RESEARCH ARTICLE

Activation of IL5R and CRTH2 on Human Eosinophils Elicit a Similar Molecular Response and Reveal a Synergistic Effect

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Eosinophils represent a key cell driving inflammatory processes in diseases such as asthma and eosinophilic esophagitis. The entire process of migration and activation of eosinophils is orchestrated by a myriad of molecules, among which interleukin 5 (IL5) plays a crucial role. Another receptor expressed on eosinophils is chemo-attractant receptor like expressed on Th2 cells (CRTH2). CRTH2 activation has a prominent role in stimulating Th2 and type 2 innate lymphoid cells to produce IL5 in disease tissue, but also induces eosinophils migration and adhesion. Prostaglandin D2 is the endogenous ligand of CRTH2, predominately released by activated mast cells, another key cell involved in eosinophilic diseases. Consequently, the IL5 and CRTH2 pathway are likely triggered simultaneously in eosinophils in disease tissue. Understanding the molecular effects of CRTH2 and IL5 receptor (IL5R) activated eosinophils might provide new insights for efficient eosinophils blocking.

We performed gene expression profiling with isolated human eosinophils to compare the molecular signatures of CRTH2 and IL5R activation. The identified genes were confirmed using an orthogonal technology, and simultaneous IL5R and CRTH2 activation was investigated on the protein level.

This study reveals that IL5R and CRTH2 activation trigger a similar molecular response in eosinophils with stronger IL5R effects. C-C chemokine ligand 2 was produced synergistically upon IL5R and CRTH2 stimulation and might contribute to monocyte and macrophage recruitment to disease tissue. The effect on C-C chemokine receptor 1 suggests that eosinophils maturation in the tissue might even occur in absence of IL5 via the CRTH2 pathway.

Keywords: CRTH2; IL5; CCL2; CCR1; eosinophils; FADS1

Introduction

Eosinophils represent a key cell type driving inflammatory processes in diseases such as asthma, eosinophilic esophagitis (EoE), nasal polyps and many more (Rosenberg, Dyer & Foster 2013). In pathology the attraction of eosinophils to the disease tissue is considered as an important first step (Rosenberg, Dyer & Foster 2013). The tissue eosinophils can then be further activated which causes the release of active molecules which will trigger additional immunological pathways (Rosenberg, Dyer & Foster 2013). The entire process of maturation, migration and activation of eosinophils is orchestrated by a myriad of molecules, among which interleukin 5 (IL5) plays a crucial role (Rosenberg, Dyer & Foster 2013). IL5 is a type 2 cytokine that activates the IL5 receptor (IL5R) present on eosinophils. IL5 is a particularly important mediator of eosinophils maturation in the bone marrow. Gene expression studies revealed the comprehensive molecular effects of IL5R activation (Bates et al. 2004; Temple et al. 2001). In line with the IL5 effects described, IL5-regulated genes are involved in adhesion, migration, activation and survival of eosinophils. Due to the importance of eosinophils and IL5 in disease, IL5 became an attractive target for drug development. In clinical studies with anti-IL5 antibodies, a reduction of the number of eosinophils in disease tissue of asthma and EoE patients was demonstrated, validating the pharmacological effect in diseases driven by eosinophilic inflammation (Nair et al. 2009; Straumann et al. 2010). In pivotal phase III clinical studies, anti-IL5 antibodies demonstrated clinical benefit by reducing exacerbation rates in patients with eosinophilic asthma (Ortega et al. 2014; Pavord et al. 2012), which led to approval by the FDA in late 2015.

Prostaglandin D2 receptor 2 (PTGDR2 or CRTH2), which is also expressed on eosinophils, presents itself as

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an interesting pharmacological target to pursue for the potential treatment of eosinophilic diseases. Prostaglandin D2 (PGD_2) is the endogenous ligand of CRTH2, and PGD_2 is predominately released by activated mast cells, which represents another key cell type involved in eosinophilic diseases (Dougherty et al. 2010; Otani et al. 2013). CRTH2 is not only expressed on eosinophils but also on T helper 2 (Th2) cells, type 2 innate lymphoid cells (ILC2) and basophils. Today it is well established that activation of CRTH2 on Th2 cells and ILC2 results in the release of type 2 cytokines such as IL4, IL5 and IL13 (Xue et al. 2005; Xue et al. 2014). Type 2 cytokines induce a type 2 environment which promotes eosinophilic inflammation. Today it is understood that patients with consistent high type 2 cytokine levels and eosinophil numbers exist, but the complexity of different, and overlapping inflammatory endotypes requires further investigation (Agache et al. 2017). In contrast to Th2 cells and ILC2, very little is known about the molecular effects of CRTH2 activation on eosinophils beside that CRTH2 activation results in eosinophils migration and upregulation of the adhesion molecules CD11b (Monneret et al. 2002). PGD_2 is produced by mast cells located in the tissue and can lead to the attraction of eosinophils. As a consequence, IL5 produced by PGD_2-activated Th2 cells and ILC2 in disease tissue is likely to activate eosinophils in addition to the stimulus of CRTH2. Because eosinophils represent abundant tissue cells in eosinophilic disease the consequence of eosinophils activated by PGD_2 is an important, not yet fully understood mechanism of eosinophilic diseases. In addition, it is unclear if IL5 and PGD_2 promote similar pathways in eosinophils, and if there is synergism between them.

To increase the understanding of CRTH2 activation on eosinophils, the difference between CRTH2 and IL5R activation, and potentially a synergistic effect, gene and protein expression studies were conducted using isolated human eosinophils. We observed that CRTH2 and IL5R activation share a large part of the pathway in eosinophils, with IL5 showing a more pronounced effect. We identified previously unknown molecules regulated by CRTH2 activation such as C-C chemokine ligand 2 (CCL2) and C-C chemokine receptor 1 (CCR1). CCL2, a molecule not primarily linked to eosinophilic diseases, was released by eosinophils in a CRTH2/IL5 synergistic fashion. Effects on CCR1 point to a previously unknown CRTH2-mediated effect on eosinophils maturation.

Material and methods
Gene expression array
The collection and use of eosinophils isolated from human whole blood was approved by the ethics committee of Northwest and Central Switzerland (EK179/11). After obtaining informed consent, human polymorphonuclear cells were isolated from peripheral blood (EDTA) of 6 healthy volunteers on a density gradient (Percoll plus GE, 1.09 g/l) followed by a lyses of the red blood cells. Eosinophils were then separated from the polymorphonuclear cells by negative selection using the human eosinophils isolation kit (Miltenyi Biotec). The reactivity of eosinophils to 13,14-dihydro-15-keto Prostaglandin D_2 (dk-PGD_2)

was monitored by flow cytometry as previously described (Strasser et al. 2015). In this study we used dk-PGD_2 instead of PGD_2 as it has similar affinity to CRTH2 but is specific over the Prostaglandin D2 receptor 1. Isolated eosinophils, 1.5×10^6 cells/condition, were then incubated at 37°C with three different treatments: vehicle, 1 µM dk-PGD_2 (Cayman) or 100 pM IL5 (Peprotech) for three hours. After incubation, cells were stored in TRIreagent (Ambion) ready for total RNA (totRNA) extraction. Cell incubation supernatants were stored at −20°C. TotRNA was isolated from the eosinophils using MagMax 96 microarray kit (Life Technologies, AM1839) with 40 µl elution volume. Quality controls were performed using Nanodrop ND-1000® (NanoDrop Technologies) and Bioanalyzer 2100© (Agilent Technologies) to warrant the RNA quality. When the ratio OD260/OD280 was superior to 1.8 but below 2.2 and the RNA integrity number was superior to 7.00, amplification was performed on 100 ng of totRNA with an Agilent Low RNA Input QuickAmp Labeling Kit (Agilent Technologies, 5190-2331/2305/2307) followed by the hybridization and washes using the SurePrint G3 Human GE 8 × 60 k v2 (G4851B) oligonucleotides (60-mer) directed against the human transcriptome (Agilent Technologies) according to the manufacturer's instruction. The arrays were scanned using the Agilent microarray scanner (G2565BA) and Scan Control software 8.5.1, and the data was extracted with the Feature Extraction software 11.5.1.1. The expression intensities for all oligos were normalized using vsn (variance-stabilizing normalization), a method that combines background adjustment and between-array normalization (Huber et al. 2002). The gene expression data is available in the Gene Expression Omnibus (Barrett et al. 2013; Edgar, Domrachev & Lash 2002) repository under the accession number GSE75142. Statistical analysis was performed using R version 3.3.3 and limma package on Bioconductor 3.30.13 (a moderated t-test) on different contrasts, i.e. vehicle vs dk-PGD_2, vehicle vs IL5 for comparison of the treatment effect to identify differentially expressed probes (DEP). The p-values were adjusted using the Benjamini-Hochberg procedure, threshold for false discovery rate was set at q = 0.01, i.e. the expected number of false positives was kept at 1%. As a first representation of the results we used a principal component analysis (PCA), a statistical technique used to display multivariate data in a low dimensional space, in order to easily visualize the data. A heatmap with hierarchical clustering was generated to visualize the grouped variables for all treatment conditions. Genes were reordered based on their similarity according to the Euclidean distance. Volcano plots were generated to identify the changes in the large data sets. For each probe the negative logarithm of the adjusted p-value (−log10 adjusted p-value) were plotted versus the log2 FC (LFC) on the y and x axes, respectively. A pathway analyse was run using the gProfileR R package on CRAN 0.6.1.

Gene expression NanoString nCounter, flow cytometry, Elisa
Eosinophils were isolated from 6 donors as described for the gene expression array. Reactivity towards dk-PGD_2 was confirmed by flow cytometry and cells were incubated

with two different treatments: vehicle and 1 µM dk-PGD$_2$ for three hours at 37°C. After incubation, cells were stored in TRIreagent for totRNA extraction. TotRNA extraction and quality controls were performed as described for the gene expression array. A custom panel of 43 probes and 17 selected housekeeping probes (NanoString nCounter) was designed based on the gene expression array results (supplementary Table 1). First, 100 ng of totRNA were hybridized to the probes for 20 hours at 65°C. Then, samples were transferred to the prep station for purification, immobilization and alignment of the complex on the cartridge prior reading by the digital analyzer (NanoString nCounter Analysis System). The Reporter Library File and Cartridge Definition File were uploaded to the digital analyzer to generate, for each samples, Reporter Code Count files necessary for data analysis performed using R version 3.3.3. Data were normalized using positive control probes included in the kit and the most stable housekeeping genes. A differential expression analysis on the contrast dk-PGD$_2$ vs vehicle was conducted on the log2 normalized matrix using limma version 3.30.13.

EDTA blood from 7 donors were incubated for 4 hours at 37°C with vehicle, 1 µM dk-PGD$_2$ or 1 nM IL5. After incubation the cell were processed by flow cytometry for the effect on cluster of differentiation 69 (CD69) and CCR1.

Alternatively, for concentration response and synergistic effect experiments, eosinophils were directly isolated from peripheral EDTA blood by negative selection using the MACSxpress Eosinophil Isolation kit (Miltenyi Biotec) followed by the MACSxpress Erythrocyte Depletion kit (Miltenyi Biotec). Isolated eosinophils from 5 donors were incubated for 24 hours with four different treatments: vehicle, 1 µM dk-PGD$_2$, 1 nM IL5 and the combination of both. After incubation, the cell supernatants were stored at −20°C before CCL2 level measurement. Isolated eosinophils from 2 donors were incubated for 4 hours with either four different treatments: vehicle, 1 µM dk-PGD$_2$, 1 nM IL5, and the combination of IL5 and dk-PGD$_2$, or with nine different concentrations of dk-PGD$_2$ or IL5. After incubation the eosinophils were processed by flow cytometry for CD69 and CCR1 expression.

Isolated eosinophils or eosinophils in EDTA blood were stained with anti-(C-C chemokine receptor 3 (CCR3))-PerCPCy5.5, anti-CRTH2-FITC, anti-CD69-PECy7, and anti-CCR1-AF647 (BD Biosciences) for 20 minutes at room temperature. Samples were lysed with BD FACS lysing solution (BD Biosciences) for 10 min and centrifuged for 5 min at 400 g. The supernatant was discarded and pelleted cells were re-suspended in buffer. The cells were then analyzed on a flow cytometer (FACSCanto™ II, BD Biosciences). Eosinophils were gated based on high CCR3 expression level and FSC profile. The MFI for CRTH2, CD69 and CCR1 were acquired and analyzed using GraphPad Prism v6.0 (La Jolla, USA) either applying a four parameter fitting model or data representation tool.

CCL2 levels were measured in eosinophils supernatant using a Meso Scale Discovery assay based on the Human Chemokine 9-plex Ultra-Sensitive kit. CCL2 concentration in the samples were derived from a fitted standard curve using Softmax Pro v5.4 (Molecular Devices).

Results

In the first set of experiments, cell purity was controlled by flow-cytometry, with an average eosinophils content higher than 90% (data not shown). The responsiveness was confirmed for all eosinophils preparation by the ability of dk-PGD$_2$ to induce a concentration dependent internalization of CRTH2 from the cell surface as monitored by flow-cytometry (supplementary figure 1).

Gene array profiling allowed us to analyze gene expression changes in eosinophils after stimulation of CRTH2 and the IL5R. The representation of the PCA analyses (**Figure 1A**) illustrates that both the CRTH2 and IL5R stimulated populations separated from the vehicle population on PC1 (15.1%). Setting the threshold at |LFC| ≥ 1 and the adjusted p-value < 0.01, CRTH2 activation resulted in a lower number of DEP regulated and a smaller magnitude than IL5R activation as illustrated on the respective volcano plots (**Figure 1B**). CRTH2 activation showed LFC values varying from 3.4 to −1.3 and IL5R activation from 6.2 to −3.4. Out of 50599 probes, 6 were down-regulated and 183 up-regulated by dk-PGD$_2$ treatment whereas 691 were down-regulated and 1284 were up-regulated by IL5 treatment. From those DEP, 117 were shared by both the dk-PGD$_2$ and IL5 activation pathway revealing an overlap of 62% of the CRTH2 regulated probes with the IL5 regulated probes (**Figure 1C**). The heatmap illustrates the difference between the two treatments in terms of number of modulated genes and intensity of the response (**Figure 1D**). The ten genes that showed the strongest regulation for each categories are summarized in **Table 1**. We identified CRTH2 specific genes such as *EFNB2*, *FADS1*, genes that were shared by both pathways such as *CCL2* and *CD69* and finally *CCR1* as a specific IL5 regulated gene. Pathway analysis demonstrated a typical cytokine-mediated signaling pathway, GO:0019221for IL5 (Ashburner et al. 2000; Carbon et al. 2009; The Gene Ontology 2017). CRTH2 specific genes did not point to a particular pathway.

In order to confirm the observation of the gene expression array analysis with an orthogonal technology, we selected genes regulated by dk-PGD$_2$ and/or IL5 to generate the panel of 43 genes to analyze with NanoString nCounter platform. Because of their low levels of expression, 6 probes were excluded. Several probes (10 out of 37) showed regulation upon dk-PGD$_2$ stimulation (27% have a |LFC| ≥ 1, 19% showed an adjusted p-value < 0.05, data not shown). A comparison between microarray and the NanoString nCounter platforms (**Table 2**) shows that most of the regulated genes were part of the top regulated genes in the gene expression array as for example *CCL2*, *CD69*, *CXCL16* and *CCR1*. Although *FADS1* and *EFNB2* did not pass statistical significance criteria, they confirmed the increase (supplementary figure 2) and were also reproduced in a separate gene expression array study (data not shown).

Finally, based on the probes, we selected several proteins to follow up. Several parameters were not confirmed at the protein levels such as FADS1, CXCL16 and EphB2 (data not shown). In contrast, the regulation of the CCL2 protein levels in the supernatant, and the surface
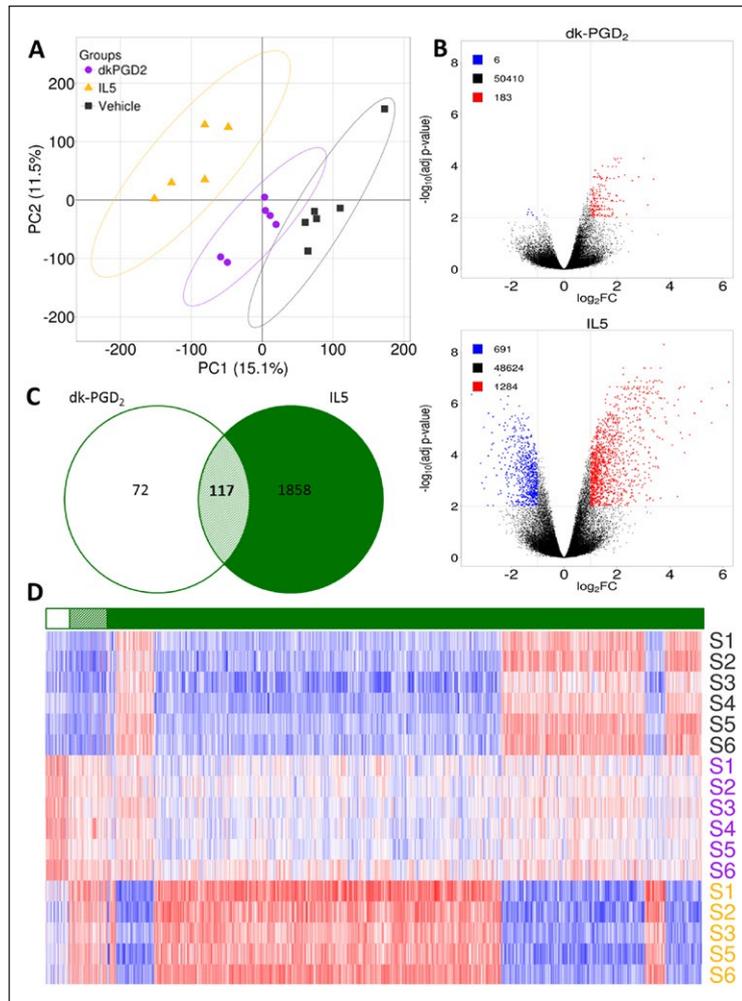
Figure 1: Gene array expression analysis. Outcome of the gene expression analysis summarized for |LFC| ≥ 1 and adjusted p-value < 0.01. The Principal Component Analysis illustrates the separation of the different treatment groups in the two first dimensions explaining 26.6% of the variation **(A)**. LFC and adjusted p-values for each contrast are shown in the Volcano plots, in blue up-regulated probes, in red down-regulated probes and in black, non-regulated probes **(B)**. The Venn diagram **(C)** represents the number of probes regulated by dk-PGD$_2$ and/or IL5. The heatmap **(D)** represents similarities between genes across samples for each area of the Venn diagram.

Table 1: Gene expression array analysis. Summarizes the 10 top regulated genes either specific to IL5R or CRTH2 activation or shared by both receptors.

dk-PGD$_2$		dk-PGD$_2$ / IL5			IL5	
Gene name	LFC	Gene name	LFC dk-PGD$_2$	LFC IL5	Gene name	LFC
EFNB2	2.3	CCL2	3.4	6.2	LOC100506810	6.2
VMO1	2.0	DUSP6	2.6	4.5	SLC45A3	5.6
ZNF331	2.0	PLK3	1.8	4.4	HSPA1B	5.6
FADS1	1.9	EGR3	2.8	3.9	RASD1	5.2
C19orf59	1.9	GK	1.3	3.8	EGR2	4.9
SH2B2	1.8	MPZL2	1.5	3.7	CD180	4.7
NAP1L5	1.8	ARHGAP18	1.7	3.6	LOC344887	4.6
NAB2	1.8	CD69	1.3	3.5	CCR1	4.5
TYMS	1.7	A_24_P510377	1.3	3.4	DCUN1D3	4.4
XLOC_008263	1.6	LOC100507672	1.5	3.3	SYN2	4.4

adjusted p-value < 0.009.

Table 2: Comparison of the gene expression array and NanoString nCounter results. Summarizes the comparison of the CRTH2 activation between the gene expression array and the gene expression NanoString.

Gene name/probe	Gene expression array		Gene expression NanoString	
	LFC dk-PGD$_2$	adj. p-value	LFC dk-PGD$_2$	adj. p-value
CCL2	3.4	0.0003	2.5	0.03
EGR3	2.8	0.0003	2.1	0.05
CCR1	2.4	0.0178	1.6	0.22
ZNF331	2.0	0.0010	1.5	0.11
EFNB2	2.3	0.0020	1.3	0.17
EGR1	3.6	0.0457	1.3	0.05
NAB2	1.8	0.0020	1.2	0.03
CD69	1.3	0.0019	1.1	0.05
CXCL16	1.2	0.0010	1.1	0.05
FADS1	1.9	0.0001	1.1	0.17
ITRIP	1.1	0.0010	0.8	0.03

expression of CD69 and CCR1 were confirmed. **Figure 2A** illustrates the behavior of the corresponding genes in gene expression array and **Figure 2B** summarizes the results of those genes in the gene expression NanoString. We observed an increase in CCL2 levels in the cells supernatant collected from the original gene expression array experiment, which was more pronounced with IL5 stimulation (40-fold) than with dk-PGD$_2$ stimulation (5-fold) (**Figure 2C**). We made the same observation for CD69 levels on eosinophils from blood incubated with either 1 μM of dk-PGD$_2$ or 1 nM of IL5 which lead to a 2.2-fold or 10.1-fold increase respectively. In the same samples we noticed a slight decrease in CCR1 expression on the eosinophils with both stimuli (1.2 and 1.4fold decrease). Again, as for the gene expression array, the response to IL5 stimuli was more pronounced than the one to dk-PGD$_2$. Intensity of the flow cytometry signal shift is illustrated in supplementary figure 1.

To ensure that we used equipotent concentrations of dk-PGD$_2$ and IL5 we performed concentration response experiments for both IL5 and dk-PGD$_2$ with isolated eosinophils. We used CD69 surface expression as a read out. For both donors we obtained similar results and were able to demonstrate that with 1 μM of dk-PGD$_2$ and 1 nM of IL5 around 90–99% receptor activation was achieved (**Figure 3A** and **3B**).

In order to investigate a potential synergistic effect of CRTH2 and IL5R activation we incubated isolated eosinophils from 5 donors with 1 μM of dk-PGD$_2$ or 1 nM IL5 or with the combination of both agonists for 24 h. We observed an increase in CCL2 levels from 1.6 to 16.4 pg/ml upon stimulation with dk-PGD$_2$ (mean 12-fold increase), and from 1.6 to 47.2 pg/ml upon stimulation with IL5 (mean 39-fold increase), and from 1.6 to 111.0 pg/ml upon stimulation with the combination (mean 103-fold increase). The combination of IL5R and CRTH2 activation elicited a larger increase in CCL2 (mean 103-fold) than the sum of the individual effects (mean 52-fold

increase) (**Figure 4A**). We also assessed a potential synergy for the CD69 and CCR1 expression on isolated eosinophils (**Figure 4B** and **C**) but did not observe this effect after testing the eosinophils of two donors. We summarized the synergistic effect observed on cells or blood for the different markers (**Figure 4D**) and concluded that the synergistic effect is only observed for CCL2.

Discussion
The majority of drugs in clinical development for immunological disorders are selectively targeting a key molecule in a defined pathway, such as IL5 in eosinophilic diseases. Hence, it has become of utmost importance to understand the contribution of the particular targeted pathway compared to other pathways known to be involved in the pathophysiology, and how they overlap or interplay. In the case of eosinophilic diseases, the focus lays on eosinophilic asthma, which represents the target population for the approved anti-IL5 therapy. In addition the type 2 immunity endotype, representing a disease driven by the type 2 cytokines IL4, IL5 and IL13, is of importance. Eosinophilic asthma and the type 2 immunity endotype are considered to be driven by the same or at least similar pathway (Agache et al. 2017; Robinson et al. 2017). Anti-IL5 antibodies and CRTH2 antagonists target this pathway at different levels with potentially overlapping and synergistic effects. Of particular interest are eosinophils as key cells involved in asthma. Eosinophils express the IL5R and CRTH2 and therefore are targeted both by anti-IL5 antibodies and CRTH2 antagonists. Beside eosinophils also mast cells, Th2 cells and ILC2 play a key role in asthma. Whereas IL5 has no effect on Th2 cells and ILC2, CRTH2 activation promotes the production of type 2 cytokines such as IL4, IL5 and IL13 (Xue et al. 2005; Xue et al. 2014). In contrast little is known about the molecular effect of CRTH2 activation on eosinophils, how it compares to IL5R activation, and if there is a synergistic effect to be anticipated.
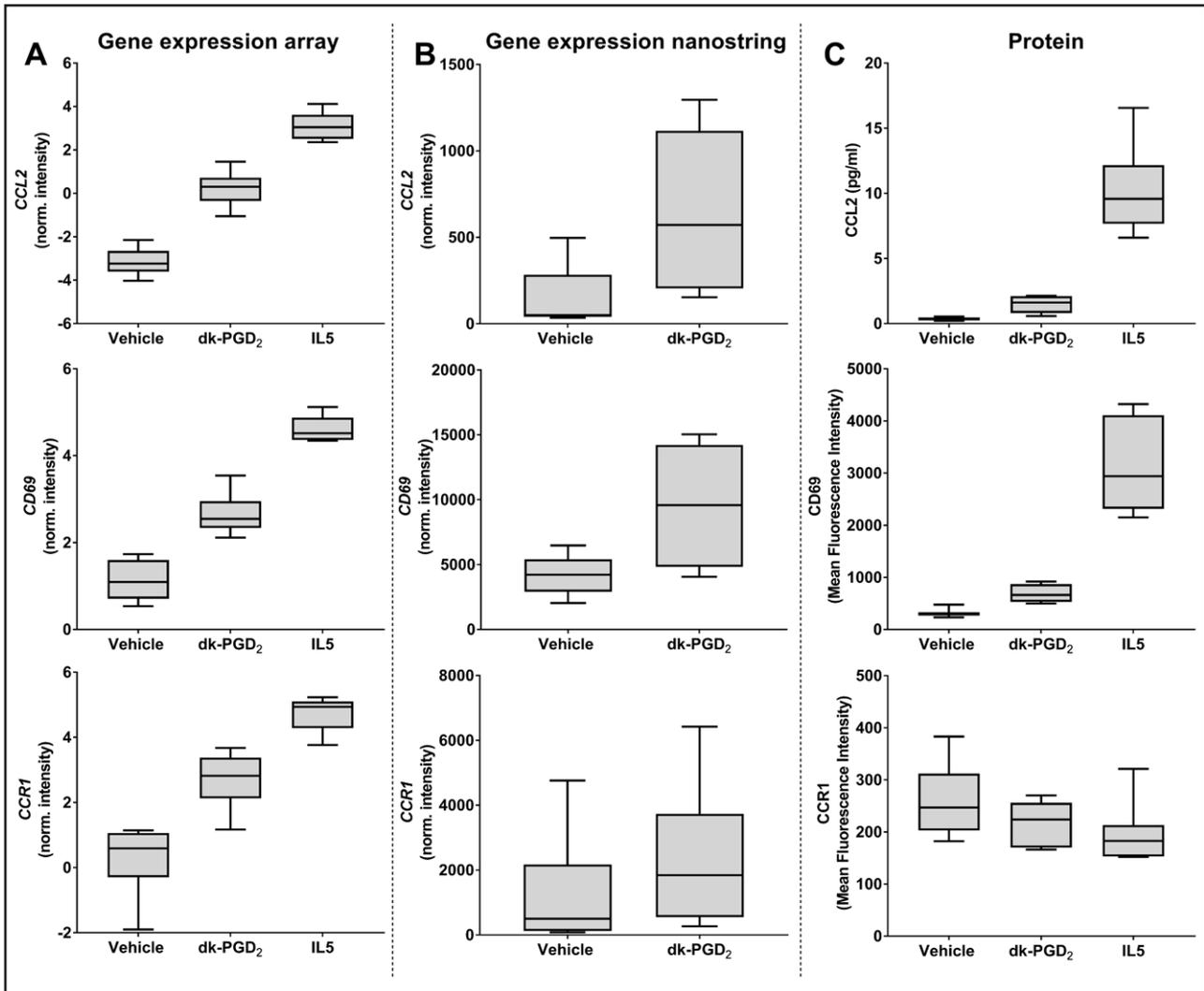
Figure 2: Translation of gene expression to protein. CRTH2 and IL5R-induced upregulation of CCL2, CD69 and CCR1 transcript by microarray analysis of eosinophils **(A)**. Confirmation of the CRTH2-induced upregulation by NanoString technology **(B)**. Effect of the stimulation translated to the protein expression either on cell supernatant for CCL2 measured by ELISA or on the cell surface for CD69 and CCR1 monitored by flow cytometry **(C)**.
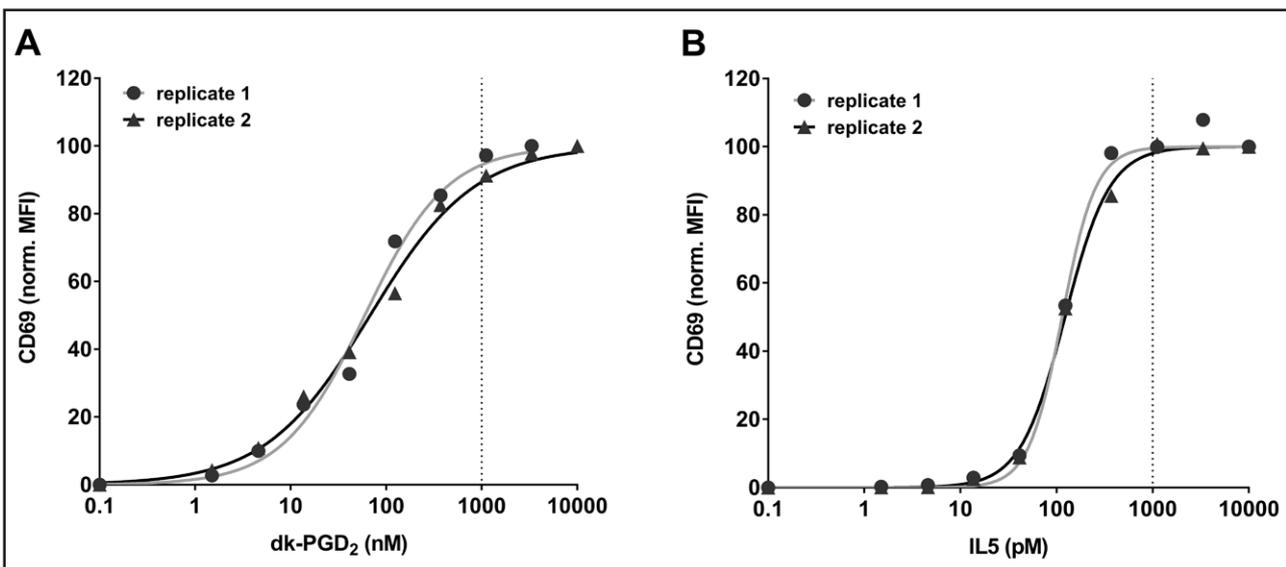
Figure 3: dk-PGD2 and IL5 concentration response curve. dk-PGD2 **(A)** and IL5 **(B)** concentration response curve on CD69 expression on isolated eosinophils monitored by flow cytometry.
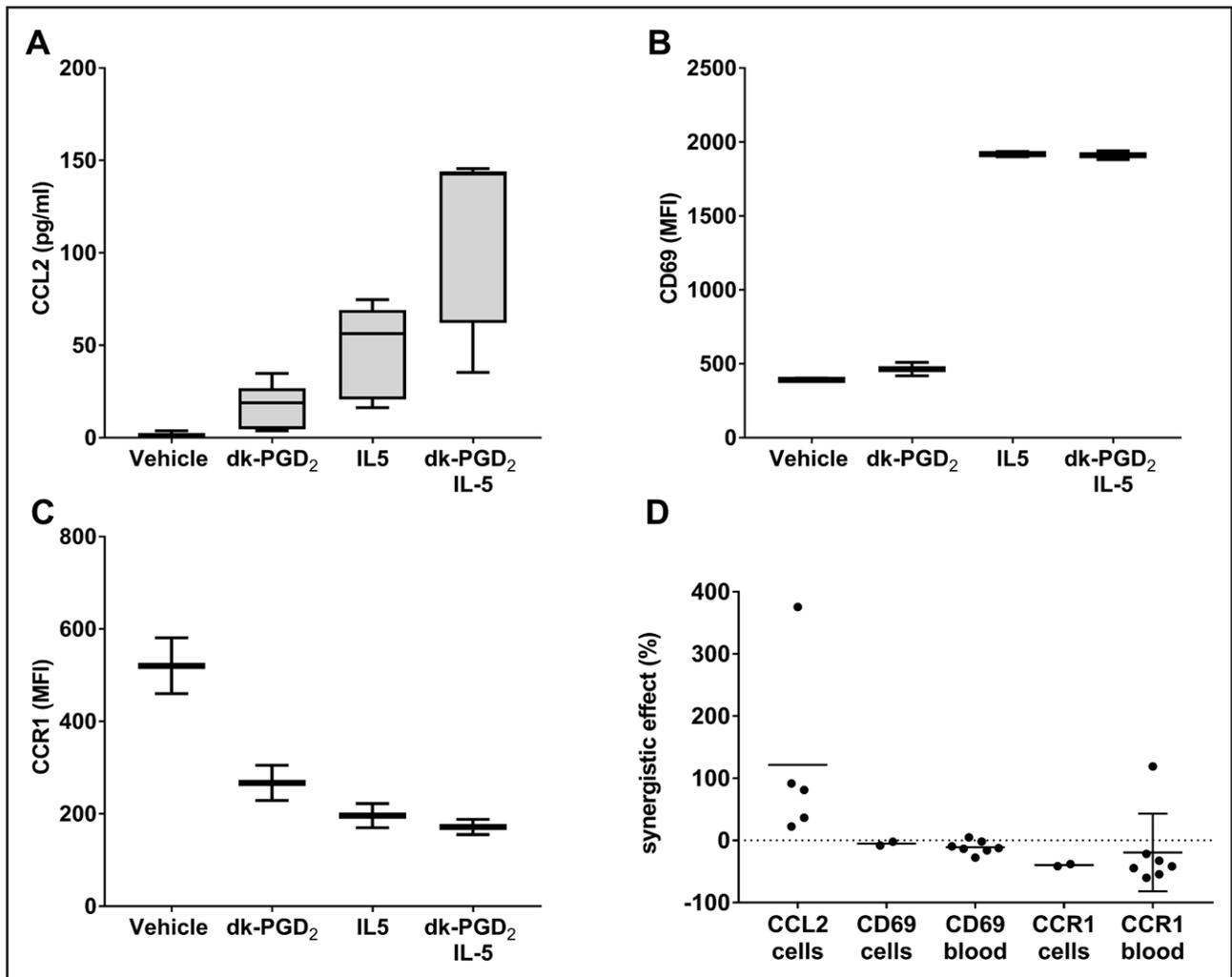
Figure 4: Synergistic effect of dk-PGD2 and IL5. Synergistic effect of CRTH2 and IL5R activation on CCL2 secretion by eosinophils after 24h of incubation expressed in concentration **(A)**. Synergistic effect of dk-PGD$_2$ and IL5 not observed on CD69 and CCR1 expression on eosinophils after 4h of incubation (**B** and **C**). Summary of the synergistic effect on CCL2, CD69 and CCR1 expressed in percentage **(D)**.

In this study, stimulation of isolated human eosinophils with IL5 produced a strong gene expression response. The modulated genes were in agreement with previously published data (Bates et al. 2004; Temple et al. 2001) and pathway analysis of the significantly regulated genes confirmed a canonical cytokine signaling (GO:0019221). Also activation of CRTH2 on eosinophils produced a consistent gene expression response. The number of regulated genes and the magnitude of the CRTH2 activation effect were smaller than for IL5R activation. The PCA clearly supported an intermediate effect of CRTH2 activation compared to IL5R activation, and the heat map analysis showed that CRTH2 activation resulted in the same pattern on most of the IL5R specific genes, but did not reach statistical significance for those genes. The genes specifically modulated by CRTH2 activation were not linked to a particular pathway when subject to pathway analysis tools.

Among the IL5R and CRTH2 top regulated genes, *CCL2*, *CD69* and *CCR1* are well known in the immunological context. *CCL2* was the top regulated gene and the CCL2 protein was quantifiable in the supernatant of stimulated

eosinophils. Isolated eosinophils produced CCL2 over the entire time-course of 24 h. As observed for the gene, IL5R activation led to a 5-fold larger increase in CCL2 compared to CRTH2 activation. CCL2 is produced by many different cell types after receiving a stimulation signal e.g. an inflammatory signal. CCL2 is primarily considered to be involved in monocyte and macrophage recruitment via CCR2. Attraction of monocytes and macrophages is not considered a typical feature of type 2 inflammation and in particular CRTH2-mediated production of CCL2 is a novel observation. In contrast the effect of CCL2 on type 2 inflammation and in particular on Th2 polarization was actually reported previously (Deshmane et al. 2009). Macrophages are important cells in inflammatory diseases such as asthma and can induce plasticity in the type of immune response (Balhara & Gounni 2012). The question arises if IL5R and CRTH2 activation of eosinophils does lead to attraction of monocytes and macrophages. Today it is well known that the asthma endotypes and phenotypes are more complex than separating asthma patients simply into the eosinophilic and non-eosinophilic phenotype (Robinson et al. 2017). Of note, beside the eosinophils,

activation of CRTH2 on Th2 cells, another key players in asthma pathology, resulted in increased levels of molecules involved in the non-typical type 2 response features e.g. neutrophil activation (Xue et al. 2015). Whereas it has been shown that non-eosinophilic asthma endotypes show little plasticity (McGrath et al. 2012) it cannot be excluded that crosstalk between different cells and stimuli can causes plasticity of the eosinophilic, type 2 endotype. Indeed it was demonstrated that more severe patients show an overlap of the eosinophilic and non-eosinophilic endotypes with systemic increases of both type 2 cytokines such as IL13, and non-type 2 molecules such as CCL2 and IL8 (Agache et al. 2016). Overall there is still a lack of understanding of the complex asthma pathology, but in particular the severe asthma patients, having the highest medical need, appear to show a complex, multidimensional pathology (Agache et al. 2017; Agache et al. 2015; Robinson et al. 2017). Therefore efforts to further characterize asthma patients are using algorithms that handle multivariate data to characterize asthma patients (Agache et al. 2017; Hinks et al. 2015; Hinks et al. 2016). In addition, further data is required on individual cellular level to understand the complex interplay in asthma.

The cell surface molecule CD69 is a known early activation marker of lymphocytes that was previously described to be expressed on the cell surface of activated eosinophils in asthma (Hartnell et al. 1993; Johansson 2014). That IL5R activation leads to upregulation of *CD69* on eosinophils is well known (Hartnell et al. 1993) but upregulation of *CD69* after CRTH2 activation was not previously described for eosinophils. The *CD69* regulation profile was very similar to the *CCL2* regulation profile with a higher activation of eosinophils by IL5, again pointing to an intermediate activation profile of CRTH2 compared to IL5R.

In the gene expression array *CCR1* was identified as IL5R specific regulated gene. Whereas the gene product increased by activation of IL5R, the protein expression on the cell surface decreased after activation of the IL5R. In addition, also CRTH2 clearly reduced the protein expression of CCR1 on the cell surface of eosinophils. This observation further shows that CRTH2 activation of eosinophils provides a very similar pattern as IL5R activation, but of weaker intensity. CCR1 is involved in leukocyte migration, enhancement of T cell activation, Th cell polarization and macrophage stimulation and therefore has caught the attention of the pharmaceutical industry (Gladue, Brown & Zwillich 2010). CCR1 is known to be expressed on lymphocytes, monocytes and dendritic cells. In eosinophils it has been reported that CCR1-expression decreases when eosinophils mature under the influence of IL5 or other chemokines such as CCL3, the cognate ligand of CCR1 (Fulkerson et al. 2014). Of note, immature eosinophils were previously described to be present in inflamed tissue of asthma patients and considering the asthma lung inflammation environment, it is likely that in-situ eosinophils maturation will contribute to persistent eosinophilia (Robinson et al. 1999).

In summary, both IL5R and CRTH2 stimulation lead to the activation of eosinophils. Both receptors appear to

stimulate similar pathways with the IL5R activation showing a more pronounced effect.

To further elaborate the interplay between IL5R and CRTH2 activation, eosinophils were isolated and stimulated in vitro using saturating concentrations of both respective ligands. We continued to focus on CCL2, CD69 and CCR1 protein and to our surprise all three revealed a different pattern. Regulation of CD69 did not demonstrate synergism for IL5R and CRTH2, and the effect of CRTH2 activation was negligible when comparing to IL5R activation. In contrast, CCL2 was increased in a synergistic manner and showed large variability between the eosinophils isolated from different healthy subjects. In particular, 1 subject showed a 4-fold higher synergistic effect on CCL2. It would be of interest to follow up on this observation in asthma patients.

CCR1 in contrast showed a less than additive effect with CRTH2 having an almost equipotent effect on CCR1 down regulation than IL5. Overall the picture shows that eosinophils in inflamed tissue, where IL5 is produced by type 2 immune cells and mast cells release CRTH2 ligands, will potentially react by the production of large quantities of CCL2. The CCR1 down-regulation as a sign of eosinophil maturation will occur even in the situation were only IL5R or CRTH2 are activated.

Of interest is that PGD_2 and LTE4, another lipid mediator released from activated mast cells and involved in inflammatory disease, synergistically induced the expression of type 2 cytokines, but also molecules involved in non-type 2 cytokine activities e.g. in neutrophil activation (Xue et al. 2015). This phenomenon further adds to the complexity of the immunological pathways and opens up for plasticity between the classical outlined endotypes such as the type 2 endotype. Hence this renders the separation of asthma patients based on endotype or phenotype difficult. In summary, in disease tissue the release of PGD_2 and Leukotrienes from mast cells will lead to the production of IL5 by resident ILC2 and Th2 cells, and potentially cause a synergistic increase in CCL2 concentration that might recruit monocytes and macrophages to the site of inflammation. Whereas a clear type 2 immunity response if foreseen the potential effect on the plasticity of the inflammatory and immune response remains to be investigated.

Among the top CRTH2-specific regulated genes were Ephrin B2 (*EFNB2*) and fatty acid desaturase 1 (*FADS1*) which have not been previously described as CRTH2 regulated. The *EFNB2* gene codes for the transmembrane receptor tyrosine kinase EphB2, which is involved in development processes, bone homeostasis, and red blood cell differentiation (Anselmo et al. 2016). The role of EphB2 in inflammation and immune response is unclear, but overall the ephrins are receiving increasing interest in the field of immunology and e.g. type 2 immune-modulatory effects were described for Ephrin-A1 (Coulthard et al. 2012; Wohlfahrt et al. 2004). The *FADS1* gene codes for the rate-limiting enzymes required for the biosynthesis of long-chain polyunsaturated fatty acids. FADS1 catalyses endogenous synthesis of arachidonic acid from

dihomo-gamma-linoleic acid. Arachidonic acid-derived eicosanoids include prostaglandins. *FADS1* knock-out mice have altered levels of tissue arachidonic acid, leading to a decrease in arachidonic acid-derived prostaglandins. There is thus evidence that FADS1 induction would increase arachidonic acid production and by this might activate the immune response. Here, the CRTH2-induced *FADS1* suggests a feedback of auto-induction of prostaglandins. Although the CRTH2 specific regulated genes *FADS1* and *EFNB2* were reproducibly identified using orthogonal technologies, the protein product was not detectable in eosinophils. EphB2 was investigated using flow cytometry and FADS1 using ELISA, IHC and western blot (data not shown). There are two possible explanations for the observed lack of translation of the gene product to protein. First of all, the available antibodies might not be specific or sensitive enough to pick up the changes. Secondly, the translation to protein might require additional triggers that were absent in the vitro test system. In summary, FADS1 and EphB2 represent interesting candidate biomarkers for CRTH2-specific activation of eosinophils, but more studies and potentially better tools are needed to clarify the potential.

Conclusion

Our study shows that IL5R and CRTH2 activation triggers a similar molecular response, in eosinophils with IL5R effects being overall more pronounced. CCL2 was produced synergistically upon IL5R and CRTH2 stimulation and might contribute to monocyte and macrophage recruitment to disease tissue. In addition, the effect on CCR1 suggests that eosinophils maturation in the tissue might even occur in absence of IL5 via the CRTH2 pathway.

Additional Files

The additional files for this article can be found as follows:

- **Supplementary Figure 1.** Flow cytometry experiment. DOI: https://doi.org/10.5334/ejmcm.1.s1
- **Supplementary Figure 2.** Comparison of gene array and Nanostring nCounter gene expression of FADS1 and EFNB2 regulation. DOI: https://doi.org/10.5334/ejmcm.1.s1
- **Supplementary Table 1.** Gene expression NanoString panel. DOI: https://doi.org/10.5334/ejmcm.1.s2

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Competing Interests

The authors have no competing interests to declare.

References

Agache, I, Strasser, DS, Klenk, A, Agache, C, Farine, H, Ciobanu, C, Groenen, PM and **Akdis, CA.** 2016. Serum IL-5 and IL-13 consistently serve as the best predictors for the blood eosinophilia phenotype in adult asthmatics. *Allergy*, 71: 1192–202. DOI: https://doi.org/10.1111/all.12906

Agache, I, Strasser, DS, Pierlot, GM, Farine, H, Izuhara, K and **Akdis, CA.** 2017. Monitoring the inflammatory heterogeneity with multiple biomarkers for multidimensional endotyping of asthma. *J Allergy Clin Immunol.* DOI: https://doi.org/10.1016/j.jaci.2017.08.027

Agache, I, Sugita, K, Morita, H, Akdis, M and **Akdis, CA.** 2015. The Complex Type 2 Endotype in Allergy and Asthma: From Laboratory to Bedside. *Curr Allergy Asthma Rep*, 15: 29. DOI: https://doi.org/10.1007/s11882-015-0529-x

Anselmo, A, Lauranzano, E, Soldani, C, Ploia, C, Angioni, R, D'Amico, G, Sarukhan, A, Mazzon, C and **Viola, A.** 2016. Identification of a novel agrin-dependent pathway in cell signaling and adhesion within the erythroid niche. *Cell Death Differ*, 23: 1322–30. DOI: https://doi.org/10.1038/cdd.2016.10

Ashburner, M, Ball, CA, Blake, JA, Botstein, D, Butler, H, Cherry, JM, Davis, AP, Dolinski, K, Dwight, SS, Eppig, JT, Harris, MA, Hill, DP, Issel-Tarver, L, Kasarskis, A, Lewis, S, Matese, JC, Richardson, JE, Ringwald, M, Rubin, GM and **Sherlock, G.** 2000. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet*, 25: 25–9. DOI: https://doi.org/10.1038/75556

Balhara, J and **Gounni, AS.** 2012. The alveolar macrophages in asthma: a double-edged sword. *Mucosal Immunol*, 5: 605–9. DOI: https://doi.org/10.1038/mi.2012.74

Barrett, T, Wilhite, SE, Ledoux, P, Evangelista, C, Kim, IF, Tomashevsky, M, Marshall, KA, Phillippy, K H, Sherman, PM, Holko, M, Yefanov, A, Lee, H, Zhang, N, Robertson, CL, Serova, N, Davis, S and **Soboleva, A.** 2013. NCBI GEO: archive for functional genomics data sets—update. *Nucleic Acids Res*, 41: D991–5. DOI: https://doi.org/10.1093/nar/gks1193

Bates, ME, Liu, LY, Esnault, S, Stout, BA, Fonkem, E, Kung, V, Sedgwick, JB, Kelly, EA, Bates, DM, Malter, JS, Busse, WW and **Bertics, PJ.** 2004. Expression of interleukin-5- and granulocyte macrophage-colony-stimulating factor-responsive genes in blood and airway eosinophils. *Am J Respir Cell Mol Biol*, 30: 736–43. DOI: https://doi.org/10.1165/rcmb.2003-0234OC

Carbon, S, Ireland, A, Mungall, CJ, Shu, S, Marshall, B, Lewis, S, Ami, GOH and **Web Presence Working Group.** 2009. AmiGO: online access to ontology and annotation data. *Bioinformatics*, 25: 288–9. DOI: https://doi.org/10.1093/bioinformatics/btn615

Coulthard, MG, Morgan, M, Woodruff, TM, Arumugam, TV, Taylor, S M, Carpenter, TC, Lackmann, M and **Boyd, AW.** 2012. Eph/Ephrin signaling in injury and inflammation. *Am J Pathol*, 181: 1493–503. DOI: https://doi.org/10.1016/j.ajpath.2012.06.043

Deshmane, SL, Kremlev, S, Amini, S and **Sawaya, BE.** 2009. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res*, 29: 313–26. DOI: https://doi.org/10.1089/jir.2008.0027

Dougherty, RH, Sidhu, SS, Raman, K, Solon, M, Solberg, OD, Caughey, GH, Woodruff, PG and **Fahy, JV.** 2010. Accumulation of intraepithelial mast cells with a unique protease phenotype in T(H)2-high asthma. *J Allergy Clin Immunol*, 125: 1046–1053. e8. DOI: https://doi.org/10.1016/j.jaci.2010.03.003

Edgar, R, Domrachev, M and **Lash, AE.** 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res*, 30: 207–10. DOI: https://doi.org/10.1093/nar/30.1.207

Fulkerson, PC, Schollaert, KL, Bouffi, C and **Rothenberg, ME.** 2014. IL-5 triggers a cooperative cytokine network that promotes eosinophil precursor maturation. *J Immunol*, 193: 4043–52. DOI: https://doi.org/10.4049/jimmunol.1400732

Gladue, R P, Brown, M F and **Zwillich, S H.** 2010. CCR1 antagonists: what have we learned from clinical trials. *Curr Top Med Chem*, 10: 1268–77. DOI: https://doi.org/10.2174/156802610791561237

Hartnell, A, Robinson, DS, Kay, AB and **Wardlaw, AJ.** 1993. CD69 is expressed by human eosinophils activated in vivo in asthma and in vitro by cytokines. *Immunology*, 80: 281–6.

Hinks, T, Zhou, X, Staples, K, Dimitrov, B, Manta, A, Petrossian, T, Lum, P, Smith, C, Ward, J, Howarth, P, Walls, A, Gadola, SD and **Djukanović, R.** 2015. Multidimensional endotypes of asthma: topological data analysis of cross-sectional clinical, pathological, and immunological data. *The Lancet*, 385: S42. DOI: https://doi.org/10.1016/S0140-6736(15)60357-9

Hinks, TS, Brown, T, Lau, LC, Rupani, H, Barber, C, Elliott, S, Ward, JA, Ono, J, Ohta, S, Izuhara, K, Djukanovic, R, Kurukulaaratchy, RJ, Chauhan, A and **Howarth, PH.** 2016. Multidimensional endotyping in patients with severe asthma reveals inflammatory heterogeneity in matrix metalloproteinases and chitinase 3-like protein 1. *J Allergy Clin Immunol*, 138: 61–75. DOI: https://doi.org/10.1016/j.jaci.2015.11.020

Huber, W, von Heydebreck, A, Sultmann, H, Poustka, A and **Vingron, M.** 2002. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics*, 18(Suppl 1): S96–104. DOI: https://doi.org/10.1093/bioinformatics/18.suppl_1.S96

Johansson, MW. 2014. Activation states of blood eosinophils in asthma. *Clin Exp Allergy*, 44: 482–98. DOI: https://doi.org/10.1111/cea.12292

McGrath, KW, Icitovic, N, Boushey, HA, Lazarus, S C, Sutherland, ER, Chinchilli, VM, Fahy, JV and **Asthma Clinical Research Network of the National Heart, Lung, and Blood Institute.** 2012. A large subgroup of mild-to-moderate asthma is persistently non-eosinophilic. *Am J Respir Crit Care Med*, 185: 612–9. DOI: https://doi.org/10.1164/rccm.201109-1640OC

Monneret, G, Li, H, Vasilescu, J, Rokach, J and **Powell, WS.** 2002. 15-Deoxy-delta 12,14-prostaglandins D2 and J2 are potent activators of human eosinophils. *J Immunol*, 168: 3563–9. DOI: https://doi.org/10.4049/jimmunol.168.7.3563

Nair, P, Pizzichini, MM, Kjarsgaard, M, Inman, MD, Efthimiadis, A, Pizzichini, E, Hargreave, FE and **O'Byrne, PM.** 2009. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N Engl J Med*, 360: 985–93. DOI: https://doi.org/10.1056/NEJMoa0805435

Ortega, HG, Liu, MC, Pavord, ID, Brusselle, GG, FitzGerald, JM, Chetta, A, Humbert, M, Katz, L E, Keene, ON, Yancey, SW, Chanez, P and **MENSA Investigators.** 2014. Mepolizumab treatment in patients with severe eosinophilic asthma. *N Engl J Med*, 371: 1198–207. DOI: https://doi.org/10.1056/NEJMoa1403290

Otani, IM, Anilkumar, AA, Newbury, RO, Bhagat, M, Beppu, LY, Dohil, R, Broide, DH and **Aceves, SS.** 2013. Anti-IL-5 therapy reduces mast cell and IL-9 cell numbers in pediatric patients with eosinophilic esophagitis. *J Allergy Clin Immunol*, 131: 1576–82. DOI: https://doi.org/10.1016/j.jaci.2013.02.042

Pavord, ID, Korn, S, Howarth, P, Bleecker, ER, Buhl, R, Keene, ON, Ortega, H and **Chanez, P.** 2012. Mepolizumab for severe eosinophilic asthma (DREAM): a multicentre, double-blind, placebo-controlled trial. *The Lancet*, 380: 651–659. DOI: https://doi.org/10.1016/S0140-6736(12)60988-X

Robinson, D, Humbert, M, Buhl, R, Cruz, AA, Inoue, H, Korom, S, Hanania, NA and **Nair, P.** 2017. Revisiting Type 2-high and Type 2-low airway inflammation in asthma: current knowledge and therapeutic implications. *Clin Exp Allergy*, 47: 161–175. DOI: https://doi.org/10.1111/cea.12880

Robinson, DS, Damia, R, Zeibecoglou, K, Molet, S, North, J, Yamada, T, Kay, AB and **Hamid, Q.** 1999. CD34(+)/interleukin-5Ralpha messenger RNA+ cells in the bronchial mucosa in asthma: Potential airway eosinophil progenitors. *Am J Respir Cell Mol Biol*, 20: 9–13. DOI: https://doi.org/10.1165/ajrcmb.20.1.3449

Rosenberg, HF, Dyer, KD and **Foster, PS.** 2013. Eosinophils: changing perspectives in health and disease. *Nat Rev Immunol*, 13: 9–22. DOI: https://doi.org/10.1038/nri3341

Strasser, DS, Farine, H, Holdener, M, Zisowsky, J, Roscher, R, Hoerner, J, Gehin, M, Sidharta, PN, Dingemanse, J and **Groenen, PM.** 2015. Development of a decision-making biomarker for CRTH2 antagonism in clinical studies. *New Horizons in Translational Medicine*, 2: 118–125. DOI: https://doi.org/10.1016/j.nhtm.2015.05.001

Straumann, A, Conus, S, Grzonka, P, Kita, H, Kephart, G, Bussmann, C, Beglinger, C, Smith, D A, Patel, J, Byrne, M and **Simon, HU.** 2010. Anti-interleukin-5 antibody treatment (mepolizumab) in active eosinophilic oesophagitis: a randomised, placebo-controlled, double-blind trial. *Gut*, 59: 21–30. DOI: https://doi.org/10.1136/gut.2009.178558

Temple, R, Allen, E, Fordham, J, Phipps, S, Schneider, HC, Lindauer, K, Hayes, I, Lockey, J, Pollock, K and **Jupp, R.** 2001. Microarray analysis of eosinophils reveals a number of candidate survival and apoptosis genes. *Am J Respir Cell Mol Biol*, 25: 425–33. DOI: https://doi.org/10.1165/ajrcmb.25.4.4456

The Gene Ontology Consortium. 2017. Expansion of the Gene Ontology knowledgebase and resources. *Nucleic Acids Res*, 45: D331–D338. DOI: https://doi.org/10.1093/nar/gkw1108

Wohlfahrt, JG, Karagiannidis, C, Kunzmann, S, Epstein, MM, Kempf, W, Blaser, K and **Schmidt-Weber, CB.** 2004. Ephrin-A1 Suppresses Th2 Cell Activation and Provides a Regulatory Link to Lung Epithelial Cells. *The Journal of Immunology*, 172: 843–850. DOI: https://doi.org/10.4049/jimmunol.172.2.843

Xue, L, Fergusson, J, Salimi, M, Panse, I, Ussher, JE, Hegazy, AN, Vinall, S L, Jackson, DG, Hunter, MG, Pettipher, R, Ogg, G and **Klenerman, P.** 2015. Prostaglandin D_2 and leukotriene E_4 synergize to stimulate diverse $T_{H}2$ functions and $T_{H}2$ cell/neutrophil crosstalk. *J Allergy Clin Immunol*, 135: 1358–66. e11. DOI: https://doi.org/10.1016/j.jaci.2014.09.006

Xue, L, Gyles, SL, Wettey, FR, Gazi, L, Townsend, E, Hunter, MG and **Pettipher, R.** 2005. Prostaglandin D_2 causes preferential induction of proinflammatory Th2 cytokine production through an action on chemoattractant receptor-like molecule expressed on Th2 cells. *J Immunol*, 175: 6531–6. DOI: https://doi.org/10.4049/jimmunol.175.10.6531

Xue, L, Salimi, M, Panse, I, Mjosberg, JM, McKenzie, AN, Spits, H, Klenerman, P and **Ogg, G.** 2014. Prostaglandin D_2 activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on $T_{H}2$ cells. *J Allergy Clin Immunol*, 133: 1184–94. DOI: https://doi.org/10.1016/j.jaci.2013.10.056