Pharmacia (Buckinghamshire, UK). Sodium butyrate was purchased from Wako Pure Chemicals (Osaka, Japan). Fluo-3AM and pluronic F-127 were purchased from Molecular Probes (Eugene, OR, USA). 13,14-Dihydro-15-Keto-Prostaglandin D_2 (DK-PGD_2) and BW245C and BW868C were purchased from Cayman (Ann Arbor, MI). Pertussis toxin (PTX) was obtained from Calbiochem (La Jolla, CA). Ramatroban, BWA868C, BWC245, and PGD_2 were dissolved in dimethyl sulfoxide (DMSO, Nacalai Tesque, Inc., Kyoto, Japan). As confirmed in preliminary experiments, the concentrations of DMSO in working dilutions used in this study (< 0.1 %) had no effect on receptor binding, Ca^{2+} mobilization and cell migration assays.

All chemicals not further specified were obtained from Wako Chemicals (Osaka, Japan).

Cloning of rat CRTH2

The mouse and human CRTH2 protein sequences were used to search for homologs in the rat EST subset of the Genbank database (http://www.ncbi.nlm.nih.gov) using the program tblastn. Three ESTs were identified, (accession numbers BE112439, BE100786,

and BE113412, all derived from heart tissue) with high homology to the 5' end of the mouse *Crth2* transcript. The ESTs were then used to search the rat high-throughput genome sequences subset of Genbank to find sequence reads from the rat *Crth2* genomic locus. The locus was identified on the sequence contig AC128991.3. After comparing the locus' open reading frame with the coding sequence of mouse Crth2, PCR primers were designed to flank the rat coding sequence for amplification and sequencing. Primer sequences used were 5'-GGGTGCCAGGTTCAGCTCTCCTTT-3' and 5'-ATGGGAGAGGCCTGGGATGTGTTG-3'.

For use as a template for amplification, cDNA was prepared from a pool of rat tissue (strain: Sprague-Dawley) total RNAs (Ambion). First-strand cDNA was synthesized with the SUPERSCRIPT™ First–Strand Synthesis System (Gibco BRL). PCR was then carried out with KOD-Plus-polymerase (Toyobo, Osaka, Japan; 2 min. at 95 ºC, 15 sec. at 94 ºC, 15 sec. at 69 ºC and 3 min. at 72 ºC) for 35 cycles. The PCR products were cloned into a pGEM T easy vector (Promega) for sequencing then subcloned into a pcDNA3.1 vector (Invitrogen, Carlsbad, CA) for expression. Clones were sequenced using the ABI Prism Dye Terminator Cycle Sequencing Reaction kit and analyzed on an ABI Prism 377 sequencing system (Applied Biosystems). The sequence is available under Genbank accession number AY228550.

Expression profiling

Total RNA samples prepared from rat tissues were purchased from Ambion (Austin, TX). First-strand cDNA was synthesized with the SUPERSCRIPT™ First–Strand Synthesis System (Gibco BRL). Semi-quantitative PCR was carried out in 20μl volume containing

20 ng of each tissues cDNA as a template and 0.5 μM of each primer using Hot Star Taq master mix kit (Qiagen). As the corresponding primers, cloning primers were used. PCR condition is as follows: All samples were pre-heated at 95 ºC for 15 min and then subjected to denaturing conditions at 95 ºC for 10 sec. After annealing at 65 C for 15 sec, genes were amplified at 72 C for 1.5 min (35 cycles).

Generation of CRTH2 stable transfectants

The rat and mouse Crth2 gene inserted into the pcDNA3.1(-) expression vector (Invitrogen) was transfected into L1.2 cell (a kind gift from Prof. Eugene Butcher, Standord, CA) by electroporation (250V/1000 mF, Gene Pulser II; Bio-Rad, Hercules, CA). Stable transfectants were selected in the presence of G418 (0.5 mg/ml; Invitrogen). Human CRTH2 stable transfectants were generated as described previously (Sugimoto et al., 2003).

Receptor binding assay

CRTH2/L1.2 cells ($2x10^5$ cells) were mixed with ^{3}H-labeled PGD_2 and various concentrations of test compounds in 100 μl binding buffer (50 mM Tris-HCl pH7.4, 40 mM $MgCl^2$, 0.1% BSA, 0.1%NaN_3) in 96-well U-bottom polypropylene plates. After incubation for 60 min at room temperature, the cell suspension was transferred to a filtration plate (#MAFB, Millipore, Bedford), and radioactivity was measured by a scintillation counter (TopCount, Perkin Elmer Life Sciences). Non-specific binding was determined by incubations in the presence of 1 μM unlabeled PGD_2.

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Ca^{2+} mobilization assay

Ca^{2+} loading buffer was prepared by mixing 1 mM Fluo-3/AM and pluronic F-127 (Molecular Probes) in Ca^{2+} assay buffer (20 mM HEPES, pH 7.6, 0.1% BSA, 1 mM probenecid, Hanks' solution). The CRTH2 transfectants established were resuspended in Ca^{2+} loading buffer at $1x10^7$ cells/ml and incubated for 60 min at room temperature. After the incubation, cells were washed and resuspended in Ca^{2+} assay buffer, then dispensed into transparent-bottomed 96-well plates (3631; Coaster, Corning, NY) at $2x10^5$ cells/well. Cells were incubated with various concentrations of ramatroban for 5 min. at room temperature. The emitted 480 nm fluorescence was measured on a FDSS6000 fluorometer (Hamamatsu Photonics, Hamamatsu, Japan).

Animal experiments

Male Brown Norway or Wistar rats (7wks) were purchased from Charles River (Yokohama, Japan). Animals were kept under standard conditions in a 12 h day/night rhythm with free access to food and water *ad libitum*. All animals received humane care in accordance with international guidelines and national law.

DK-PGD_2, supplied as a solution in methyl acetate, was evaporated under a gentle stream of nitrogen, and reconstituted in ethanol to prepare a stock solution of 30 mg/ml. Prior to injection, the adequate volume of PBS was added to obtain diluted ethanol solutions as specified in Figure legends.

Rats were injected intravenously with DK-PGD_2, or the corresponding volume of the respective solvent with or without pretreatment by ramatroban (dissolved in NaOH, pH-neutralized by HCl addition, and given in a 10% cremophor solution) under slight

anesthetization with ether. At the time points indicated after injection, peripheral blood was collected from the abdominal vein under anesthetization by i.p. injection of urethane (2 g/kg). After blood collection, animals were sacrificed by complete bleeding. Immediately after the bleeding, the left femur was isolated and the femoral head and condoles were removed. The displaceable cells were recovered by flushing the lumen of the femur shaft with 4 ml PBS. Total white blood cells in the samples were counted under the microscope using a hemocytometer. Differential cell counts were performed on the blood smears stained with May-Gruenwald's and Giemsa's solution based on standard morphologic and histological criteria (200 cells counted in total).

Statistics

Unless otherwise stated, data are expressed as means ± SD of at least three independent experiments. Statistical significance was determined using the unpaired Student's *t*-test if applicable or with the Dunnett's or Welch test if variances were non-homogeneous using commercially available statistic software (GraphPad Software, San Diego, CA). *P* values <0.05 were considered as statistically significant (*p <0.05, **p < 0.01).

Results

Molecular cloning, sequence alignment, and tissue expression of rat CRTH2

The gene encoding the rat ortholog of CRTH2 was cloned by search for homologs in the rat EST subset of the Genbank database using the program tblastn and the full cDNA sequence is shown in Figure 1. The sequence of the rat CRTH2 protein bears 89% identity with mouse CRTH2 and 75% identity with human CRTH2, the three differing primarily in their cytoplasmic tail regions (Figure 2).

Semi-quantitative RT-PCR analysis revealed that the tissue distribution of rat (Figure 3) resembles the pattern reported for the mouse CRTH2 mRNA (Abe et al., 1999), showing highest expression in the lung, brain, ovary, and spleen but differs from that in humans, where it is most highly expressed in heart, stomach, small intestine and thymus (Sawyer et al., 2002).

Pharmacological characterization of rat CRTH2

In order to pharmacologically characterize the rat CRTH2 receptor we generated stable rat CRTH2 expressing L1.2 cells and compared them to clones expressing the mouse or human ortholog. Saturation analysis experiments of [^{3}H]-labeled PGD_2 specific binding to recombinant rat, mouse and human CRTH2 transfectants were performed. The three orthologs showed similar binding affinities for PGD_2, similar total binding, and similar numbers of binding sites per cell, respectively (Figure 4, Table.1). Non-labeled PGD_2 inhibited the binding of [^{3}H]-labeled PGD_2 to rat, mouse and human CRTH2 transfectants in a concentration-dependent manner with IC_{50} values of 6.1 nM, 2.6 nM and 2.3 nM,

respectively. The CRTH2 specific agonist, DK-PGD_2, was comparable to PGD_2 in binding affinities to all three orthologs. Interestingly, BWA868C, known as a DP specific antagonist, showed weak but significant inhibition of [^{3}H]-labeled PGD_2 binding at μM concentrations. Ramatroban, a CRTH2/TP antagonist, showed inhibitory effects on the binding of [^{3}H]-labeled PGD_2 to rat, mouse and human CRTH2 transfectants with IC_{50} values around 50 nM. At concentrations equal to or exceeding 1 μM, weak but significant inhibition of PGD_2 binding to CRTH2 was noted for BW245C and ridogrel, questioning their absolute specificity for the DP and TP receptor, respectively (Figure 5, Table 2).

To determine the functional expression of rat CRTH2, PGD_2, DK-PGD_2 and BW245C-stimulated calcium mobilization was monitored. PGD_2 and DK-PGD_2 induced Ca^{2+} mobilization in a concentration-dependent manner with EC_{50} values of 6.9 nM and 4.9 nM in human CRTH2 transfectants, 16 nM and 40 nM in mouse CRTH2 transfectants and 39 nM and 90 nM in rat CRTH2 transfectants, respectively. These effects were completely suppressed by pretreatment with a $G_{\alpha i}$ inhibitor, pertussis toxin (PTX). The DP specific agonist BW245C failed to induce Ca^{2+} mobilization in all three transfectants (Figure 6A and data not shown). Ramatroban concentration-dependently inhibited PGD_2-induced Ca^{2+} mobilization in rat, mouse and human CRTH2 transfectants with similar IC_{50} values in the range of 120-130 nM. In line with its weak inhibitory effect on PGD_2 binding BW868C diminished the calcium response at 10 μM. Again, ridogrel was without any effect (Figure 6B, Table 3). Unlike indomethacin, ramatroban did not show any agonistic effects on Ca^{2+} signaling in CRTH2 transfectants (data not shown).

Induction of leukocyte recruitment from the bone marrow by the CRTH2 agonist, DK-PGD_2

PGD_2 stimulation evoked a weak but significant migratory response of CRTH2 transfected L1.2 cells which was fully abrogated by ramatroban (data not shown). Splenocytes and bone marrow cells isolated from rats showed a distinct mRNA signal for CRTH2. Exposure of the cells to PGD_2 or DK-PGD_2 however did not trigger significant cell migration while a response to other chemoattractants such as platelet activation factor, leukotriene B_4 or IL-5 was observed. In addition, pretreatment of cells with IL-5 did not prime leukocytes for CRTH2 agonists nor did DK-PGD_2 preincubation modify the responses to any chemoattractant investigated (data not shown).

Due to these weak responses *in vitro* we sought to investigate CRTH2-triggered migration *in vivo*. A bolus injection of PGD_2 (not shown) or the CRTH2 specific agonist, DK-PGD_2, led to a time- and dose-dependent leukocyte recruitment from the bone marrow in rats (Figures 7, 8), mice, and guinea pigs (not shown). In all species investigated, the maximum increases in leukocyte numbers in peripheral blood were reached 60 min after injection (Figure 7). Increases in peripheral blood cell counts were paralleled, but not fully compensated, by a drop of cell counts in the bone marrow (Figure 8). Significant effects were seen at DK-PGD_2 doses equal or higher to 100 µg per animal (data not shown). As expected, eosinophils and lymphocytes were affected in particular as exemplified in Figure 7, however, neutrophils also showed a similar tendency.

We then set out to examine whether this DK-PGD_2-induced peripheral blood eosinophilia was mediated by CRTH2 and pretreated rats with ramatroban before DK-PGD_2 injection. Indeed, a dose-dependent reduction of total leukocytes and eosinophils in the peripheral blood was noted in rats pretreated with the CRTH2 antagonist. At the highest ramatroban dose tested, i.e. at 30 mg/kg, cell counts were comparable to levels of solvent controls proving that blockade of the CRTH2 receptor in vivo abrogates DK-PGD_2-induced cell recruitment (Figure 9).

Discussion

In the present study, we identified the rat CRTH2 gene, examined the organ expression pattern and the homology of this gene to its human and mouse counterparts, characterized its pharmacology using gene-transfected cells, and, finally, established an *in vivo* animal model suitable for CRTH2 antagonist evaluation.

Overall identity between the human and rat CRTH2 sequences was 75.6 %, and 96 % between the mouse and rat sequences. Phylogenetic homology comparison studies evidenced high overall similarity to other rat chemoattractant receptors such as LTB_4, N-formyl peptide, C3a, and C5a (not shown).

RT-PCR analysis revealed that rat CRTH2 mRNA was expressed in various tissues including kidney, embryo, liver, brain, thymus, heart, lung, spleen, testis and ovary. Like the mouse (Abe et al., 1999), the rat CRTH2 mRNA signal was strongest in lung, brain and spleen suggesting – together with the high percentage of sequence identity - high functional similarity between rat and mouse CRTH2. The tissue expression pattern of rodents however differs from the human tissue expression profile (Sawyer et al., 2002) pointing towards potential physiological and/or pathophysiological disparities. Indeed, some biological responses reported for its selective agonist, DK-PGD_2, in secretory organs and tissues (Larsen et al., 2002), suggest that CRTH2 has more functions than inducing the migration of leukocytes, inducing shape changes, and regulating the expression of adhesion molecules in Th2 cells, eosinophils and/or basophils (Hirai et al., 2001) (Heinemann et al., 2003). Nevertheless, these well-established functions of CRTH2

fit very nicely into the concept of PGD_2 being a crucial player in the pathogenesis of asthma (Murray et al., 1985; Murray et al., 1986) (Wenzel et al., 1991) (Fujitani et al., 2002) (Matsuoka et al., 2000). Since various animal models for asthma exist to study the underlying disease processes, the investigation of rodent CRTH2 receptor biology and its comparison to human CRTH2 is very helpful and might lead to a better understanding of PGD_2's function under physiological and pathophysiological conditions.

With regard to ligand binding, ligand specificity, G-protein coupling, second messenger generation, and induction of migration CRTH2 receptors from all species studied so far seem to behave alike (c.f. Figures 4-6, Tables 1-3). Rat CRTH2 binds PGD_2 with high affinity (Kd = 11 nM), comparable to human (Kd = 6.3 nM) and mouse (Kd = 9.1 nM). Equilibrium competition binding assays confirmed that DK-PGD_2 is a high affinity ligand for CRTH2. Interestingly, BWA868C, reported to be a selective DP antagonist, showed competitive binding to CRTH2 at high concentrations (c.f. Table 2). This partial antagonism might explain why BWA868C showed a weak inhibitory effect on PGD_2-induced Ca^{2+} flux (Figure 6B) and human eosinophil migration (Sugimoto et al., 2003).

Ligand-triggered calcium mobilization was observed in rat, mouse and human CRTH2 transfectants after PGD_2 or DK-PGD_2 stimulation, respectively. Very recently, Hata *et al.* failed to detect changes in intracellular calcium in their mouse transfectants using ER293 host cells. The host cells we used, L1.2 mouse B cell leukemia cells, clearly are capable of generating a calcium flux in response to chemokine ligand (Gallatin et al., 1983) (Yoshida et al., 1998) and thus CRTH2-triggered calcium mobilization was

comparatively easy to measure. All other data presented in the report by Hata et al. however nicely fit to the results we obtained for murine CRTH2 (Hata et al., 2003).

As expected from studies performed in human and mouse cells, chemotaxis of rat CRTH2 transfectants was observed after PGD_2 stimulation, however the response was comparatively weak (data not shown). The fact that chemotaxis was completely abolished in the presence of ramatroban confirms the general role of CRTH2 as a chemoattractant receptor. Why primary rat cells failed to respond to CRTH2 agonists remains obscure. Either the CRTH2 receptor protein is quickly downregulated during cell isolation or during the pre-incubation period in culture, or some additional unknown factors come into play.

In vivo, however, the migratory response was obvious. Based on our in vitro results performed in transfectants and on a recent report by Heinemann and colleagues, who demonstrated eosinophil recruitment from the bone marrow in the bloodstream following Δ12-PGJ_2 injection into the hind limbs of guinea pigs (Heinemann et al., 2003), we challenged rats intravenously with DK-PGD_2. In addition to cell recruitment from the bone marrow which accounts for approximately 50% of the total cell increase in the blood, DK-PGD_2 seems to recruit leukocytes from additional, not yet identified sources of the body. Although the specificity of this CRTH2 agonist is unknown in vivo and additional effects cannot be excluded, the leukocyte recruitment observed was clearly CRTH2-mediated, since ramatroban pretreatment dose-dependently blocked eosinophilia in this model (c.f. Figures 7-9). Preliminary data obtained in our laboratory argue for a

direct, IL-5-independent mechanism of eosinophil recruitment from the bone marrow via CRTH2 activation however further studies are required for final clarification.

Collectively, this report characterizes the CRTH2 receptor of the rat, presents evidence for an in vivo role of CRTH2 in leukocyte recruitment, and confirms ramatroban as a potent small molecule antagonist and a useful tool to study CRTH2 biology.

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Footnotes

[1] These authors contributed equally to this work.

Legends for figures

Figure 1: Rat CRTH2 cDNA gene sequence

Full cDNA sequence of the rat CRTH2 gene. The sequence is available under Genbank accession number AY228550. For details regarding the cloning procedure see *Methods* section.

Figure 2: Rat CRTH2 cDNA amino acid sequence alignment

Amino acid sequence alignment of human, mouse, and rat CRTH2. Identical residues are shown with white backgrounds, similar residues with gray backgrounds, and dissimilar residues with black backgrounds. Identity between the human and mouse sequences is 77.5%, between the human and rat sequences is 75.6%, and between the mouse and rat sequences is 96%. The GenBank accession numbers for the sequences are human, NP_004769; mouse, NP_034092; and rat, AAP57088.

Figure 3: RT-PCR detection of rat *Crth2* expression in tissue cDNA

Rat *Crth2* was amplified by RT-PCR (35 cycles) using equal amounts of total RNA derived from each of the tissues shown to give a product of 1297 bp. Amplification performed with no template cDNA added (designated H_2O) is shown as a negative control. The housekeeping genes β2 microglobulin (β2m) and Gapdh (GDH) were

amplified from the same samples for 20 PCR cycles to ensure that the starting cDNA amounts for each sample were approximately equivalent.

Figure 4: Saturation binding of [^{3}H]-labeled PGD_2 to rat CRTH2 transfectants

2 x 10^5 rat CRTH2 transfectants were incubated in a total volume of 100 µl with various concentrations of [^{3}H]-labeled PGD_2 in the absence (total-binding) or presence (non-specific binding) of 1 µM of cold PGD_2. Binding of labeled PGD_2 to the CRTH2 expressing cells was assessed by scintillation counting after washing of the filters twice (see *Methods* for details), calculated based on the cpm standard curve obtained by labeled PGD_2 and expressed as fmoles/cell equivalents. Means of each data point determined in duplicate is shown. Graph represents one out of two independently performed experiments with very similar results.

Figure 5: Competitive binding of [^{3}H]-labeled PGD_2 to rat CRTH2 transfectants

A. Competitive binding of [^{3}H]-labeled PGD_2 (1 nM) to rat CRTH2 by non-labeled PGD_2 (n = 4), DK-PGD_2 (n = 4), and BW245C (n = 3). B. Effects of ramatroban (n = 4), BWA868C (n = 4) and ridogrel (n = 3) are shown. Experiments were performed as described in the *Methods* section. Data represent mean values ± SD. Significant differences between test compound-treated and untreated binding was analyzed by Student's *t* test ; *$p < 0.05$, **$p < 0.01$.

Figure 6: PGD_2- and DK-PGD_2-induced Ca^{2+} mobilization in rat CRTH2 transfectants

A. Concentration-response of Ca^{2+} mobilization in rat CRTH2 transfectants induced by PGD_2 (n = 3) and DK-PGD_2 (n = 3), and the effect of PTX (1 μg/ml, pre-incubated for 2 h) on this response (n = 2). B. Effects of ramatroban (n = 5), BWA868C (n = 3) and ridogrel (n = 3) on PGD_2 (10 nM)-induced Ca^{2+} mobilization in rat CRTH2 transfectants. Data represent mean values ± SD. Significant differences between test compound-treated and untreated Ca^{2+} mobilization was analyzed by Student's *t* test ; **p <0.01.

Figure 7: Changes in peripheral blood leukocyte counts in rats following injection of DK-PGD_2

Rats were intravenously injected with DK-PGD_2 (300 μg/rat) or the corresponding volume of vehicle (2% EtOH in PBS). At the time points indicated, animals were killed and heparinized blood was collected for the determination of total white blood cell numbers and differential leukocyte staining. Data are mean values ± SEM of 3 (vehicle) or 5 (DK-PGD_2) animals each. Statistical differences were analyzed using Dunnett's method (*P<0.05, **P<0.01).

Figure 8: Changes in bone marrow leukocyte counts in rats following injection of DK-PGD_2

Rats were treated as described in legend to Figure 7, and bone marrow was obtained as detailed in the *Methods* section. Data are mean values ± SEM of 3 (vehicle) or 5 (DK-

PGD_2) animals each. Statistical differences were analyzed using Dunnett's method (**P*<0.05, ***P*<0.01).

Figure 9: Ramatroban prevents eosinophilia induced by CRTH2 activation

Rats were intravenously injected with various doses of ramatroban or the corresponding volume of vehicle (10% cremophor in pH neutralized NaOH) prior to injection of DK-PGD_2 (250 μg/rat) or its solvent (1 ml of 0.83% ethanol/PBS). Sixty min later, heparinized blood was collected from the abdominal vein for the determination of total white blood cell numbers and differential leukocyte staining. Data are mean values ± SEM of 3 (solvent controls, S) or 5 (DK-PGD_2 (DK) with or without ramatroban) animals each. Statistical differences were analyzed using Dunnett's method (**P*<0.05, ***P*<0.01).

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Table 1: Binding characteristics of [^{3}H]PGD_2 to recombinant CRTH2 expressed on L1.2 cells

	Rat	Human	Mouse
Kd (nM)	11	6.3	9.1
Bmax (pM)	530	450	600
Binding sites/cell	1.6×10^6	1.4×10^6	1.8×10^6

CRTH2 transfectants (2×10^5 in 100 μl for rat, 3×10^5 cells in 100 μl for human and mouse) were incubated with various concentrations of [^{3}H]-labeled PGD_2 in the presence of 1 μM cold PGD_2. Binding sites were calculated from Bmax values and the cell numbers used in the experiments. Each data point was determined in duplicate. Data represent the mean of two independent experiments.

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Table 2: Determination of IC_{50} values (nM) for various ligands in inhibiting [^{3}H]PGD_2 binding to CRTH2

	Rat	Human	Mouse
PGD_2	6.1 ± 3.4	2.6 ± 1.2	2.3 ± 0.2
DK-PGD_2	9.4 ± 1.5	18.1 ± 5.3	6.1 ± 0.5
BW245C	> 10000	> 10000	> 10000
Ramatroban	45 ± 12	47 ± 13	60 ± 13
BW868C	1039 ± 372	3945 ± 689	930 ± 216
Ridogrel	> 10000	> 10000	> 10000

Various concentrations of non-labeled ligands were added to the reaction mixture of 1 nM [^{3}H]-labeled PGD_2 and CRTH2 transfectants. Data are expressed as mean values ± SD from 3-4 independent determinations.

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Table 3: Inhibition of PGD_2-induced Ca^{2+} mobilization in CRTH2 transfectants by various receptor antagonists

	Rat	Human	Mouse
Ramatroban	123 ± 28	118 ± 26	133 ± 37
BW868C	> 10000	> 10000	7959 ± 881
Ridogrel	> 10000	> 10000	> 10000

Various concentrations of test compounds were added to rat, human or mouse CRTH2 transfectants before exposure of cells to PGD_2 (10 nM). Where calculable, data are expressed as mean values ± SD (nM) from 3 independent determinations.

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```
   1  ATGGCCAACA TCACACTGAA GCCGCTCTGT CCACTCTTGG AGGAGATGGT
      M  A  N  I   T  L  K   P  L  C   P  L  L  E   E  M  V
  51  CCAGCTTCCA AACCACAGCA ACTCTAGCCT CCGCTATATT GACCACGTGT
       Q  L  P   N  H  S  N   S  S  L   R  Y  I   D  H  V  S
 101  CGGTGCTGTT GCACGGGCTG GCCTCCCTGC TGGGCCTGGT GGAAAACGGA
        V  L  L   H  G  L   A  S  L  L   G  L  V   E  N  G
 151  CTCATCCTGT TTGTGGTGGG CTGTCGCATG CGCCAGACGG TGGTCACTAC
      L  I  L  F   V  V  G   C  R  M   R  Q  T  V   V  T  T
 201  CTGGGTGCTG CACCTGGCGC TGTCCGACTT GTTAGCCGCT GCCTCTCTGC
       W  V  L   H  L  A  L   S  D  L   L  A  A   A  S  L  P
 251  CTTTCTTCAC CTACTTTCTG GCAGTGGGCC ACTCGTGGGA ACTGGGCACC
        F  F  T   Y  F  L   A  V  G  H   S  W  E   L  G  T
 301  ACTTTCTGCA AGCTACATTC CTCGGTCTTC TTCCTCAACA TGTTTGCCAG
      T  F  C  K   L  H  S   S  V  F   F  L  N  M   F  A  S
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        G  F  L   L  S  A  I   S  L  D   R  C  L   Q  V  V  R
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      C  L  M  L   W  A  L   A  V  L   N  T  V  P   Y  F  V
 501  GTTCAGGGAC ACCATCCCGC GGCGTGATGG CCGCATCATG TGCTATTACA
       F  R  D   T  I  P  R   R  D  G   R  I  M   C  Y  Y  N
 551  ACATGCTGCT CTTGAATCCA GGGTCTGACC GCGACACCAC GTGCGACTAC
       M  L  L   L  N  P   G  S  D  R   D  T  T   C  D  Y
 601  CGTCAGAAGG CCCTGGCGGT CAGCAAGTTC CTGTTGGCCT TCATGGTACC
      R  Q  K  A   L  A  V   S  K  F   L  L  A  F   M  V  P
 651  TCTGGCCATC ATCGCCTCGA GCCACGTGGC TGTGAGCCTA CAACTGCATC
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       R  R  V   R  E  Q   S  Q  E  K   Q  G  S   L  S  C
1201  ACTTTGGATT AG
      T  L  D  *
```

Figure 1, Shichijo et al.

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CRTH2 amino acid seuqence alignment:

```
                  1                                                  50
Human_CRTH2     (1) MSANATLKPLCPILEQMSRLQSHSNTSIRYIDHAAVLLHGLASLLGLVEN
Mouse_CRTH2     (1) M-ANVTLKPLCPLLEEMVQLPNHSNSSLRYIDHVSVLLHGLASLLGLVEN
  Rat_CRTH2     (1) M-ANITLKPLCPLLEEMVQLPNHSNSSLRYIDHVSVLLHGLASLLGLVEN

                  51                                                 100
Human_CRTH2    (51) GVILFVVGCRMRQTVVTTWVLHLALSDLLASASLPFFTYFLAVGHSWELG
Mouse_CRTH2    (50) GLILFVVGCRMRQTVVTTWVLHLALSDLLAAASLPFFTYFLAVGHSWELG
  Rat_CRTH2    (50) GLILFVVGCRMRQTVVTTWVLHLALSDLLAAASLPFFTYFLAVGHSWELG

                 101                                                 150
Human_CRTH2   (101) TTFCKLHSSIFFLNMFASGFLLSAISLDRCLQVVRPVWAQNHRTVAAAHK
Mouse_CRTH2   (100) TTFCKLHSSVFFLNMFASGFLLSAISLDRCLQVVRPVWAQNHRTVAVAHR
  Rat_CRTH2   (100) TTFCKLHSSVFFLNMFASGFLLSAISLDRCLQVVRPVWAQNHRTVAAAHR

                 151                                                 200
Human_CRTH2   (151) VCLVLWALAVLNTVPYFVFRDTISRLDGRIMCYYNVLLLNPGPDRDATCN
Mouse_CRTH2   (150) VCLMLWALAVLNTIPYFVFRDTIPRLDGRIMCYYNLLLWNPGPDRDTTCD
  Rat_CRTH2   (150) VCLMLWALAVLNTVPYFVFRDTIPRRDGRIMCYYNMLLLNPGSDRDTTCD

                 201                                                 250
Human_CRTH2   (201) SRQAALAVSKFLLAFLVPLAIIASSHAAVSLRLQHRGRRRPGRFVRLVAA
Mouse_CRTH2   (200) YRQKALAVSKFLLAFMVPLAIIASSHVAVSLRLHHRGRQRTGRFVRLVAA
  Rat_CRTH2   (200) YRQKALAVSKFLLAFMVPLAIIASSHVAVSLQLHHRGRQRTGRFVRLVAA

                 251                                                 300
Human_CRTH2   (251) VVAAFALCWGPYHVFSLLEARAHANPGLRPLVWRGLPFVTSLAFFNSVAN
Mouse_CRTH2   (250) IVVAFVLCWGPYHIFSLLEARAHSVTTLRQLASRGLPFVTSLAFFNSVVN
  Rat_CRTH2   (250) IVVAFILCWGPYHIFSLLEARAHSVTTLRQLASRGLPFVTSLAFFNSVVN

                 301                                                 350
Human_CRTH2   (301) PVLYVLTCPDMLRKLRRSLRTVLESVLVDDSELGGA-----GSSRRRRTS
Mouse_CRTH2   (300) PLLYVFTCPDMLYKLRRSLRAVLESVLVEDSDQSGG-----LRNRRRRAS
  Rat_CRTH2   (300) PLLYVLTCPDMLHKLRRSLLTVLESVLVEDSDLSTGPGKRCRRRHRRRAS

                 351                                                 400
Human_CRTH2   (346) STARSASPLALCSRPEEPRGPARLLGWLLGSCAASPQTGPLNRALSSTSS
Mouse_CRTH2   (345) STATPASTLLLADRIPQLR-PTRLIGWMRRGSAEVPQRV-----------
  Rat_CRTH2   (350) STTTPASTLLLADRFPQLR-PARLIGWMRRGSAELPRRVREQSQEKQGSL

                 401
Human_CRTH2   (396) -----
Mouse_CRTH2   (383) -----
  Rat_CRTH2   (399) SCTLD
```

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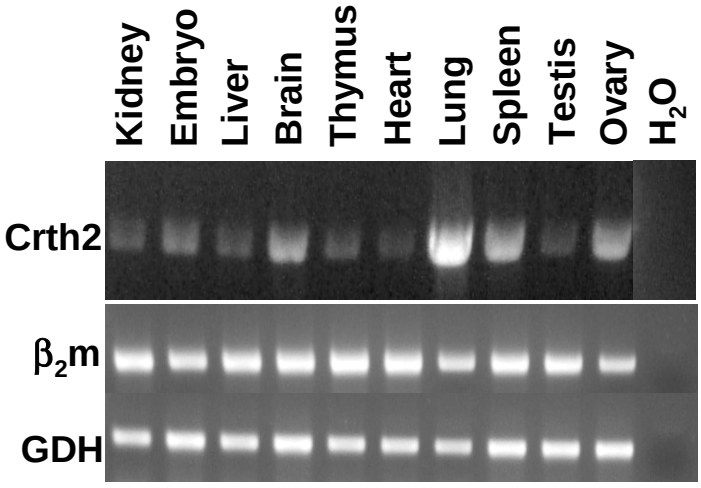

JPET #55442 Figure 3, Shichijo et al.

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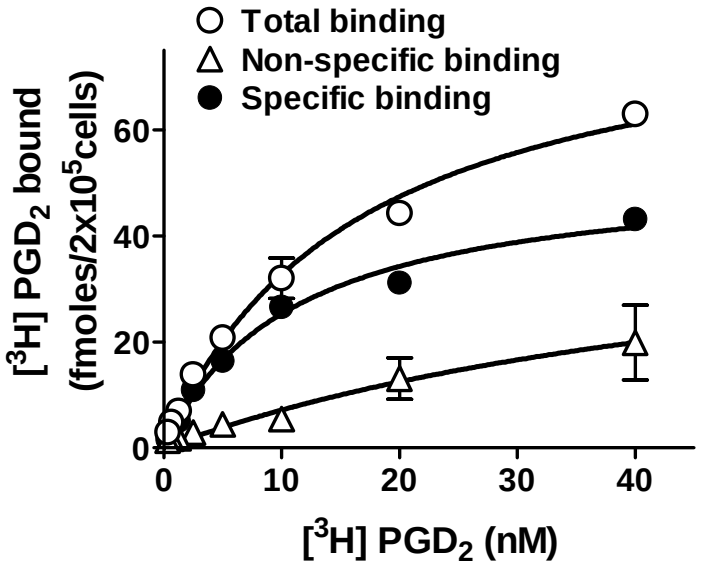

JPET #55442 Figure 4, Shichijo et al.

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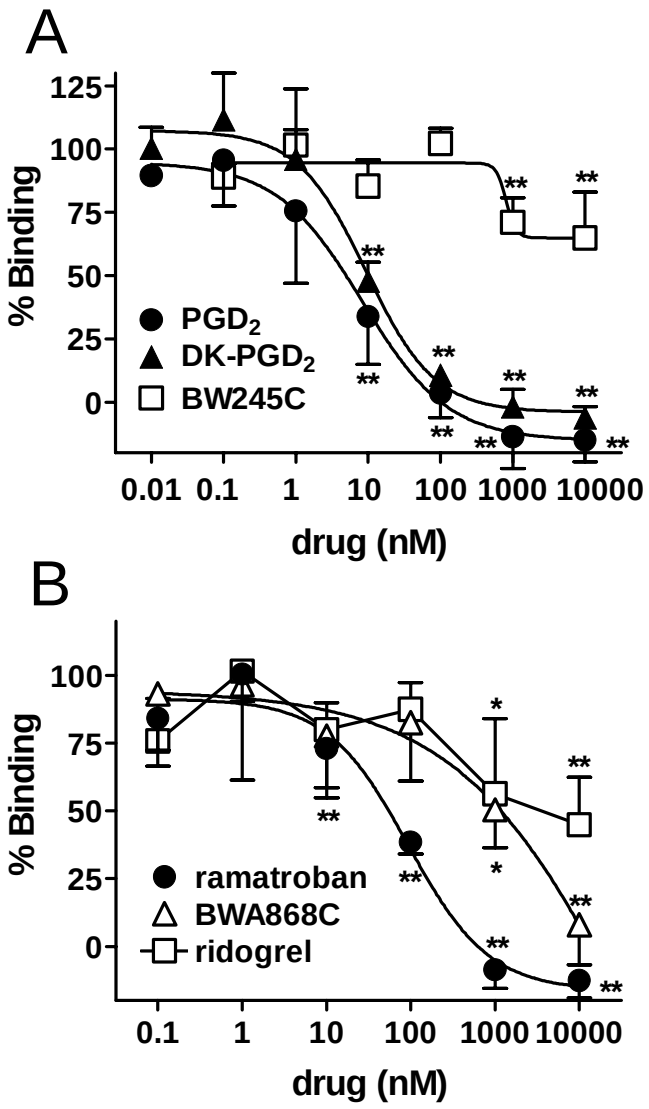

JPET #55442, Figure 5, Shichijo et al.

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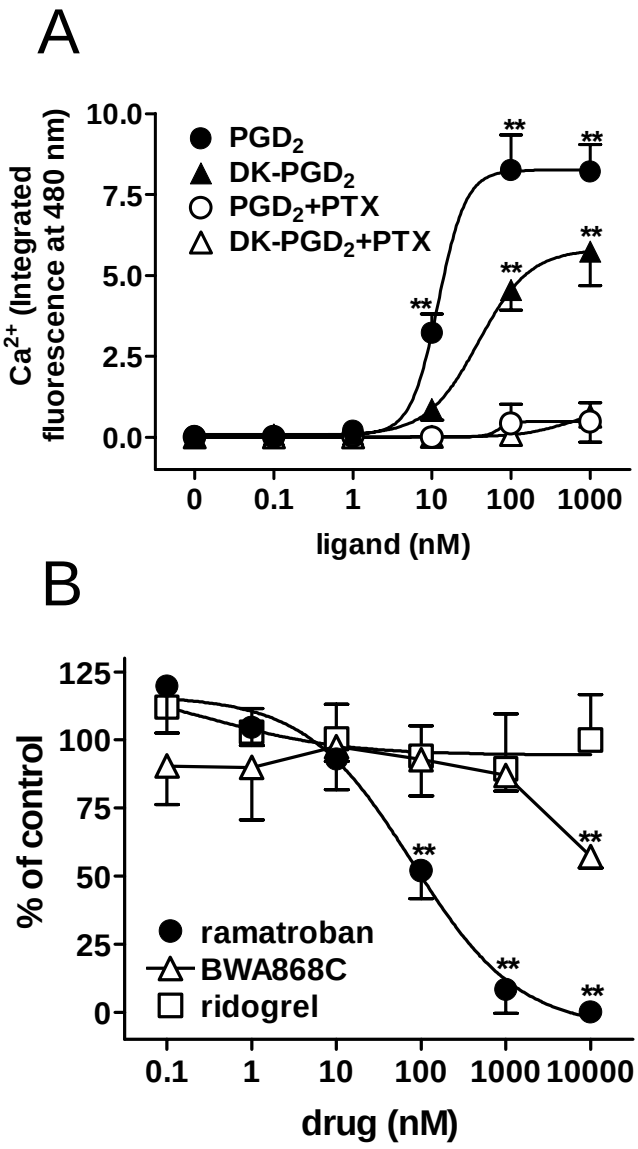

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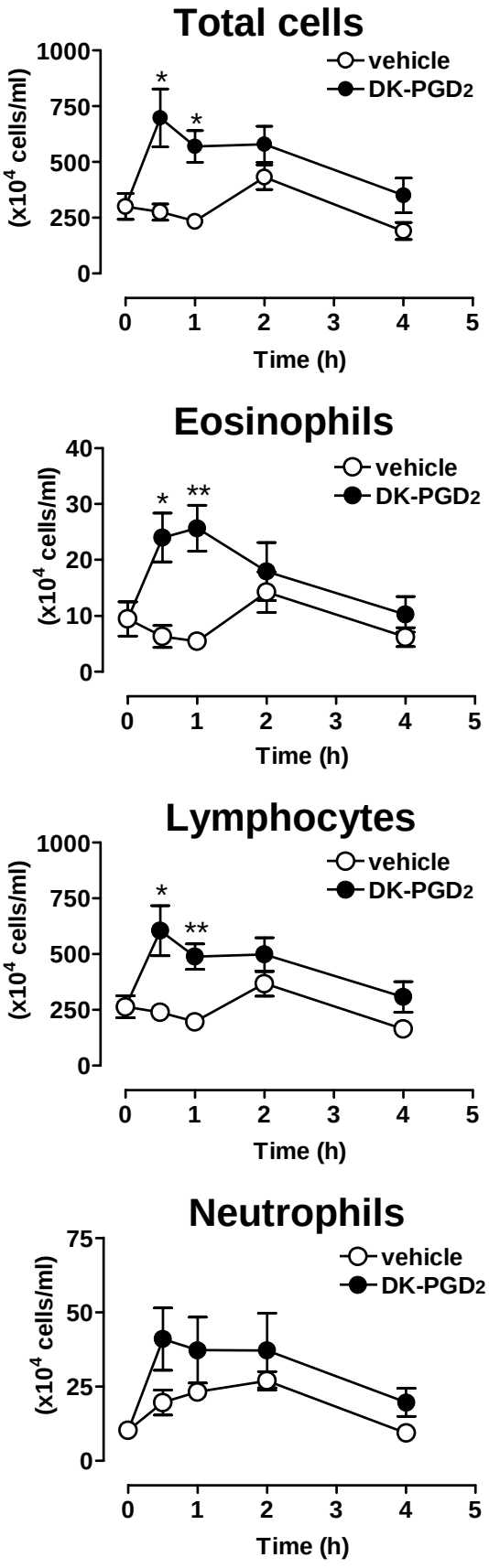

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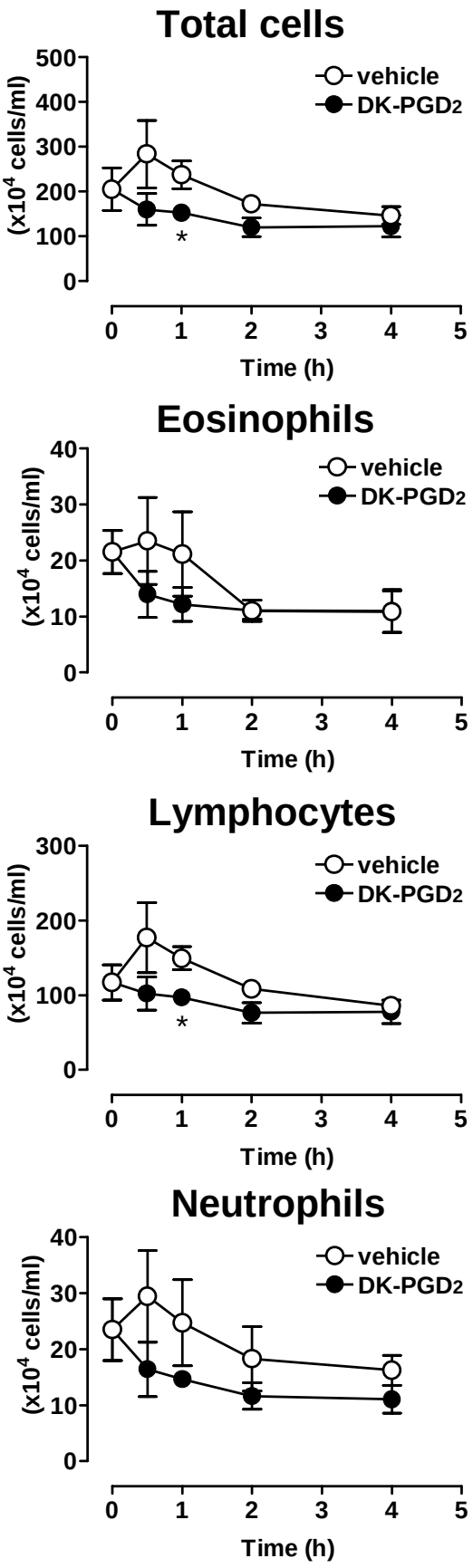

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