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ORIGINAL ARTICLE

The mRNA level of Charcot–Leyden crystal protein/galectin-10 is a marker for CRTH2 activation in human whole blood *in vitro*

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Abstract

CRTH2 is one of the prostaglandin D_2 receptors and plays a proinflammatory role in allergic diseases. Gene expression markers in whole blood induced by CRTH2 activation have not previously been reported. Using microarray analyses of 54 675 genes, we revealed modest gene expression changes in human whole blood stimulated *in vitro* by a selective CRTH2 agonist, DK-PGD_2. Five genes were found to exhibit 1.5- to 2.6-fold changes in expression. The expression of Charcot–Leyden crystal protein/galectin-10 (*CLC*/Gal-10) in particular was consistently enhanced in human whole blood stimulated by DK-PGD_2, as confirmed by quantitative real-time polymerase chain reaction analyses. DK-PGD_2-induced increases in blood *CLC*/Gal-10 mRNA levels were largely attenuated by the CRTH2 antagonist CAY10471.Thus, the DK-PGD_2-induced *CLC*/Gal-10 mRNA level can serve as a potential marker for monitoring pharmacodynamic effects of blood exposure to CRTH2 modulating agents.

Keywords: *Prostaglandin D_2; allergic diseases; eosinophils; basophils; Th2 cells; microarray*

Introduction

Prostaglandin D_2 (PGD_2), produced primarily by IgE-activated mast cells upon allergen exposure, has been recognized as one of the key inflammatory mediators in atopic diseases such as asthma, allergic rhinitis, allergic conjunctivitis and atopic dermatitis (Fujishima et al. 2005, Luster & Tager 2004, Martin & Peebles 2006, Matsuoka et al. 2000, Torres et al. 2008). The biological responses of PGD_2 are largely mediated through its interaction with two G protein-coupled receptors, the D-type prostanoid receptor (DP or DP1) and the chemoattractant receptor homologous molecule expressed on T helper (Th) 2 cells (CRTH2 or DP2). CRTH2 is preferentially expressed on haematological cells such as Th2 cells, eosinophils, basophils, mast cells and monocytes (Nagata & Hirai 2003, Nagata et al. 1999), whereas DP is more widely expressed and is present in both haematological and non-haematological cell types (Breyer et al. 2001, Hata & Breyer 2004, Powell 2003). DP and CRTH2 regulate distinct cellular responses with opposing effects in complex inflammatory processes (Kostenis & Ulven 2006, Sandig et al. 2007). While the role of DP in allergic inflammation is unclear, CRTH2 has been shown to play proinflammatory roles in promoting the accumulation of lymphocytes and eosinophils (Satoh et al 2006, Shiraishi et al. 2005, Shirasaki et al. 2009) and the production of Th2 cytokines and IgE during allergic inflammation (Satoh et al. 2006). Sequence variants of the gene encoding CRTH2, which differentially influence its mRNA stability, have also been shown to be associated with asthma (Huang et al. 2004, Wang et al. 2009). Therefore, developing selective antagonists of CRTH2 is desirable for the potential treatment of atopic disorders.

Pharmacodynamic markers in whole blood are widely used to aid drug discovery and development. CRTH2 is known to mediate several PGD_2-induced responses such as cell shape change, chemotaxis, CD11b upregulation

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and Th2 cytokine production in several types of blood leukocytes, including basophils, eosinophils and Th2 cells (Hirai et al. 2001, Monneret et al. 2001, Tanaka et al. 2004, Xue et al 2005). In whole blood samples, eosinophils are preferred cells for detecting CRTH2 activation due to their relative abundance and high granularity compared with those of basophils and Th2 cells. Consequently, eosinophil cell shape change and CD11b upregulation have been utilized as biomarkers in whole blood for evaluating ligand effects on CRTH2 activation *in vitro* (Monneret et al. 2001, Sandham et al. 2007, 2009). These functional responses are induced rapidly, usually within 5–15 min of CRTH2 activation. They are detected using flow cytometry initially to identify the eosinophil population in whole blood samples, followed by analysing changes in mean value of forward scatter for cell shape change or by comparing fluorescence intensity after labelling blood cells with fluorochrome-conjugated antibody for CD11b surface expression levels.

Alterations of gene expression in whole blood have been practically applied as disease markers and for monitoring pharmacodynamic activity of drug exposure; however, detectable gene expression changes in human whole blood induced by CRTH2 activationhave not been reported. In the present studies, we aim to identify genes that are differentially expressed in human whole blood upon CRTH2 activation *in vitro* by conducting a genome-wide gene expression profiling analysis using Affymetrix gene chips, followed by confirmation using quantitative real-time polymerase chain reaction (PCR). We have discovered that the expression of at least one gene, Charcot–Leyden crystal protein/galectin-10 (*CLC*/Gal-10), is consistently induced in human whole blood upon stimulation by a selective CRTH2 agonist, 13,14-dihydro-15-keto prostaglandin D_2 (DK-PGD_2) (Hirai et al. 2001). The increase in *CLC*/Gal-10 mRNA level induced by CRTH2 activation in whole blood is largely attenuated in the presence of a CRTH2 antagonist *in vitro*. Thus, the level of *CLC*/Gal-10 mRNA in whole blood induced by DK-PGD_2 can serve as a potential biomarker for monitoring pharmacodynamic effects of exposure to a CRTH2 modulating agent.

Methods

Collection of human blood and CRTH2 activation in vitro

Human whole blood samples were collected from healthy volunteers after obtaining informed consent using BD Vacutainer® venous blood collection tubes with K_2EDTA (BD Biosciences, San Diego, CA, USA; cat. no. 367861). The whole blood samples were kept at room temperature and used for experiments within 1 h of collection. For activation of CRTH2 *in vitro*, human whole blood samples were pre-incubated at 37°C for 10 min before adding 1 μM of selective CRTH2 agonist DK-PGD_2 (Cayman Chemical, Ann Arbor, MI, USA;cat. no. 12610) or 0.005% DMSO as vehicle control. The blood samples were then incubated at 37°C for various times as indicated below for the eosinophil shape change assay or RNA isolation for either microarray or quantitative real-time PCR analysis. For some samples, the prewarmed blood was first incubated with 10 μM of selective CRTH2 antagonist CAY10471 (Ulven & Kostenis 2005) for 10 min at 37°C before adding DK-PGD_2. CAY10471 was obtained from Cayman Chemical (cat. no. 10006735).

Eosinophil shape change assay

Whole blood samples were evaluated for changes in eosinophil shape to ensure CRTH2 activation before carrying out microarray or quantitative real-time PCR analysis on the same samples. The procedure used was similar to the whole blood gated autofluorescence forward scatter (GAFS) assay described previously (Bryan et al. 2002, Schratl et al. 2007). Ten minutes after the addition of DK-PGD_2 (or vehicle), blood samples (100 μl) were removed from the incubation tubes and added to 250 μl of ice-cold fixation buffer (0.25% paraformaldehyde in Dulbecco's phosphate-buffered saline). After incubation on ice for 5 min, red blood cells were lysed by adding 1.75 ml of ice-cold lysis buffer (150 mM NH_4Cl and 10 mM $KHCO_3$, pH 7.2) and incubating on ice for an additional 10 min. The remaining leukocytes were then washed once and re-suspended in 250 μl of ice-cold fixation buffer for the GAFS assay using a FACSAria flow cytometer (BD Biosciences). Eosinophils were identified and gated by their high autofluorescence. Shape changes induced by DK-PGD2 were quantified as increases in forward scatter compared with that of vehicle control.

Microarray analysis

Blood was sampled at 1, 3 and 24 h after the addition of DK-PGD_2 for total RNA isolation for the microarray analyses. Total RNA was extracted using a PAXgene™ Blood RNA Kit (PreAnalytiX GmbH, Hombrechtikon, Switzerland) and concentrated with a GeneChip® Blood RNA Concentration Kit (Affymetrix, Santa Clara, CA, USA). To improve sensitivity of gene expression analysis on GeneChips arrays, total RNA was converted to cDNA according to the Affymetrix Globin Reduction protocol using the human globin-reduction primers from IDT (Integrated DNA Technologies, Coralville, IO, USA) and Affymetrix One-Cycle cDNA Synthesis Kit with the addition of reagents from the Poly-A RNA Control Kit. cRNA was produced with the Affymetrix IVT Labeling Kit. Hybridization Mix was created using the

Hybridization Control Kit (Affymetrix, P/N 900454) and included Control Oligo B2. The samples were hybridized on AffymetrixGeneChip® Human Genome U133 Plus 2.0 Array. Staining and washing steps were performed as suggested by the manufacturer. Each hybridized array was scanned with a GeneChip Scanner 3000 7G (Agilent/Affymetrix). Image analysis was performed with Affymetrix GCOS software.

Microarray data from 72 samples (blood from each donor was treated with vehicle or DK-PGD_2 in duplicate at three time points, resulting in 12 samples from each of six donors, for 72 samples in total) were QC via bioconductor packages. A complete randomized block design was invoked to assign experimental samples to the different treatments/time points. In this design each donor served as a blocking factor, and blood samples from each donor were treated with both vehicle and CRTH2 agonist at all the time points. The normalized data (RMA, quantile–quantile) were analysed at the probe set level using a mixed model with treatment, time, and the interaction between treatment and time as fixed factors, and donor as the random effect. The least square mean of each treatment/time combination was compared using *t*-statistics. The genes/probe sets with p-values<0.05 were filtered out as potential differentially expressed genes. This less stringent statistical cut-off reflects the moderate gene expression change induced by CRTH2 activation in highly heterogeneous samples in which the responsive cell types account for only a small portion of total cells.

Quantitative real-time polymerase chain reaction

The expression levels of three genes, *CLC*/Gal-10, Nedd4 family interacting protein 2 (*NDFIP2*) and purinergic receptor P2Y14 (*P2RY14*), all identified from microarrary analysis, were chosen for quantitative real-time PCR confirmation studies. To identify a suitable reference gene, we examined the expression of three genes, β-actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-glucuronidase (*GUSB*). *GUSB* was found to have the most stable expression across all conditions and replicates and therefore was chosen as the reference control gene. Blood was sampled 3 h after the addition of DK-PGD_2. Total RNA was extracted using the same PAXgene™ Blood RNA Kit mentioned above and converted to cDNA using the Ovation™ RNA Amplification System V2 with Ovation™ WB reagent (NuGen, San Carlos, CA, USA). Real-time PCR reactions were performed in triplicate in a 384-well optical plate using an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with the following conditions: 45°C for 2 min, then 95°C for 3 min, followed by 40 cycles at 95°C for 15 s and 60°C for 45 s. The reaction mixture (20 µl) consisted of10 µl PerfeCTa®qPCRFastMix, ROX™ (Quanta, Gaithersburg, MD, USA), 1 µl of primers (10 µM) and dye-labelled TaqMan® probe (10 µM), 2 µl of RNAase-free water, and 7 µl of cDNA (10 $ng\mu l^{-1}$). The primers and dye-labelled TaqMan® probes were either custom designed or are commercially available from Applied Biosystems. Their oligonucleotide sequences or Applied Biosystems catalogue numbers are as follows: *CLC*/Gal-10 (NM_001828): forward primer (328–349) GGATGGCCAAGAATTTGAACTG, reverse primer (409–388)GGTGTAAGAGGATTGGCCATTG, probe (355–386) CCATTACCTGGTACTTATCTGGCAGCACTGAG; *P2RY14* (NM_001081455): forward primer (2261–2280) CAGCAACTTCCCCTGTTCAA, reverse primer (2359–2335) TCCCATTCGCCAGTAGATTAATATA, probe (2302–2328) ACTGGGAAAAAGACACACCCACACCGT; *NDFIP2* (NM_019080): Applied Biosystems, cat. no. Hs00324851_ml; *GUSB* (NM_000181): Applied Biosystems, cat. no. Hs99999908_ml.

The expression levels of target genes were normalized to the reference gene (*GUSB*) levels and represented as relative expression (E), $E=2^{(\Delta Ct)}$, where ΔCt is the difference between reference and target gene cycles at which the amplification exceeds an arbitrary threshold.

Results

Gene expression profile in human whole blood after CRTH2 activation in vitro

Whole blood samples were obtained from six healthy volunteers and stimulated with a selective CRTH2 agonist, DK-PGD_2 (1 µM), *in vitro*. These blood samples were initially evaluated for eosinophil shape change at

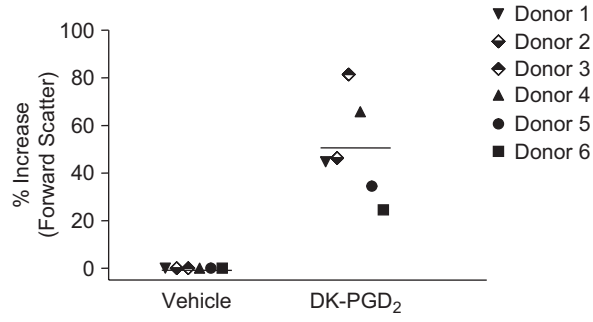

Figure 1. Eosinophil shape change induced by 13,14-dihydro-15-keto prostaglandin D_2 (DK-PGD_2).Samples of uncoagulated whole blood obtained from six healthy volunteers were stimulated with DK-PGD_2 (1 µM) or vehicle (0.005% DMSO) at 37°C for 10 min.Eosinophil shape change was measured by the gated autofluorescence forward scatter assay as described in Methods. Data are expressed as percentage increase in forward scatter comparing DK-PGD_2-treated samples to vehicle controls from the same donor and are mean values of duplicate determinations.

10 min after the addition of DK-PGD_2. As indicated in Figure 1, shape change of eosinophils as measured by mean value of forward scatter was increased in the whole blood from all six donors after the addition of DK-PGD_2. We then proceeded with the genome-wide microarray analysis for these samples to profile gene expression changes in whole blood at 1, 3 and 24 h after the addition of the CRTH2 agonist. We found that DK-PGD_2 induced consistent mild to moderate changes in gene expression in whole blood. Among the 54 675 Affymetrix probe sets tested, the following numbers of probe sets changed significantly in their intensities (with $p<0.05$, *t*-test): 248 probe sets at 1 h, 260 probe sets at 3 h, and 1819 probe sets at 24 h (Figure 2). Only nine genes at 1 h, 18 genes at 3 h and 28 genes at 24 h were found to have greater than 1.3-fold change in their expression after CRTH2 activation in whole blood.

The hierarchical analysis of 18 top differentially expressed genes induced by DK-PGD_2 in whole blood is summarized in Figure 3. Notably, early growth response 3 (*EGR3*, 1.59-fold) and early growth response 1 (*EGR1*, 1.56-fold), were the two most upregulated genes at 1 h after DK-PGD_2 stimulation. *CLC*/Gal-10 (2.56-fold) was the most upregulated gene (Figure 3) followed by *NDFIP2*(1.58-fold) and *P2RY14*(1.53-fold) at 3 h after DK-PGD_2 stimulation. None of the genes that were upregulated at 24 h reached 1.5-fold.

To examine further the changes in CLC/Gal-10 expression, the microarray expression levels for the protein in all six donors are re-plotted in Figure 4. DK-PGD_2-induced CLC/Gal-10 expression can be observed as early as 1 h after stimulation although the fold change is small (Figure 4). The *CLC*/Gal-10 mRNA level reached a peak at 3 h and returned to the level of vehicle control at 24 h after stimulation (Figure 4).

Confirmation of CLC expression induced by DK-PGD2 in human whole blood

We conducted quantitative real-time PCR to confirm the finding of the top three upregulated genes, *CLC*/Gal-10, *NDFIP2* and *P2RY14* that were identified from the Affymetrix gene chip study at 3 h after DK-PGD_2 stimulation. A selective CRTH2 antagonist, CAY10471 (Ulven&Kostenis 2005), was included in the study design to verify that the induction of gene expression by DK-PGD_2 was mediated by CRTH2.

Whole blood samples were obtained from five additional donors and evaluated for change in forward scatter (shape) of eosinophils 10 min after DK-PGD_2 stimulation. Similar to the data observed in the previous six donors, DK-PGD_2 induced increases in forward scatter of eosinophils in these five donors, which could be blocked by CRTH2 antagonist CAY10471 (Figure 5). We then proceeded with the quantitative real-time PCR analysis to determine expression levels of the three selected genes at 3 h after DK-PGD2 stimulation.

As shown in Figure 6, the expression of the *CLC*/Gal-10 gene was consistently increased in all five donors 3 h after DK-PGD_2 stimulation when measured using quantitative real-timePCR. The fold increases ranged from 1.63- to 4.12-fold depending on the donor. The

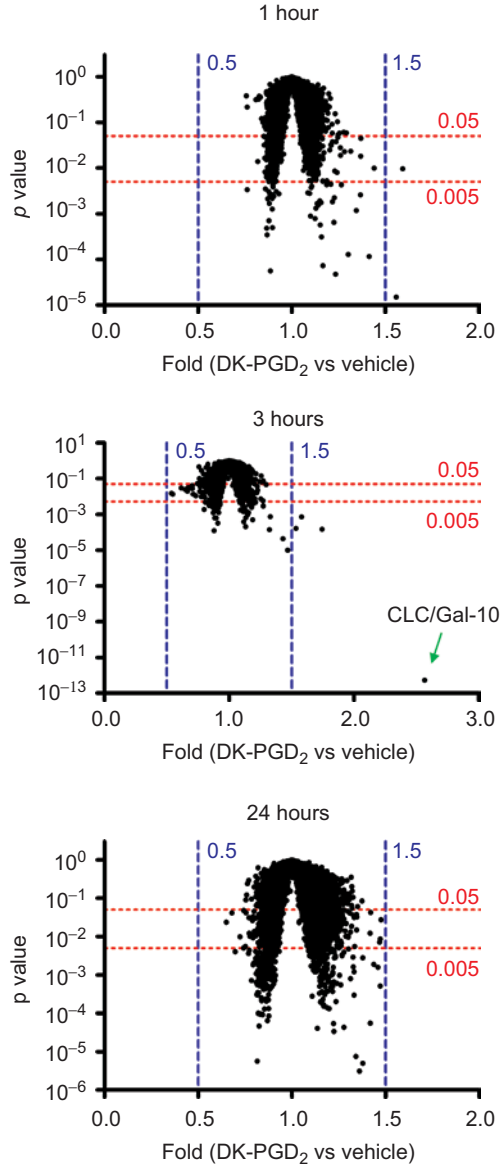

Figure 2. Whole blood gene expression change induced by 13,14-dihydro-15-keto prostaglandin D_2 (DK-PGD_2). Samples of uncoagulated human peripheral blood obtained from six healthy volunteers were stimulated with DK-PGD_2 (1 μM) or vehicle (0.005% DMSO) at 37°C for 1 h, 3 h or 24 h, as indicated.The levels of cellular mRNA were quantified in duplicate by microarrary analysis using AffymetrixGeneChip® Human Genome U133 Plus 2.0 Array as described in Methods. *p*-Values, comparing DK-PGD_2 treatment versus vehicle control from six donors using a *t*-test, were calculated for all 54 675 probe sets and plotted against average fold changes (DK-PGD_2/vehicle) from six donors. The green arrow indicates the *CLC*/Gal-10 probe set.

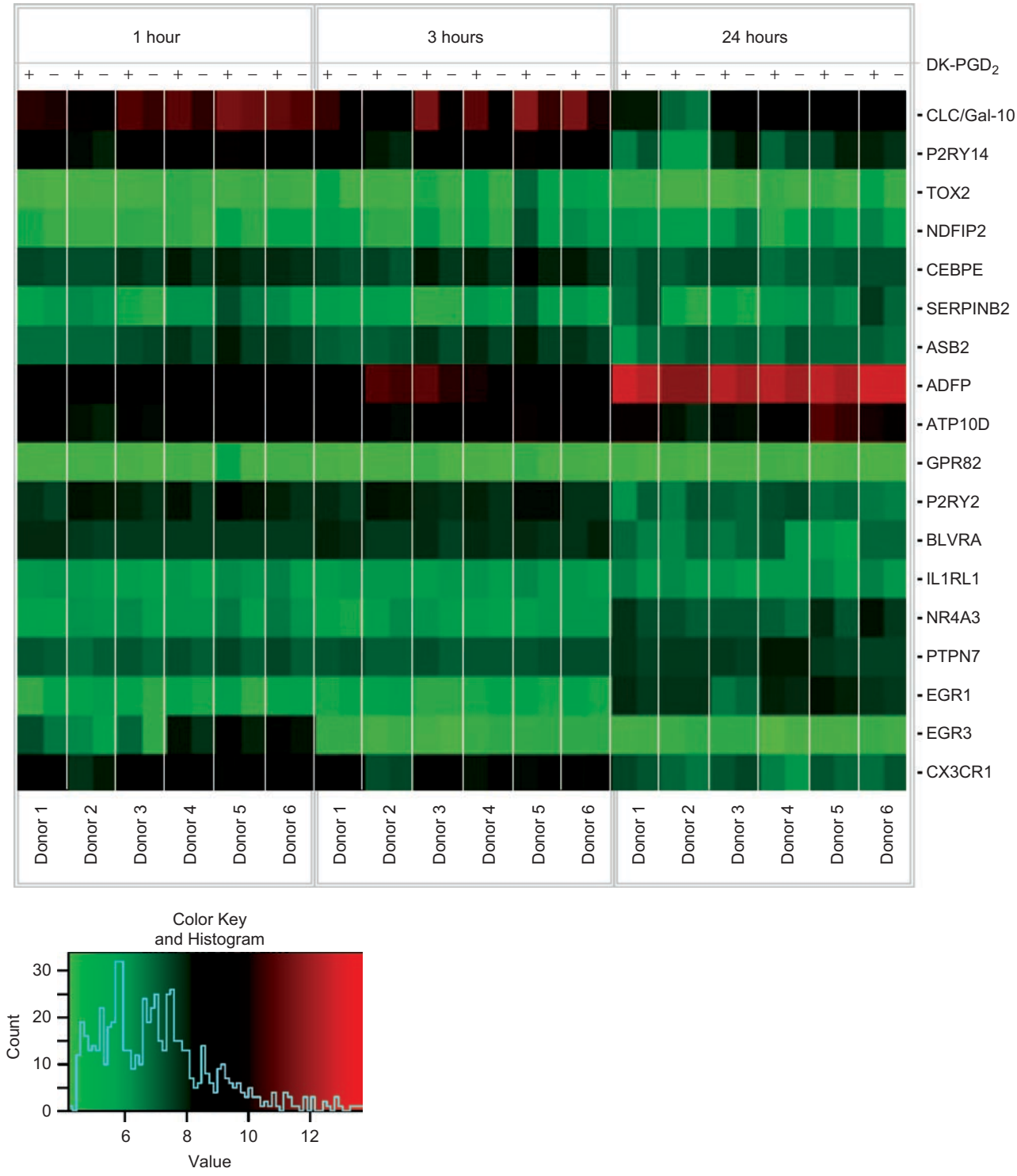

Figure 3. Expression intensity heat map of 18 differentially expressed genes. Each row represents a microarray expression value of a gene as indicated, and each column represents a donor sample treated with 13,14-dihydro-15-keto prostaglandin D_2 (DK-PGD_2) at 1 μM (+) or vehicle (0.005% DMSO) (−) at the time point indicated. The lower panel is a colour intensity key representing expression values from low (green) to high (red). The gene names listed are: *CLC*/Gal-10, Charcot–Leyden crystal protein/galectin-10; *P2RY14*, purinergic receptor P2Y, G-protein coupled, 14; *TOX2*, TOX high mobility group box family member 2; *NDFIP2*, Nedd4 family interacting protein 2; *CEBPE*, CCAAT/enhancer binding protein (C/EBP), epsilon; *SERPINB2*, serpin peptidase inhibitor, clade B (ovalbumin), member 2; *ASB2*, ankyrin repeat and SOCS box-containing 2; *ADFP*, adipose differentiation-related protein; *ATP10D*, ATPase, class V, type 10D; *GPR82*, G protein-coupled receptor 82; *P2RY2*, purinergic receptor P2Y, G-protein coupled, 2; *BLVRA*, biliverdin reductase A; *IL1RL1*, interleukin 1 receptor-like 1; *NR4A3*, nuclear receptor subfamily 4, group A, member 3; *PTPN7*, protein tyrosine phosphatase, non-receptor type 7; *EGR1*, early growth response 1; *EGR3*, early growth response 3; *CX3CR1*, chemokine (C-X3-C motif) receptor 1.

DK-PGD_2-induced increases in *CLC*/Gal-10 expression were suppressed by the CRTH2 antagonist CAY10471 in four out of five donors. Paradoxically, both DK-PGD_2 (agonist) and CAY10471 (antagonist) induced similar increases in *CLC*/Gal-10 expression by 3- to 4-fold in one of the donors (donor 8, Figure 6). The effect of CRTH2 activation on the expression of *NDFIP2* and *P2RY14* in whole blood samples was less pronounced when measured using quantitative real-time PCR. Among the five donors analysed, only *NDFIP2* from two donors and *P2RY14* from one donor reached greater than 1.5-fold increase in expression at 3 h after DK-PGD_2 stimulation.

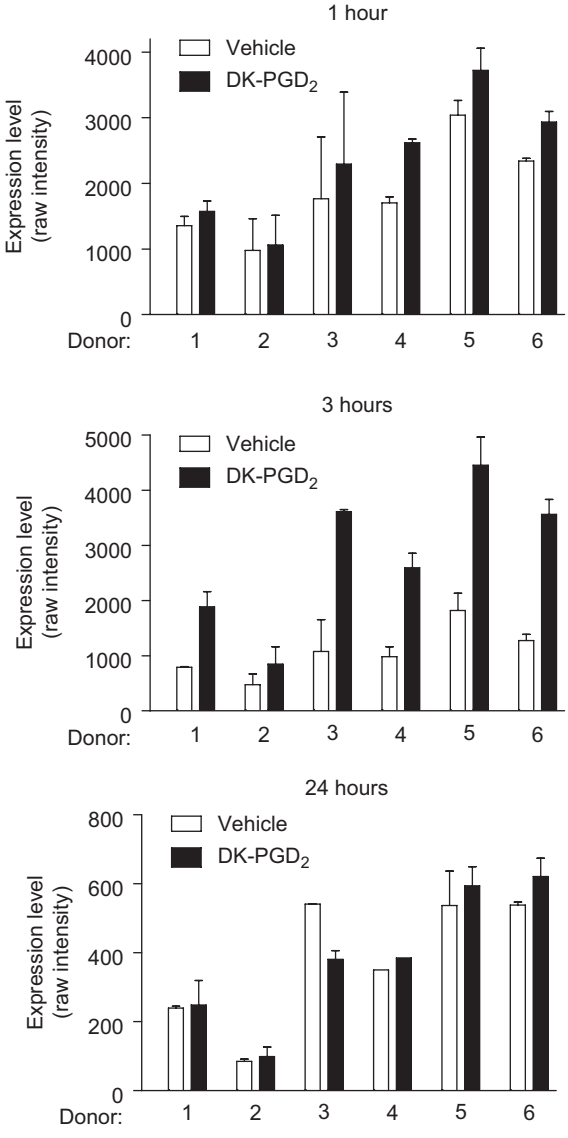

Figure 4. Changes in *CLC*/Gal-10 expression induced by 13,14-dihydro-15-keto prostaglandin D_2 (DK-PGD_2) in human whole blood. Expression levels of the *CLC*/Gal-10 gene in human blood as quantified by microarray analysis are shown for each donor. Data are mean values of duplicate determinations for each donor.

Discussion

Using a genome-wide microarray analysis, we have revealed a modest change in gene expression in human whole blood upon activation *in vitro* by a selective CRTH2 agonist, DK-PGD_2. The change of expression levels of nearly all the mRNAs that are significantly enhanced or suppressed by DK-PGD_2 stimulation is less than 1.5-fold when compared with those of the vehicle control. Since DK-PGD_2 does not have agonist activity for DP1 (Hirai et al. 2001, Sawyer et al. 2002), this modest change in gene expression is probably a reflection of relative low abundance of cell types, such as eosinophils, basophils and Th2 cells, that express CRTH2 in whole blood from healthy volunteers. The DK-PGD_2-induced increases in the levels of mRNAs in these cell types are likely to be diluted by the levels of the same mRNAs expressed in the majority of leukocytes in whole blood that do not express CRTH2. We hypothesize that only the messages that are selectively expressed in CRTH2-expressing cells can be differentiated in whole blood upon CRTH2 activation. Consistent with this hypothesis, we identify the expression level of only one gene, *CLC*/Gal-10, that reaches greater than 2-fold of that of vehicle control in whole blood upon DK-PGD_2 stimulation for 3 h. *CLC*/Gal-10 has been reported to be highly and specifically expressed in eosinophils and basophils (Ackerman et al.1982, 1993, Archer & Blackwood 1965). The expression levels of other genes such as *EGR1*, *EGR3*, *NDFIP2* and *P2RY14* that are not selectively expressed in CRTH2-expressing cell types, only reach about 1.5-fold of those of vehicle control in DK-PGD2-treated whole blood.

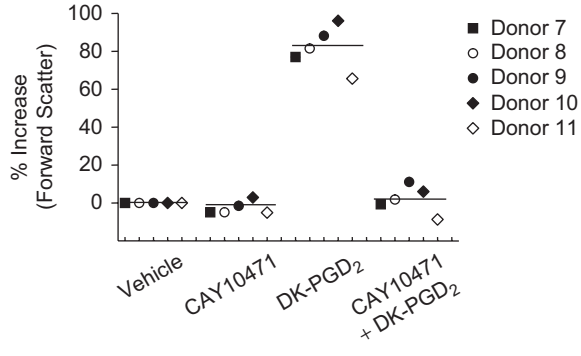

Figure 5. 13,14-Dihydro-15-keto prostaglandin D_2 (DK-PGD_2)-induced eosinophil shape change is blocked by CAY10471. Samples of uncoagulated human peripheral blood from five healthy volunteers were incubated with CAY10471 (10 μM) for 10 min prior to DK-PGD_2 (1 μM) stimulation for 10 min. Eosinophil shape change was measured by the gated autofluorescence forward scatter assay as described in Methods.Data are expressed as percentage increase in forward scatter comparing CAY10471- and/or DK-PGD_2-treated samples versus vehicle controls from the same donor and are mean values of duplicate determinations.

We confirm that the expression of the *CLC*/Gal-10 gene is consistently upregulated in DK-PGD_2-treated whole blood using quantitative real-time PCR. The CLC/Gal-10 protein possesses mannose binding activity (Swaminathan et al. 1999) and is not an eosinophil lysophospholipase (Ackerman et al. 2002). It is a major constituent representing an estimated 7–10% of the total cellular protein of eosinophils. Similar high amounts of CLC/Gal-10 protein are expressed in basophils (Ackerman et al. 1980, 1982). The protein forms hexagonal bipyramidal crystals (Charcot–Leyden crystal) and can be found in a variety of tissues and body fluids as a hallmark of allergic inflammation involving eosinophils or basophils. For example, the CLC/Gal-10 protein is significantly elevated in sputum specimens from patients with acute asthma and from patients with other respiratory diseases associated with bronchopulmonary infection (Dor et al. 1984). It is one of the major proteins elevated in nasal lavage fluid of patients with allergic rhinitis (Ghafouri et al. 2006), and genetic variation in CLC/Gal-10 has been found to be associated with allergic rhinitis (Brybornet al. 2010). Expression of CLC/Gal-10 is also elevated in the peripheral blood of

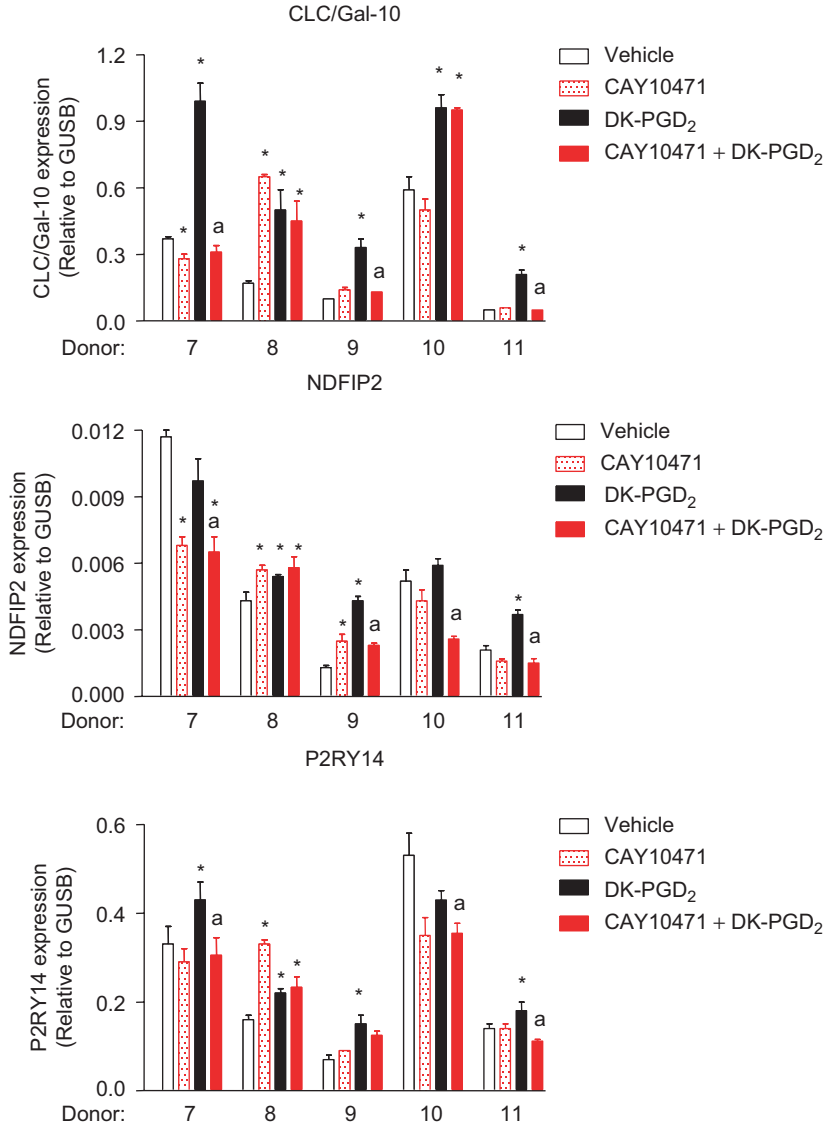

Figure 6. Expression of *CLC*/Gal-10, *NDFIP2* and *P2RY14* in human whole blood treated with CRTH2 ligands.Samples of uncoagulated human peripheral blood from five healthy volunteers were stimulated with 13,14-dihydro-15-keto prostaglandin D_2 (DK-PGD_2) (1 μM) for 3 h before quantifying the levels of mRNAs for *CLC*/Gal-10, *NDFIP2* and *P2RY14* using quantitative real-time polymerase chain reaction as described in Methods. For some conditions as indicated, the blood samples were incubated with CAY10471 (10 μM) for 10 min prior to DK-PGD_2 stimulation. The relative expressions of the three targeted genes were normalized to that of *GUSB* according to the formula described in Methods.Data are means ± SD from triplicate determinations; *$p<0.005$, comparingwith vehicle-treated sample by *t*-test; [a]$p<0.005$, comparing with DK-PGD2-treated sample by *t*-test.

aspirin-induced asthmatic patients (Devouassoux et al. 2008). Regulation and function of CLC/Gal-10 protein in eosinophils and basophils is still not clear. Our identification of increased *CLC*/Gal-10 expression in human blood upon CRTH2 activation *in vitro* reveals the first regulatory mechanism of *CLC*/Gal-10 expression, and further supports the proinflammatory role of CRTH2 in allergic diseases. Other galectins, such as galectin-1 and galectin-3, are involved in inflammation-related diseases and allergic disease models (Camby et al. 2006, Ge et al. 2010). Our microarrary study did not identify upregulation of other galectins upon CRTH2 stimulation in whole blood inferring specific regulation of *CLC*/Gal-10 expression by CRTH2.

In addition to expression in eosinophils and basophils, CLC/Gal-10 protein is constitutively expressed in human CD4+CD25+Foxp3+ regulatory T (Treg) cells, while they are nearly absent in resting and activated CD4+CD25- T cells. The protein has been identified as a novel marker essential for the anergy and suppressive function of human Treg cells (Kubach et al. 2007). The DK-PGD_2-induced increases in CLC/Gal-10 expression seen in human blood are unlikely to originate from Treg cells as there are no reports of CRTH2 expression in these T cells.

DK-PGD_2-induced *CLC*/Gal-10 expression in whole blood was largely suppressed by the CRTH2 antagonist CAY10471, verifying that the induction is mediated by CRTH2. We observed that the expression of *CLC*/Gal-10 was enhanced by both an agonist and an antagonist of CRTH2 in one of the donors, despite the fact that eosinophil cell shape change was not increased by the antagonist in the same donor. Although mechanisms of both cell shape change and *CLC*/Gal-10 expression are probably mediated by CRTH2 after agonist or antagonist treatment, we cannot explain this paradox at the current time. It has been demonstrated that CRTH2 signals through at least two distinct mechanisms, G-proteins (Gαi) and β-arrestin 2, and that CAY10471 can inhibit both mechanisms, but not necessarily to the same degree (Mathiesen et al. 2006). It is possible that the molecular mechanisms for CRTH2-mediated eosinophil shape change and *CLC*/Gal-10 expression are distinct in CRTH2-expressing cells, resulting in the different effects observed for CAY10471 on these two functions in donor 8.

In conclusion, we have discovered that the expression of *CLC*/Gal-10 is upregulated in human whole blood upon CRTH2 activation *in vitro*, and this upregulation can be blocked by a selective CRTH2 antagonist. Therefore, DK-PGD_2-induced increases in the *CLC*/Gal-10 mRNA level can be applied as a potential pharmacodynamic marker for monitoring the exposure of human peripheral blood to CRTH2 modulating agents. Although eosinophil shape change in whole blood has been widely applied for evaluating potency of CRTH2 modulating agents (Armer et al. 2005, Sandham et al. 2007, 2009), the discovery of CRTH2-mediated *CLC*/Gal-10 mRNA upregulation provides an alternative marker which may prove useful in clinical settings in which flow cytometry instruments may not be readily available.

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Declaration of interest

The authors are either permanent or temporary employees of Hoffmann-La Roche Inc. The authors report no declarations of interest.

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