Masterthesis

Signaling of the Prostaglandin D_2 Receptors DP and CRTH2

submitted by

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Statutory Declaration

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Abbreviations

AHR	**A**irway **h**yperreactivity
BHV-1	**B**ovine **h**erpes**v**irus **1**
BSA	**B**ovine **s**erum **a**lbumin
cAMP	**C**yclic **a**denosine **m**ono**p**hosphate
CCD	**C**harge **c**oupled **d**evice
COX 1&2	**C**yclo**ox**ygenase 1 & 2
CREB	**c**AMP **r**esponse **e**lement **b**inding **p**rotein
CRTH2	**c**hemoattractant **r**eceptor-homologous molecule expressed on **Th2** cells
DAPI	4',6-**d**i**a**midino-2-**p**heny**l**indole
DK-PGD2	13,14-**d**ihydro,15-**k**eto **p**rosta**g**landin **D2**
DMEM	**D**ulbecco's **m**odified **E**agle's **M**edium
DN	**D**ominant **n**egative
DNA	**D**esoxyribo**n**ucleic **a**cid
DP	**D**-type **p**rostanoid receptor
dsRNA	**D**ouble-**s**tranded **r**ibo**n**ucleic **a**cid
EC_{50}	Half maximal **e**ffective **c**oncentration
ECP	**E**osinophil-**c**ationic **p**rotein
EDN	**E**osinophil-**d**erived **n**eurotoxin
ERK1/2	**E**xtracellular signal-**r**elated **kinase** s 1/2
FBS	**F**etal **b**ovine **s**erum
FCS	**F**orward **sc**atter
FL-1	**Fl**uorescence **c**hannel 1
GEF	**G**uanine **e**xchange **f**actor
GM-CSF	**G**ranulocyte-**m**acrophage **c**olony-**s**timulating **f**actor
GPCR	**G**-**p**rotein-**c**oupled **r**eceptor
HEK293	**H**uman **e**mbyonic **k**indney cells 293
HEPES	4-(2-**h**ydroxy**e**thyl)-1-**p**iperazine**e**thane**s**ulfonic acid
hFGF-B	**H**uman **f**ibroblast **g**rowth **f**actor-**b**asic
hGEF	**H**emagglutination **e**nhancing **f**actor
HMVEC-L	**H**uman **m**icrovascular **e**ndothelial **c**ells-**l**ung
IFNγ	**I**nter**f**ero**n** **γ**
IgE	**I**mmuno**g**lobulin **E**

IGF	**I**nsulin **g**rowth **f**actor
IL	**I**nter**l**eukin
LPI	**L**yso**p**hosphatidyl**i**nositol
LPS	**L**ipo**p**oly**s**accharide
NFAT	**N**uclear **f**actor of **a**ctivated **T** cells
OVA	**Ova**lbumin
PBMC	**P**eripheral **b**lood **m**ononuclear **c**ells
PBS	**P**hosphate-**b**uffered **s**aline
PGD_2	**P**rosta**g**landin **D_2**
PI	**P**hosphatidyl**i**nositol
PMNL	**P**oly**m**orpho**n**uclear **l**eukocytes
RhoA	**R**as **h**omolog gene family, member **A**
RLU	**R**elative **l**ight **u**nits
ROCK	**R**ho-associated protein **kinase**
SNP	**S**ingle **n**ucleotide **p**olymorphism
SRE	**S**erum **r**esponse **e**lement
SSC	**S**ide **sc**atter
STAT6	**S**ignal **t**ransducer and **a**ctivator of **t**ranscription 6
TAC	**T**etrameric **a**ntibody **c**omplexes
TCF	**T**ernary **c**omplex **f**actor
TF	**T**ranscription **f**actor
TNFα	**T**umor **n**ecrosis **f**actor **α**
TXA2	**T**hrombo**x**ane **A_2**
VGEF	**V**ascular **e**ndothelial **gr**owth **f**actor
WHO	**W**orld **H**ealth **O**rganization

Abstract

Prostaglandin D_2 (PGD_2) is the major lipid mediator released by allergen-induced cross-linking of IgE-activated mast cells. During allergic responses it allegedly accounts for vasodilation, bronchoconstriction, infiltration of eosinophils and Th2 lymphocytes into the site of inflammation and consequently evokes the pathophysiology of allergic diseases such as bronchial asthma. Mast cell-derived PGD_2 recruits and activates Th2 cells as well as eosinophils and thereby might provide the link between the early- and the late-phase of allergic responses. PGD_2 elicits its biological effects via two distinct G-protein coupled receptors, the D-type prostanoid receptor (DP) and the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). Besides immune cells, DP is widely expressed in the whole organism and controversially, pro- and anti-inflammatory effects have been reported for this receptor. On the one hand, genetic depletion of DP prevents from airway hyperreactivity and decreased eosinophil infiltration into the airways of ovalbumin-challenged mice. On the other hand, DP mediates the inhibition of effector cell function and regulates the cytokine release of immune cells. *In vivo*, inhalation of a DP agonist prevents from asthma in allergen challenged mice. CRTH2 is specifically expressed on immune cells and generally accounts for pro-inflammatory cell responses *in vitro* and *in vivo*. For instance, CRTH2 mediates the infiltration of eosinophils into the lungs. Both receptors are co-expressed in eosinophils and are assumed to regulate their effector functions in allergic responses. Recently our group reported that DP and CRTH2 are able to interact with each other. They form heteromeric signaling units in recombinant HEK293 cell lines and alter each other's Ca^{2+} signaling capacities. The significance of GPCR heteromerization in drug development has been recognized lately. I hypothesized that the interaction of DP and CRTH2 might be an important regulatory mechanism in the activation of the serum response element (SRE), the nuclear factor of activated T cells (NFAT) and the cAMP response element binding protein (CREB) as these elements are highly implicated in the regulation of inflammatory reactions. I found that, in a recombinant cell line, activation SRE and NFAT underlies the cross-talk of DP and CRTH2 whereas CREB-activation is solely mediated by DP. CRTH2-mediated SRE and NFAT activation is dependent (i) on the co-expression and (ii) on the functionality of DP. Contrarily, DP was able to induce signal transduction independently from CRTH2. Furthermore, DP and CRTH2 contribute in phosphatidylinositol (PI) 3-kinase and ERK1/2 (extracellular signal-regulated kinases 1/2) involving signal transduction whereas DP might activate an additional pathway via RhoA.

As SRE regulates adhesion and migration of leukocytes, I performed experiments that aimed to elucidate the DP- and CRTH2-mediated regulation of those cellular functions in eosinophils. Both, DP and CRTH2 contributed to actin cytoskeletal rearrangement, whereas CRTH2 was the overall more dominant receptor. Taken together the obtained data demonstrate a close interaction of DP and CRTH2 in the recombinant cell model for DP and CRTH2 interaction as well as in primary cells. In general, CRTH2 was shown to often dominate over DP-mediated signaling. However, this thesis reveals that powerful signaling of CRTH2 seems to rely on the functionality of DP, which might be important to consider in approaches to treatment of inflammatory diseases such as bronchial asthma. Integrating the knowledge of receptor cross-talk and heteromerization provides the possibility to target pathologies of the immune system more precisely and to avoid potential side effects.

Zusammenfassung

Prostaglandin D_2 (PGD_2) ist der wichtigste Lipidmediator, der durch Allergen-induziertes Vernetzen von oberflächengebundenen IgE auf Mastzellen freigesetzt wird. In allergischen Reaktionen verursacht PGD_2 Vasodilation, Bronchokonstriktion und die Einwanderung von Eosinophilen an den Entzündungsherd. Da aus Mastzellen freigesetztes PGD_2 –Lymphozyten und Eosinophile rekrutiert und aktiviert, könnte es die Verbindung zwischen der Früh- und der Spätphase von allergischen Reaktionen erklären. Die biologischen Effekte von PGD_2 werden über zwei G-Protein gekoppelte Rezeptoren (GPCR), dem D-Typ Prostanoid Rezeptor (DP) und dem Chemoattractant Rezeptor-Homolog exprimiert auf Th2 Zellen (CRTH2), vermittelt. Außer von Immunzellen wird DP im gesamten Organismus exprimiert. Bemerkenswerterweise wurde DP mit pro- und antiinflammatorischen Effekten in Verbindung gebracht. Einerseits wurde durch genetische Deletion von DP die Hyperreaktivität der Atemwege und die Einwanderung von Eosinophilen in die Lungen von Ovalbumin-sensibilisierten Mäusen verringert, andererseits wurde gezeigt, dass DP die Inhibierung von Effektorzellfunktionen vermittelt und die Ausschüttung von Zytokinen reguliert. CRTH2 wird spezifisch von Immunzellen exprimiert und vermittelt pro-imflammatorische Reaktionen *in vitro* und *in vivo*. Beispielsweise, vermittelt CRTH2 die Einwanderung von Eosinophilen in Allergen-sensibilisierte Lungen. Vor kurzem konnte gezeigt werden, dass DP und CRTH2 miteinander interagieren. Die Rezeptoren bilden heteromere Einheiten, die die Signalweiterleitung regulieren und ihre Ca^{2+} Signalwege beeinflussen. Die Signifikanz von GPCR-Heteromerisierung bei der Entwicklung von neuen Medikamenten wurde in den letzten Jahren erkannt. Die Hypothese dieser Arbeit war, dass die Interaktion zwischen DP und CRTH2 wichtige regulatorische Elemente (Serum Response Element (SRE), Nukleärer Factor aktivierter T-Zellen (NFAT), cAMP Response Element Bindungsprotein (CREB) in entzündlichen Prozessen regulieren könnte. Diese Arbeit zeigt, dass die Aktivierung von SRE und NFAT in einer rekombinanten Zellinie stark von der Interaktion beider Rezeptoren untereinander abhängig ist, wohingegen die Aktivierung von CREB von DP vermittelt wird. Die CRTH2-vermittelte Aktivierung von SRE und NFAT ist erstens von der Anwesenheit und zweitens von der Funktionalität von DP abhängig. Andererseits kann DP unabhängig von CRTH2 die nötigen Signalwege aktivieren. Weiters konnte gezeigt werden, dass DP und CRTH2 in der Aktivierung von Phosphatidylinositol (PI) 3-Kinase- und ERK1/2-abhängigen Signalwegen zusammenarbeiten und dass DP zusätzlich über RhoA SRE induzieren kann.

Da SRE die Adhäsion und Migration von Leukozyten reguliert, wurden Experimente durchgeführt, die aufklären sollten, ob DP und CRTH2 auch diese zellulären Funktionen regulieren. Beide Rezeptoren sind auf Eosinophilen exprimiert, die neben ihren Effektorfunktionen bei parasitären Infektionen, auch für die späte Phase von allergischen Reaktionen verantwortlich sind. DP und CRTH2 waren beide an der Umformung des Aktin–Zytoskeletts beteiligt, wobei CRTH2 im Allgemeinen der dominantere Rezeptor war. Diese Arbeit konnte aufzeigen, dass CRTH2-vermittele Signale von der Funktionalität von DP abhängig sein könnten, was bei der Behandlung von allergischen Erkrankungen bedacht werden sollte. Das Wissen über Rezeptor-Interaktionen und Heteromerisierung ermöglicht es, Fehlfunktionen des Immunsystems präziser und effizienter zu behandeln, dadurch das systemische Gleichgewicht nicht zu stören und auch Nebenwirkungen zu vermeiden.

1. Introduction

1.1. Allergic Disease

Allergic diseases, characterized by an intolerance of the immune system against environmental substances, are highly prevalent all over the world. The World Health Organization (WHO) estimates that up to 20 % of the population are affected by hypersensitivity-caused disease such as bronchial asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis and anaphylaxis[1]. Interestingly, the reported cases have continuously increased in the last three decades, especially in western regions like Australia, the USA or the Unites Kingdom[2].

Genetic predispositions have been shown to remarkably impact the development of allergies. For instance, single nucleotide polymorphisms (SNPs) in the genes coding for interleukin 4 (IL-4)[3], the IL-4 receptor[4], tumor necrosis factor α (TNFα)[5], interferon γ (IFNγ)[6] or the signal transducer and activator of transcription 6 (STAT6)[4] are correlated with asthma[7]. In contrast, "The Hygiene Hypothesis" postulates that contact with infectious agents, like bacteria or parasites but also with commensal microorganism in the childhood, is beneficial for the prevention of allergic disease. If these stimuli are absent, the immune system might develop a Th2 cell-polarized- instead of a Th1 cell-dominant response to antigens. However, the final proofs are still missing and the mechanisms behind remain to be elucidated. Allergic diseases are classically divided into four categories[8], whereas the most prevalent allergic responses belong to the immediate hypersensitivity type (type I) and appear at the interface between the external- and the internal space respectively in the respiratory tract, the gastro-intestinal tract and the skin[9].

1.1.1. Immediate Hypersensitivity (Type I)

Upon allergen-induced cross-linking of IgE bound to their surface, mast cells release biogenic amines (e.g. histamines), lipid mediators (e.g. PGD_2, platelet activation factor, leukotrienes), cytokines (e.g. TNFα) and enzymes (e.g. tryptase). These substances cause plasma extravasation, bronchoconstriction, intestinal hypermotility, inflammation and tissue-damage. Histamine and TNFα also activate endothelial cells to open the vascular barrier and express adhesion molecules which promotes the recruitment of Th2 cells and eosinophils to the site of inflammation (late phase of immediate hypersensitivity).[10]

Repeated immediate hypersensitivity and late phase reactions are prominent features of allergic disease, like bronchial asthma. Airway cells of asthmatic patients show a predominant Th2 cytokine profile (IL-4, IL-5, IL-9, IL-13). The pathology of asthma is characterized by airway obstruction, chronic bronchial inflammation, airway hyperreactivity (AHR), airway remodeling, airway eosinophilia and increased mucus production[11].

1.2. Eosinophil Granulocytes and their Role in Allergic Responses

Paul Ehrlich was the first who identified eosinophil granulocytes in 1879. By staining white blood cells with the acidic red dye eosin he identified a population of leukocytes that contain alkaloid (eosinophilic) granules in the cellular cytoplasm. Due to this characteristic feature he named these population eosinophil granulocytes. Eosinophil granulocytes are derived from stem cell precursors in the bone marrow[12]. Several stimuli like granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3 and IL-5 promote the maturation of eosinophils from myeloid precursors. Under healthy conditions they represent about 2-5 % of the circulating blood cells whereas the majority is present in tissues surrounding the respiratory tract, the gut and the urogenital epithelium ready to destroy invading organisms[12]. Their granules contain major basic peptides, eosinophil cationic proteins, eosinophil-derived-neurotoxins and eosinophil peroxidase. Those basic proteins are assigned to destroy parasitic invaders, in case of a helminthic infection for instance. Under atopic conditions those effector-proteins are released upon allergen stimuli and are toxic against normal tissue. Besides tissue damage, eosinophils release a variety of cytokines, chemokines and growth factors (i.e. IL-4, IL-5, IL-13, IFNγ, eotaxin, GM-CSF) that regulate the immune response. Eosinophils contribute to the late phase of allergic responses as they are recruited to the site of inflammation by mediators released from allergen-activated mast cells[11].

1.3. Prostaglandins

Prostaglandins are lipid mediators with distinct roles and physiological functions. Together with another group of these hormone-like substances, the leukotrienes, they are of high interest for pharmacological studies. *S. Bergström, B. Samuelson* and *J.R. Vane* early recognized the significance of prostaglandin metabolism and were honored with the Nobel Prize for Medicine and Physiology "*for their discoveries concerning prostaglandins and related biologically active substances"* in 1982[13]. Their fundamental research elucidated the biosynthesis and the metabolism of prostaglandins including the important discovery that anti-inflammatory drugs, like Aspirin act through the inhibition of the prostaglandin biosynthesis. Their profound work provided the basis for the advanced and recent research field of prostaglandins and their respective receptors. These cardinal lipid mediators are ubiquitously present in the mammalian organism and regulate the physiology of inflammatory responses, blood pressure, blood clotting, pain and fever, for instance. Thus, they are of high interest for pharmacologic research[14].

1.3.1. Biosynthesis of Prostaglandins

Prostaglandins are derived from the arachidonic acid. Due to events that destabilize the cell membrane such as contact with histamine, cytokines, bacterial lipopolysaccharide (LPS), allergens or mechanical stimuli, the phospholipid bilayer is irritated which activates and enables the phospholipase A2 to catalyse the liberation of arachidonic acid from phospholipids[15]. Arachidonic acid, a 20 carbon polyunsaturated fatty acid with 4 double bounds, is oxidized by cyclooxygenase 1 & 2 (COX1 & 2) to PGG_2 followed by reduction to the unstable precursor PGH_2. This intermediate is the substrate of five individual enzymes (PGE-, PGF-, PGD-, PGI- and thromboxane synthase), which trigger the formation of the respective prostaglandins PGE_2, $PGF_{2\alpha}$, PGD_2, PGD_2 and TXA_2 (thromboxane A_2) (Figure 1)[14].

Although nearly every tissue in the human body is able to produce one or more types of prostaglandins their effects are highly dependent on the expression of specific receptors for every single prostaglandin or thromboxane. Prostaglandins stimulate cells in an autocrine or juxtacrine manner and have divers and multifaceted effects depending on the kind of prostaglandin or tissue and reason for its synthesis[14].

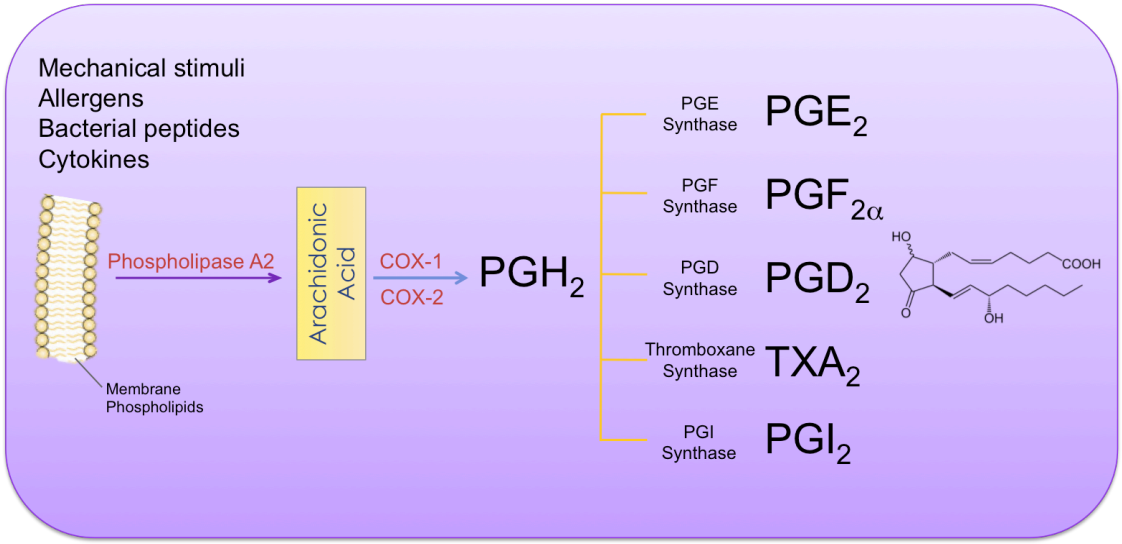


Figure 1. PGD_2 synthesis. Phospholipase A2 generates arachidonic acid from phospholipids in response to mechanical stimuli, bacterial peptides, allergens or cytokines. The prostaglandin precursor PGH_2 is then derived from arachidonic acid by cyclooxygenase 1 and 2. Among other prostaglandins, PGD_2 is then produced out of its precursor by its specific prostaglandin D synthases.

1.4. Prostaglandin D_2

Prostaglandin D_2 is synthesized in the brain where it has important functions in the regulation of sleep and pain perception for instance. In the peripheral tissues it is mainly released by immunoglobulin E (IgE)-activated mast cells in response to allergen exposure. Furthermore, other cells like macrophages, dendritic cells, Th2 cells, endothelial cells and platelets produce small amounts of endogenous PGD_2[16].

PGD_2 plays a key role in inflammatory processes where it can elicit both, controversial effects depending on the tissue and the inflammatory stimulus[16]. For instance, it promotes eosinophil infiltration into the lungs of mice in response to allergen exposure which leads to pulmonary eosinophilic inflammation[17] but can also cause neuronal protection in case of neuronal injury[18]. Mast cell-derived PGD_2 recruits and activates eosinophils as well as Th2 cells and provokes the pathophysiological effects of vasodilation, bronchoconstriction and airway narrowing. PGD_2 is therefore an essential promoter of the late phase of allergic response[19].

1.4.1. Prostaglandin D_2 Receptors

PGD_2 elicits its biological effects via G protein-coupled receptors (GPCRs), the D-type Prostanoid Receptor (DP), the chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes (CRTH2) and binds in high doses the thromboxane receptor A2 (TXA_2)[14].

1.4.1.1. D-type Prostanoid Receptor (DP)

DP (or DP_1) was the first receptor identified for PGD_2. Besides its expression on immune cells like Th1- and Th2 cells, mast cells, macrophages, basophils, eosinophils, it is widely expressed in the organism, for instance in smooth muscles, the vasculature and the central nervous system[20,21]. Upon activation, DP accounts for vascular and pulmonary smooth muscle relaxation, vasodilation[22], mucin secretion[23], arterial hypertension and inhibition of platelet aggregeation[24]. Controversially, pro- and anti-inflammatory effects have been linked to DP activation.

DP activation is counteracting inflammation by inhibition of effector functions[25,26] and cytokine release of immune cells[27]. DP agonist inhalation prevents asthma in an *in vivo* mouse model.[25,28] It was reported that DP couples to Gαs-proteins, which consequently increase the intracellular cAMP level through adenylyl cyclase activation[14]. Elevated cAMP is generally linked to inhibition of immune cell function[29]. In recombinant HEK293 cell lines expressing the human DP receptor, DP was also shown to activate Gαq/11-proteins leading to increased intracellular Ca^{2+} levels[20].

In contrast, DP also accounts for pro-inflammatory effects. *In vivo,* genetic deletion of DP was protective against airway hyperreactivity and decreased eosinophils infiltration into the lungs of ovalbumin (OVA) challenged mice[30]. Oral administration of a specific DP antagonist was beneficial for the treatment of allergic rhinitis as it prohibited early nasal response (sneezing) and mucus production in guinea pigs[31].

1.4.1.2. Chemoattractant Receptor Homologous-Molecule Expressed on Th2 lymphocytes (CRTH2)

CRTH2 is the most recently identified receptor for PGD_2 and is specifically expressed on hematopoietic cells such as Th2 lymphocytes, monocytes, macrophages, eosinophils, basophils and dendritic cells and generally accounts for pro-inflammatory cell responses[32]. It mediates activation and cytokine release (IL-3, IL-5, IL-13) from Th2 cells[33,34] and induces chemotaxis of eosinophils, basophils and Th2 cells[35].

In vivo, CRTH2 induced eosinophil accumulation and inflammation in the lungs of ovalbumin challenged mice[36]. Atopic dermatitis, pollen- or dust mite sensitivity is associated with increased CRTH2 expression on $CD4^+$ T cells in humans[15]. By coupling to Gαi-proteins, CRTH2 activation leads to inhibition of adenylyl cyclase and consequently lowers cAMP- and raises Ca^{2+} levels within the cells[35]. Furthermore, there is evidence that CRTH2 can also

activate Gαq-proteins at least in eosinophils and basophils leading to shape change or PI 3-kinase activation for instance[37].

1.4.1.3. DP and CRTH2 Interplay

Although DP- and CRTH2-mediated signaling seems to be controversial, both receptors are co-expressed on immune cells (Th2 cells, dendritic cells, basophils, eosinophils) and regulate their cellular responses to PGD_2[16]. In general, CRTH2 often dominates the PGD_2 induced cell functions[33,38] but there is evidence that DP regulates the signaling of CRTH2 what might be explained by up- and down-regulation of DP and CRTH2 expression due to an unknown regulatory mechanism[39,40]. In eosinophils, CRTH2 accounts for chemotaxis[35] and degranulation[38], whereas DP activation prolongs eosinophil survival by inhibiting apoptosis[38]. Recently our group reported that DP and CRTH2 are able to interact with each other[41]. In a recombinant HEK293 cell line DP and CRTH2 were able to form heteromeric units. The DP receptor was shown to amplify the CRTH2-mediated Ca^{2+} release, coincidentally losing its own potency to respond to selective agonists. Further on, desensitization or pharmacological blockade of DP prevented signaling via CRTH2. It was also proposed that CRTH2-mediated Ca^{2+} release depends on Gαi- and Gαq/11- proteins whereas DP signaling solely required Gαq/11-protein activation[41]. Together, the authors suggest that CRTH2 and DP receptors interfere with each other and that DP might transactivate CRTH2 signaling.

1.4.2. Impact of Prostaglandin D_2 Signaling in Viral Infection

Besides its famous role in allergen-caused inflammation and in cellular defense against parasites, PGD_2 accounts to host reactions in response to viral invasion.

Recent studies report an important role of PGD_2 in viral infections. Similar to the pro- and anti-inflammatory effects of PGD_2 in allergic inflammation, in case of viral infections PGD_2 plays a controversial role. For instance PGD_2 is increasingly expressed in the lungs of old mice. This correlates with a decreased activation and migration of respiratory dendritic cells (DC). As a successful T cell-mediated response to virus infection is dependent on effective DC activation and migration, the anti-viral T-cell response is consequently impaired[42]. Antagonistic blocking of PGD_2 restored the effective T cell-mediated anti-viral immune response. Furthermore it was shown that this effect was mainly DP mediated. PGD_2 was therefore suggested as a treatment for respiratory viral infections in older humans[43].

Moreover, it was shown that PGD_2 links allergic inflammation with enhanced risk for viral infection. Administration of dsRNA into lungs of ovalbumin (OVA) challenged mice increased the levels of PGD_2 which consequently led to the infiltration and accumulation of eosinophils into the lungs. Importantly, pharmacological blockade or genetic deletion of the CRTH2 receptor prevented the dsRNA-induced exacerbation of eosinophilic airway inflammation[44].

It has long been known that prostaglandins have anti-viral properties[45]. More recently it was reported that the PGD-synthase is a target of the immediate early protein (BICP0) from bovine herpesvirus 1 (BHV-1). BICP0 triggers the expression of a variety of cellular and viral proteins and accounts for BHV-1 replication. Since PGD_2 impaired the viral protein replication, the authors suggest that this is due to an inhibition of BICP0[46].

Taken together PGD_2 and the receptors DP and CRTH2 have an important role in the host response to viral infections. However, the explicit role of each receptor and the impacts of receptor interference has not yet been proven.

1.5. Signaling of G-protein Coupled Receptors (GPCR)

GPCRs connect the intracellular with the extracellular space by transmitting signals via G-proteins that activate the respective cellular response. By definition, these integral membrane proteins consist of seven transmembrane-spanning α-helices connected by intra- and extracellular loops. The ligand-binding pocket is located in the extracellular region, whereas a G-protein is C-terminally coupled to the receptor. Mainly heterotrimeric G-proteins but also small GTPases like Rho, Ras or Rac couple to this family of receptors and activate a variety of signaling pathways. Heteromeric G-proteins consist of three subunits named Gα, Gβ and Gγ, whereas the β- and the γ- subunits are usually connected to each other (Gβγ). Classically, upon ligand binding the receptor undergoes a conformational change that enables the GEF (guanine exchange factor) domain of the receptor to replace the Gα-subunit bound GDP (guanosin diphosphate) with GTP (guanosin triphosphate). Consequently, Gα dissociates from Gβγ. Both, the Gα- and the Gβγ-subunits are potent signal transducer and activate several signaling cascades[47]. The diversity and complexity of GPCR signaling is additionally provided by at least 18 different Gα-subunits discovered in humans up to now [48]. Regarding that GPCRs are further able to induce G-protein-independent signaling events and that receptor di- or oligomerization can essentially alter the ligand-binding and signaling

properties, the incredible variety of signaling possibilities mediated by this protein family becomes obvious[49].

Their pharmacological and clinical significance is further demonstrated by the fact that approximately 36 % off all current drugs on the market target GPCRs[50]. Recently, the impact of homo- or heteromerization of GPCRs was realized. In the past, it was assumed that GPCRs exist only as monomeric proteins and transmit signals independently, whereas recent research elucidated that di-/oligoformation is a common feature within this receptor family. This feature provides a completely new therapeutic potential of drugs targeting GPCRs[51,52].

1.5.1. Functional Consequences of Heteromerization

Heteromerization can lead to essential functional changes within the receptor-signaling unit. Upon contact with a second receptor, the first receptor can undergo allosteric modulations that might alter the response to its ligand. Heteromerization upon co-expression of a second receptor can lead the formation of functional signaling units or to increased activation of associated G-proteins, for instance[53,54]. The presence of agonists for both receptors may lead to either enhanced or decreased activity of one receptor. On the one hand, blockade of one receptor by a selective antagonist may prevent agonist binding (i.e. trans-inhibition) or promotes the agonist binding (i.e. trans-activation) of the second receptor. Furthermore, the receptor trafficking can be altered in heteromers, for instance ligand binding of one receptor can induce endocytosis of both.[49,55] Heteromerization can also influence signaling capacities at the G-protein level. Recently, it was shown that the dopamine receptors D1 and D2 form heteromers in the brain and hence activate Gαq/11. However, in the monomeric form, the receptor D1 is coupled to Gαs- and D2 to Gαi-proteins[56]. In this case the signaling is switched from adenylyl cyclase modulation to activation of the phospholipase C what leads to a massive Ca^{2+} release from the endoplasmic reticulum. This demonstrates that heteromerization can form completely new signaling units and alter the Ca^{2+} signaling capacities of GPCRs[57].

1.6. Transcriptional Regulation of Allergic Mechanisms

Little is known about the activation of transcription factors activated by PGD_2 via DP and CRTH2 and the involved signaling pathways. I was particularly interested in elucidating the involvement of NFAT (nuclear factor of activated T cells), SRE (serum response element) and CREB (cAMP response element binding protein) in DP- and CRTH2- mediated

signaling. Those regulatory elements are highly implicated in allergic responses. The serum response factor (SRF) binds to SRE, which regulates cytoskeletal reorganization and is highly involved in the cellular functions of adhesion and migration[58]. The serum response factor (SRF) and the ternary complex factor (TCF) are mainly responsible for the induction of SRE. SRF is capable of binding and activating SRE alone or in a complex with TCF[59]. The activation and translocation of these transcription factors may be triggered by protein kinase C (PKC) or by small RhoGTPase-dependent pathways among others[59-61]. As we and others found that PGD_2 is involved in eosinophil shape change[62] and cytoskeletal rearrangement in eosinophils[37] and HEK-CRTH2+DP cells (unpublished data) I examined the induction of SRE by DP and CRTH2.

Increased calcium or cAMP levels can lead to the activation of CREB by proteinkinase A, which in turn leads to the transcription of target genes with cAMP-responsive elements (CRE) within their promoters[63,64]. CRE regulates a wide range of cellular functions such as survival or growth and can be activated via cAMP increase followed by PKA activation or by MAPK-associated pathways[65].

NFAT regulates gene expression of immune cells during early immune response and hypersensitivity. Expression of cytokines (i.e. IL-2, IL-4, IL-5) and surface molecules on immune cells were shown to be NFAT-dependent[66]. Dominant negative NFAT-transgenic mice show less pulmonary inflammation and accumulation of eosinophils in the bronchoalveolar lavage of these mice is delayed[67]. Further on, the expression of eosinophil-cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) is enhanced by NFAT activation[68]. NFAT is involved in the development of allergic inflammation and regulates apoptosis through induction of the Fas ligand[69].

2. Specific Aim of the Thesis Project

Aim I:

To elucidate whether and how the PGD_2 receptors DP and CRTH2 regulate the activation of the transcription regulatory elements CREB, NFAT and SRE in recombinant HEK293 cell lines.

I will particularly investigate the cross-talk between DP and CRTH2 in luciferase reporter gene assays using specific receptor agonists, antagonists and pharmacological inhibitors involved in the respective signaling pathways.

Aim II:

To verify the data obtained from Aim I by performing functional assays in human peripheral blood eosinophils.

3. Material and Methods

3.1. Materials

3.1.1. Chemicals

Aqua bidestillata	Mayerhofer Pharmazeutika, Linz, Austria
BD Cell FIX	BD Biosciences, San Jose, CA, USA
BD FACS Flow	BD Biosciences, San Jose, CA, USA
BD FACS Lysing Solution	BD Biosciences, San Jose, CA, USA
Bovine serum albumin	Sigma-Aldrich, St. Louis, USA
Dextran	Sigma-Aldrich, St. Louis, USA
Dulbecco's Modified Eagle's Medium	Invitrogen, Lofer, Austria
Dulbecco's PBS 1x liquid - $CaCl_2$, Mg_2	Invitrogen, Lofer, Austria
Dulbecco's PBS 1x liquid +$CaCl_2$, Mg_2	Invitrogen, Lofer, Austria
EDTA solution	Sigma-Aldrich, St. Louis, USA
EGM-2 MV Bullet medium	Lonza, Basel, Switzerland
FBS	Hyclone Distributor VWR, Logan, USA
Fibronectin	Sigma-Aldrich, St. Louis, USA
Formaldehyde, 37 %	Carl Roth GMBH & Co KG, Karlsruhe, Germany
Gelatine Type B, cell culture tested	Sigma-Aldrich, St. Louis, USA
Geneticin Selective Antibiotic	Invitrogen, Karlsruhe, Germany
Glucose	Sigma-Aldrich, St. Louis, USA
Glycerol	Sigma-Aldrich, St. Louis, USA
HBSS	Invitrogen, Lofer, Austria
HEPES cell culture tested	Sigma-Aldrich, St. Louis, USA
Histopaque	Sigma-Aldrich, St. Louis, USA
Immersion Oil for Microscopy, Type A	Nikon Instruments, Badhoevedorp, NL
Lipofectamin2000	Invitrogen, Lofer, Austria
Mautner-Markhof Ethanol absolut	Lactan GMBH &Co.KG
NaCl	Carl Roth GMBH&Co.KG
NH_4Cl	Sigma-Aldrich, St. Louis, USA
OPTIMEM	Invitrogen, Lofer, Austria
PBS	Invitrogen, Lofer, Austria

Phalloidin-Texas Red	Invitrogen, Lofer, Austria
Poly-D-Lysine	Sigma-Aldrich, St. Louis, USA
Texas-Red Phalloidin	Invitrogen, Lofer, Austria
Triton-X 100	Sigma-Aldrich, St. Louis, USA
Vectashield Mounting Medium	Vector Laboratories, Burlingam, CA, USA
Zeocin	Invitrogen, Lofer, Austria

3.1.2. Buffers/Solutions

Assay buffer	PBS (with or without Ca^{2+}/Mg^{2+})	
	0.1 % Bovine Serum Albumine	
	10 mM Glucose	
	10 mM HEPES	adjusted to pH 7.4 with NaOH

Fixative Solution	1 ml BD CellFix solution
	30 ml FACSFlow
	10 ml Aqua dest.

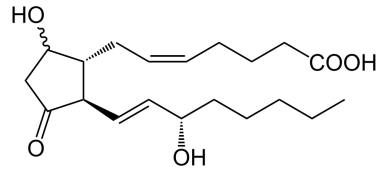
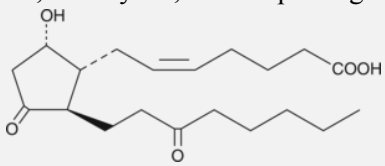
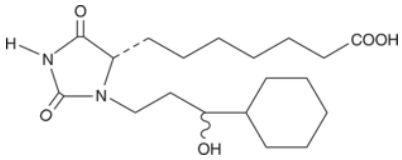
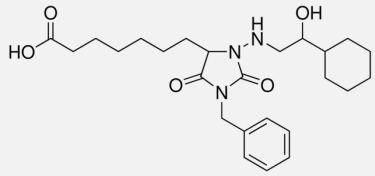
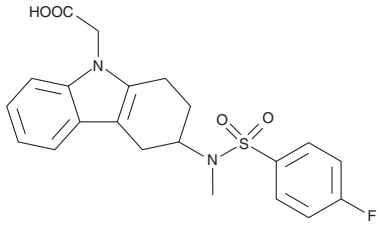
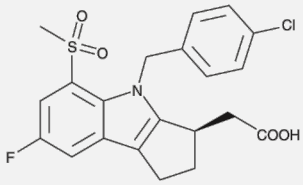
3.1.3. Kits and Enzymes

Complete Protease Inhibitor Cocktail (Roche Applied Science, USA)
Eosinophil Isolation Kit (StemCell Technologies, Vancouver, Canada)
Fast SYBR Green PCR Master Mix (Applied Biosystems, Vienna, Austria)
FLIPR® Calcium 4 Assay Kit (Molecular Devices, Sunnyvale, CA, USA)
HiSpeed Plasmid Maxi Kit (QIAGEN, Hilden, Germany)
Steadylite Plus Kit (Packard Instrument Company, Meriden, CT, USA)

3.1.4. Plasmids

The plasmids pCREB-luc and pNFAT-luc for luciferase reporter gene assays were purchased from Stratagene, La Jolla, CA, USA. pSRE-luc was a kind gift from Silvio J. Gutkind form the National Institutes of Health (NIH), Bethesda, MA, USA.

3.1.5. Pharmacological Substances

PGD_2	Prostaglandin D_2 	Cayman Chemicals (Ann Arbor, MI, USA)
DK-PGD_2 (Selective CRTH2 agonist)	13,14-dihydro,15-keto prostaglandin D_2 	Cayman Chemicals (Ann Arbor, MI, USA)
BW 245c (Selective DP agonist)	(4S)-(3-[(3R,S)-3-cyclohexyl-3-hydroxypropyl]-2,5-dioxo)-4-imidazolidin heptanoic acid 	Cayman Chemicals (Ann Arbor, MI, USA)
BW A868c (Selective DP antagonist)	3-[(2-cyclohexyl-2-hydroxyethyl)amino]-2,5-dioxo-1-(phenylmethyl)-4-imidazolidine-heptanoic acid 	Cayman Chemicals (Ann Arbor, MI, USA)
CAY10471 (Selective CRTH2 antagonist)	(+)-3-[[(fluorophenyl)sulfonyl] methyl amino]-1,2,3,4-tetrahydro-9H-carbazole-9-acetic acid 	Cayman Chemicals (Ann Arbor, MI, USA)
MK 0524 (Selective DP antagonist; Laropiprant)	4-[(4-chlorophenyl)methyl]-7-fluoro- 1,2,3,4-tetrahydro-5-(methylsulfonyl)-cyclopent[b]indole-3-acetic acid 	Cayman Chemicals (Ann Arbor, MI, USA)

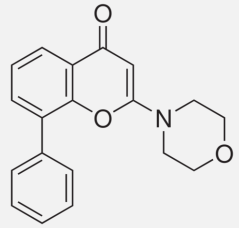
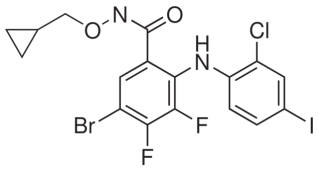
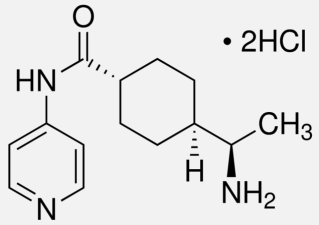
Rho Inhibitor I	Exoenzyme C-3 Transferase	Cytoskeleton. Inc. (Denver, Colorado, USA).
Ly-294.002 (PI 3-kinase Inhibitor)	2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride 	Sigma-Aldrich (St. Louis, USA)
PD-184161 (ERK1/2 Inhibitor)	5-Bromo-2-[(2-chloro-4-iodophenyl)amino]-N-(cyclopropylmethoxy)-3,4-difluoro-benzamide 	Sigma-Aldrich (St. Louis, USA)
Y-27632 (ROCK Inhibitor)	(*R*)-(+)-*trans*-4-(1-Aminoethyl)-N-(4-Pyridyl) cyclohexanecarboxamide dihydrochloride 	Sigma-Aldrich (St. Louis, USA)
MH-362-63-8 ($G\alpha_q$ Inhibitor) was a kind gift from Ed Cannon, Unigen Inc. (Lacey, Washington, USA).		

Table 1. Pharmacological Substances

3.1.6. Antibodies

Alexa Fluor 488 IgG_1 and Alexa Fluor 488 IgG_{2B} were purchased from Invitrogen, Karlsruhe, Germany. Mouse monoclonal (9E10) ab32 anti-Myc IgG_1 and mouse monoclonal M1 anti-Flag IgG_{2b} were purchased from Abcam, Cambridge, UK.

3.2. Methods

3.2.1. Cells and Cell Lines

3.2.1.1. Human Embryonic Kidney Cells (HEK 293)

To determine the activation of the transcription factors cAMP response element binding protein (CREB), nuclear factor of activated T cells (NFAT) and serum response element (SRE) we used clones of HEK293 cell lines that stably express the Myc-tagged human CRTH2 (HEK-CRTH2), the Flag-tagged human DP (HEK-DP) or both receptors in combination (HEK-CRTH2+DP) as described previously[41,70]. HEK-CRTH2 codes for the resistance gene for neomycin and HEK-DP for zeocin.

HEK293 cells were kept in serum containing selection medium (DMEM+10 % FBS) with either neomycin (0.2 %) or zeocin (0.4 %) or both. Cells were propagated in 75 cm^2 cell culture flasks and medium was changed every second day. Cells were harvested after 3-4 days of culturing when they were grown to 90 % confluence.

3.2.1.2. Human Lung Microvascular Endothelial Cells (HMVEC-L)

Human lung microvascular endothelial cells are isolated cells from small vessels of normal lung tissue. They consist of primary blood and lymphatic endothelial cell populations and are suitable to study vascular pathology, inflammation or pulmonary diseases for instance. In this case, HMVEC-L were cultured on VenaEC^{TM} biochips to elucidate the role of DP and CRTH2 in adhesion of human eosinophils to endothelial cells under the condition of physiological shear stress.

HMVEC-L cells were maintained in medium supplemented with hemagglutination enhancing factor (hGEF), Gentamicin (GA-1000), hydrocortisone, FBS 5 % (fetal bovine serum) vascular endothelial growth factor (VGEF), human fibroblast growth factor-basic (hFGF-B), insulin growth factor (R3-IGF-1) and ascorbic acid (EGM-2 MV Bullet medium). Cells were grown to about 90 % confluence and used for experiments from passage six to nine. Medium was changed every second day.

3.2.1.3. Purification of Human Peripheral Blood Eosinophils

Human eosinophil granulocytes were isolated from blood taken from healthy volunteers according to a local ethics committee-approved protocol. After taking the blood sample, the coagulation was prevented by binding the Ca^{2+} ions with sodium citrate (3,8 %). Most of the

erythrocytes were removed by dextran treatment. As dextran is a glucose polymer that crosslinks the erythrocytes, which then sediment to the bottom of the test tube. To split the leukocytes into peripheral blood mononuclear cells (PBMC) and polymorphonuclear leukocytes (PMNL), cells were subjected to a density gradient centrifugation using histopaque, which is a polysuccrose solution with a defined density of 1.077.

Protocol

To separate the blood cells from plasma 4.4 ml of 3.8 % sodium citrate were filled up to 40 ml with blood and centrifuged at 400 g for 20 min. For the dextran sedimentation 6 ml of 6 % dextran was added, filled up to 50 ml with 0.9 % saline and incubated at room temperature for 30 min. To perform the density gradient centrifugation the upper phase of dextran sedimentation was placed onto 15 ml of histopaque. Then the blood cells were centrifuged at 400 g for 20 min. This step separates the blood cells into the PBMC and the PMNL. PMNL were resuspended in washing buffer and the erythrocytes were lysed by adding 0.2 % saline and 1.6 % saline in the same amount. After a washing step with PBS (phosphate buffered saline) the PMNL were stained with Kimura and the number of cells was determined.

Human eosinophil granulocytes were separated from the polymorphonuclear-rich fraction by negative magnetic selection. The cells were immunomagnetically labeled with a cocktail of monoclonal antibodies bound in tetrameric antibody complexes (TAC) that bind to cell surface markers of hematopoietic cells (specific for CD2, CD14, CD19, CD56 and glycophorin A) and to magnetic dextran iron particles (Figure 2). These antibodies do not bind to eosinophils, what allows them to pass through the magnetic separation column placed into a magnetic field. All the other cells are retained in the column. The purity and viability of isolated eosinophils was > 97 %.

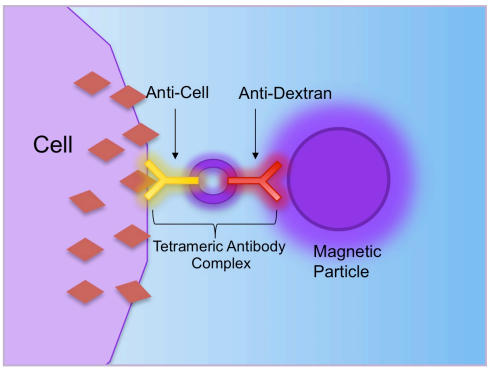


Figure 2. Negative selection. StemSep© Tetrameric Antibodies Complexes label hematopoietic cells except from eosinophil granulocytes.

3.2.2. Flow Cytometric Analysis of Receptor Expression

Flow cytometry gives the opportunity to analyze small particles or cells due to their form and structure. This method allows simultaneously quantification of more than one property of an intact cell at the same time. A flow cytometer measures the light scattering that occurs if a single cell in suspension is passed through a beam of light of a single wavelength. The light

scattering induced by a particle or cell lead to changes in the light spectrum what several detectors recognize in turn. The detector in line with the laser determines the forward scatter (FSC) which corresponds to the volume and surface structure of a cell, whereas the side scatter (SSC) detector is positioned in a 90° angle to the light beam and is related to cytoplasmatic granularity. Additional fluorescence detectors observe the emitted light when a single cell passes through the laser beam. Hence, a cell can be analyzed due to its size, granularity and fluorescent properties at the same time.

The level of CRTH2 and DP receptor expression on HEK-CRTH2, HEK-DP and HEK-CRTH2+DP cells was determined by staining the cells with anti-Myc IgG_1 or anti-Flag M1 IgG_{2b} antibodies followed by flow cytometric analysis on a BD FACSCalibur flow cytometer. (Table 2)

Protocol

Cells were seeded in 6-well plates in DMEM + 10 % FCS medium, grown to confluence and harvested in 750 µl of PBS supplemented with Ca^{2+} and Mg^{2+}. The cells were centrifuged at 400 g for 5 min, and blocked in 250 µl of 1 % BSA for 30 min at 4 °C. Subsequently, the cells were centrifuged (400 g, 7 min), resuspended in 50 µl of the 1st antibody (1 µg/mL in 1 % BSA) and incubated for 60 min at 4 °C.

Cells were washed with 250 µl of PBS followed by staining with 50 µl of the 2nd antibody (2µg/mL in 1 % BSA) for 45 min at 4 °C. Then, cells were washed twice, fixed (CellFIX) and analyzed by flow cytometry.

Receptor	Tag	1st Antibody	2nd Antibody
CRTH2	Myc	Anti-MYC IgG_1	Alexa Fluor® 488 IgG1
DP	FLAG	Anti-FLAG IgG_{2B}	Alexa Fluor® 488 IgG_{2B}

Table 2. Antibody combinations for flow cytometric analysis of receptor expression in HEK293 cells

3.2.3. Luciferase Reporter Gene Assays

3.2.3.1. Transfection

In order to analyze agonist-induced activation of the transcription factors (TF), CREB, NFAT and SRE in HEK-CRTH2, HEK-DP, and HEK-CRTH2+DP cells, cells were transfected with plasmids encoding the respective TF under the control of the luciferase reporter gene driven by a basic promoter element (TATA box) plus a defined inducible cis-enhancer element. With

the help of liposomes the plasmid DNA was incorporated into the cells. The positively charged lipids form complexes with the negatively charged nucleic acid. As both consist of a phospholipid bilayer, liposomes easily merge with the cell membrane. Lipofectamine2000 was purchased from Invitrogen. Plasmids were replicated in *E.coli* and isolated with Qiagen Plasmid Maxi Kit.

Protocol

Cells were seeded into 96-well plates at a density of 60,000 cells/well and grown to approximately 90 % confluence in non-selective medium (DMEM + 10 % FCS). Then, medium was replaced with serum-free medium (Opti-MEM®) and cells were transfected with the plasmids pCREB-luc (200 ng/well), pNFAT-luc (200 ng/well) or pSRE-luc (100 ng/well), respectively. For efficient transfection 0.6 µl Lipofectamin2000/ x ng of DNA were added. After incubating the cells for 5 h at 37 °C the medium was again changed to serum-containing medium (DMEM + 10 % FCS).

For the gene dose experiment with dominant negative (DN) RhoA in combination with pNFAT-luc the cells were transfected with increasing amounts of T19N (DN RhoA) in combination with 100 ng pNFAT-luc. To treat every sample equally plasmid DNA was filled up to 200 ng with pcDNA 3.1.

3.2.3.2. Luciferase Reporter Gene Assay

Agonist-induced activation of the TF was determined using the Steadylite Plus Kit as previously described[71] by measuring the amount of expressed reporter gene (*luc*). If the TF gets activated, it binds to the specific enhancer elements on the plasmid and triggers the expression of firefly luciferase. By lysing the cells and adding D-luciferin as a substrate the luciferase converts it into oxyluciferin and light.

$$\text{D-Luciferin} \xrightarrow[\text{ATP} + \text{O}_2 + \text{Mg}^{2+}]{\text{Firefly luciferase}} \text{Oxyluciferin} + \text{LIGHT} + \text{PP}_i + \text{AMP} +$$

The amount of emitted light is relative to the promoter activity. The relative light units (RLU) were measured immediately in a chemiluminescence plate reader (TopCount NXT). (Figure 3)

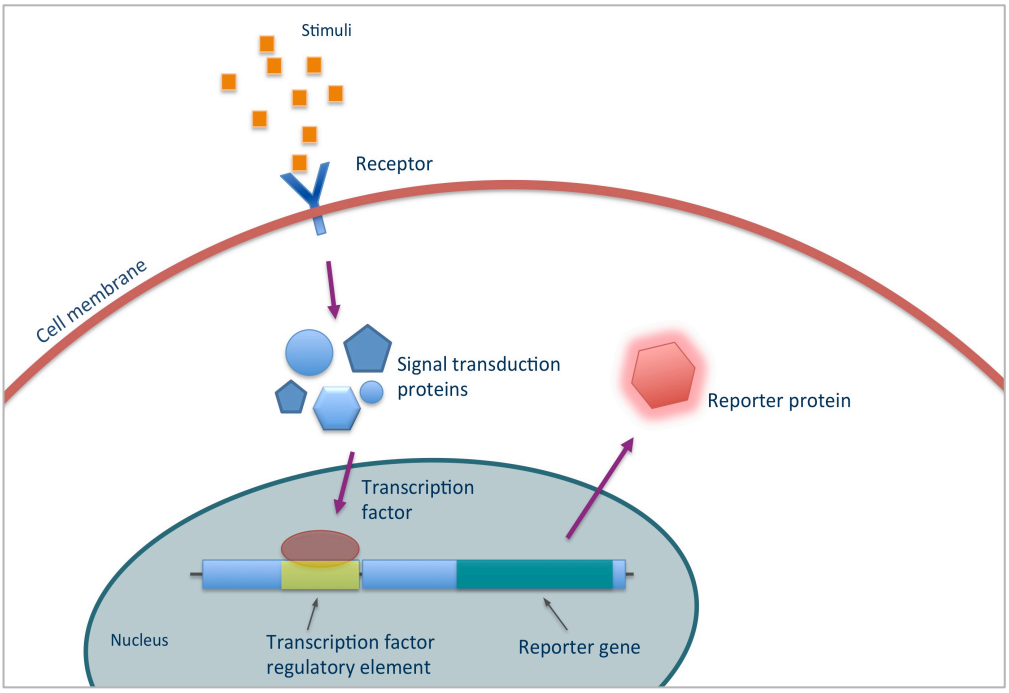


Figure 3. Luciferase reporter gene assay. In response to the stimuli the receptor triggers the signal transduction leading to the activation of the transcription factor (TF) of interest. The activated TF translocates to the nucleus where it binds to the respective enhancer element in the promotor of the reporter plasmid; this in turn triggers the expression of the reporter gene.

Protocol

Agonists/antagonists/inhibitors were prepared in Opti-MEM® and diluted in 1:10 steps from 10^{-5} to 10^{-11} M. Medium was sucked off and 100 µl per well of the dilutions was added to the cells. Usually, cells were pre-treated with antagonist or inhibitor for 20 min followed by agonist stimulation at a concentration of 1 µM for 3 h at 37 °C. Subsequently medium was sucked off, 100 µl PBS was added and cell numbers were determined in a FlexStation II (Molecular Devices). Luminescence was determined by the Stadyliteplus High Sensitivity Luminescence Reporter Gene Assay kit purchased from PerkinElmer. Cells were incubated with 100 µl of the substrate dissolved in buffer for 10 min and the RLU were measured. The cell lines used are listed in Table 3.

Cell lines:	***Expressed receptors:***
HEK-CRTH2-DP	DP and CRTH2
HEK-DP	DP
HEK-CRTH2	CRTH2

Table 3. List of cell lines used in the luciferase reporter gene assays.

3.2.4. Eosinophil Adhesion to Endothelial Cells under Flow Conditions

In order to study the role of DP and CRTH2 receptors in cell-cell interactions between endothelial cells and eosinophils the PGD_2 receptor-mediated adhesion of eosinophils to endothelial cells under flow conditions was investigated as described before [72]. Thus, a micro flow system that allows the determination of cell adhesion under physiological shear stress was used (Cellix). The Cellix system consists of a bright field phase contrast microscope, a Mirus™ 2.0 Nanopump and a CCD camera to monitor cell-cell interactions. For these experiments, primary human microvascular lung endothelial cells (HMVEC-L) were grown to confluent monolayers on biochips, and freshly isolated human eosinophils were perfused across the endothelial monolayer under distinct shear stress of 0.5 – 2 dyne/cm^2 that mimics physiological flow conditions of microvascular vessels. The amount of adhesion was recorded by microscopy. (Figure 4)

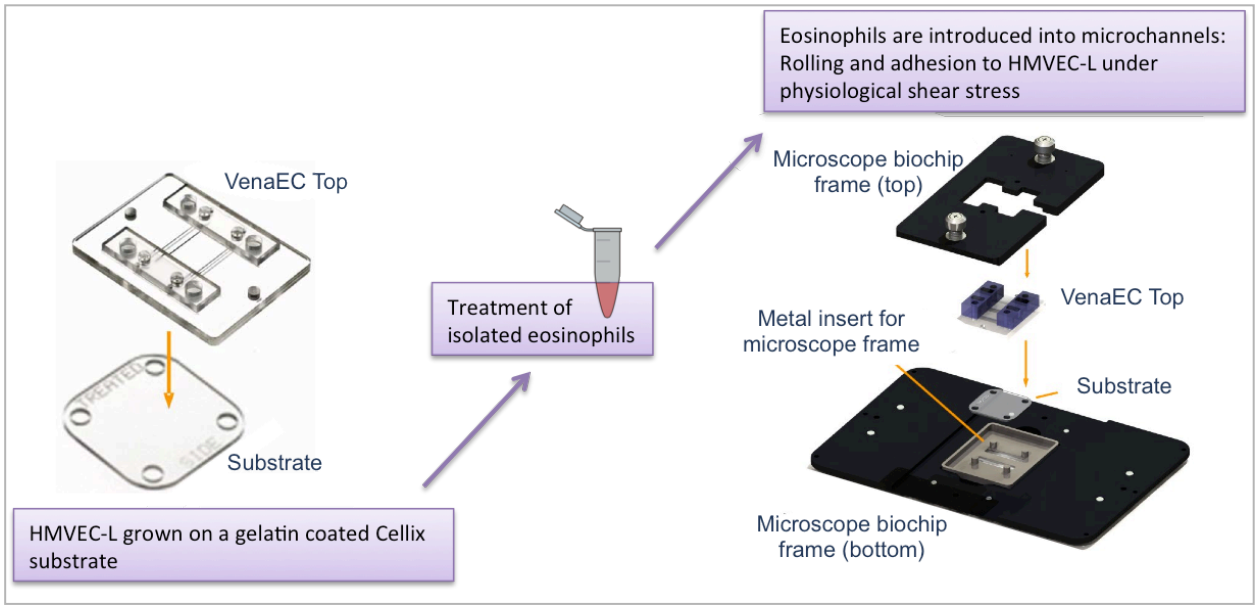


Figure 4. Workflow – Eosinophil adhesion to endothelial cells under flow conditions. Adapted from Cellix Ldt.

3.2.4.1. Seeding and Culturing Endothelial Cells on VenaEC™ Substrates

VenaEC™ substrates are designed to grow endothelial monolayer on their surface. They were sterilized with UV light for 20 min and subsequently coated with 1 % gelatin. HMVEC-L were seeded at a density of 400,000 cells / substrate and grown to confluent monolayers.

3.2.4.2. Measuring Adhesion under Shear Stress

The monolayer was stimulated with 10 pM of TNFα or vehicle for 4 hours. The substrates were then assembled to the flow chamber and freshly isolated eosinophils ($3x10^5$) were perfused across the cell layer. Eosinophils were preincubated with the antagonist for 5 min, directly followed by stimulation with the agonist for 5 min before the perfusion. Adhesion of eosinophils was recorded with a charge coupled device (CCD) camera using 10x magnification for 2.5 min under a defined shear stress of 2 dyne/cm^2.

3.2.5. Phalloidin Staining of Eosinophil F-Actin

Phalloidin belongs to the group of toxins derived from *Amanita phalloides*. It binds to actin polymers (F-actin) more tightly than to actin monomers (G-actin) and inhibits its depolymerization. Fluorescence labeled phalloidin is used as an imaging tool for actin filaments *in vitro*.

F-actin staining was generally performed as described previously [73]. Eosinophils were isolated freshly as described above (see 1.4). Glass coverslips were coated with fibronectin (5 µg/ml) for 1 h at 37 °C in 24 well plates. Fibronectin was sucked off and coverslips were washed

twice with assay buffer (PBS with Ca^{2+} and Mg^{2+} supplemented with 0.1 % BSA, 10 mM HEPES and 10 mM glucose, pH 7.4). Eosinophils maintained in assay buffer were pre-treated with the respective antagonist (10 min), inhibitor (45 min) or vehicle, seeded on the coverslips (250,000 per well) and were allowed to adhere in presence of the agonist for 10 min at 37 °C. The following steps were performed at room temperature. After washing, eosinophils were fixed with 500 µl of 3.7 % formaldehyde solution (in PBS) for 10 min. Cells were then washed 3 times followed by permeabilization with 500 µl of 0.1 % Triton-X-100 (in PBS) for 5 min. After 3 washing steps the cells were blocked with 1 % BSA in PBS for 25 min to prevent non-specific binding. Cells were then stained with 0.4 U of Phalloidin-Texas-Red for 20 min in the dark followed by three washing steps. The coverslips were mounted with Vectashield DAPI (4',6-diamidino-2-phenylindole) mounting medium to stain the nuclear fraction. To visualize actin polymerization, cells were analysed using an Olympus DP50-CU digital camera and Olympus Cell˙P software (Olympus, Lake Success, NY, USA).

3.2.6. Leukocyte Shape Change Assay

Due to their activation leukocyte change their shape what allows them to adhere and migrate through surrounding tissues like the endothelium of a blood vessel. The flow cytometric determination of the diameter of the cell allows the quantification of this cellular function. Changes in the volume or cell shape of eosinophils in response to PGD_2 or selective DP and/or CRTH2 agonists in combination with selective antagonists or inhibitors were specified by the changes in the forward scatter.

Leukocyte shape change assay was performed as described before[74]. Human eosinophils were freshly isolated as described above (see 3.2.1.3). Cells were treated with the respective antagonist, inhibitor or vehicle for 10 min or 45 min at room temperature in assay buffer (PBS with Ca^{2+}/Mg^{2+} supplemented with 0.1 % BSA, 10 mM HEPES and 10 mM glucose, pH 7,4) followed by a 4 min incubation with increasing concentrations of agonists or vehicle at 37 °C in a water bath. Samples were then put on ice and fixed with 150 µl of CellFIX and immediately analysed by measuring the changes in the forward scatter on a FACSCalibur flow cytometer (BD Biosciences). Data represent the percent increase in forward scatter compared to vehicle.

3.2.7. Statistics

Data shown represent means ± S.E.M. of *n* independent experiments. Statistical analysis was performed by ANOVA followed by Bonferroni post test using GraphPad Prism software (Graph Pad, San Diego, CA, USA). P values < 0.05 were considered to be significant.

4. Results

4.1. DP and CRTH2 Regulate Inflammation-related Transcription Factors in Differential Manners

4.1.1. Surface Expression of DP and CRTH2 on HEK 293 Cells

The surface expression of DP and CRTH2 on HEK-CRTH2+DP, HEK-CRTH2 and HEK-DP cells was determined by the use of antibodies specific for the Flag- (DP) and the Myc-tag (CRTH2) and measured by flow cytometry. The histograms show the expression of CRTH2 (red) and DP (blue) compared to the specific isotype control (Alexa Fluor® 488 IgG1 for CRTH2; Alexa Fluor® 488 IgG_{2B} for DP) (Figure 5). The shift in FL-1 (fluorescence channel 1) compared to the respective isotype control specifies the receptor expression on the cell surface.

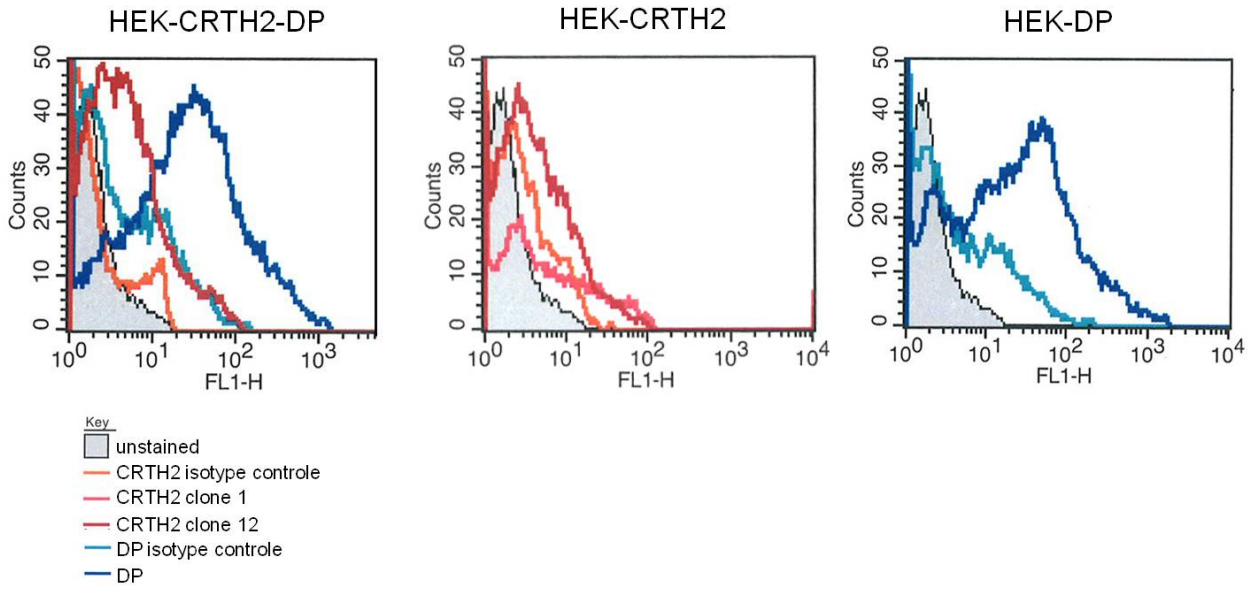


Figure 5. DP and/or CRTH2 are expressed on the recombinant HEK293 cell lines HEK-CRTH2+DP, HEK-CRTH2 and HEK+DP. Receptor expression was determined with anti-Myc (Myc-CRTH2) and/ or anti-Flag (Flag-DP) antibodies and was compared to respective isotype-control antibodies by flow cytometry. HEK293 cells stably transfected with Myc-CRTH2 and/or Flag-DP express DP and/ or CRTH2 on the respective cell surface. HEK-CRTH2 clone 12 was used for further experiments.

4.1.2. Downstream Signaling Events of DP and CRTH2

Little is known about the activation of transcription factors activated by PGD_2 via DP and CRTH2 and the involved signaling pathways. Hence, I investigated the involvement of NFAT (nuclear factor of activated T cells), SRE (serum response element) and CREB (cAMP response element binding protein) in DP- and CRTH2-mediated signaling as those regulatory elements are highly implicated in allergic responses.
The DP and CRTH2 mediated activation of transcription factors was elucidated with luciferase reporter gene assays.

4.1.2.1. DP Activates CREB

PGD_2 induced CREB activation in HEK-CRTH2+DP, which was approximately 10-fold higher as compared to vehicle (EC_{50}=0,13 nM see Table 4). In HEK-DP cells, CREB activation was lower but still up to 4-fold higher as compared to vehicle (Figure 6 A). The selective DP agonist BW245c mimicked the effect of PGD_2 in HEK-CRTH2+DP and in HEK-DP cells suggesting that DP mainly activates the pathway leading to CRE induction. In HEK-CRTH2 cells PGD_2 failed to activate CREB. The fact that in cells co-expressing both receptors CREB activation is significantly higher than in cells expressing solely DP indicates a supportive and DP-dependent role of CRTH2 (Figure 6 A and C). The selective CRTH2 agonist DK-PGD_2 activated CREB in HEK-CRTH2+DP up to 5-fold over vehicle but not below concentrations of 1 µM. This activation can be considered as a non-selective, probably DP-mediated effect regarding the EC_{50} value in the 100 nM to 1 µM range (Figure 6 B).

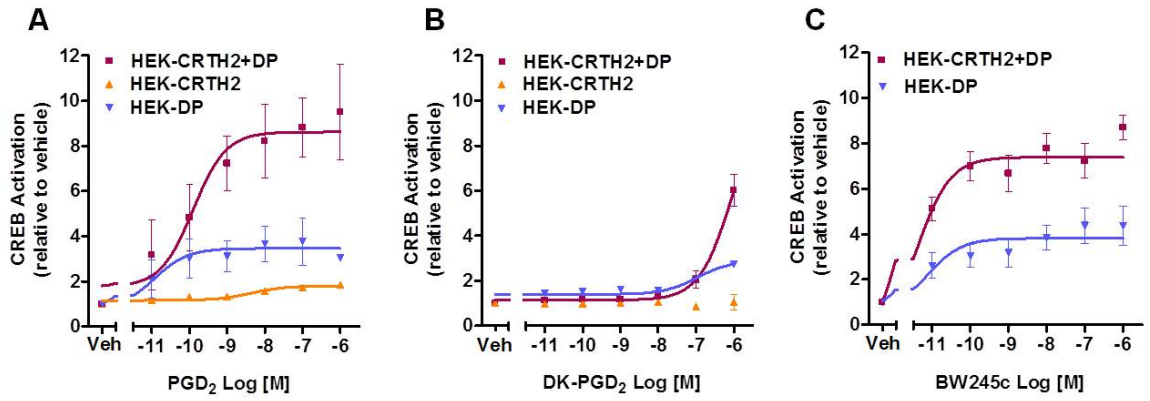


Figure 6. DP mediates CREB activation. Activation of CREB in response to DP and/or CRTH2 agonists was measured in luciferase reporter gene assays in HEK-CRTH2+DP, HEK-CRTH2 and HEK-DP cells. HEK293 cells were transfected with plasmid DNA containing enhancer sites for CREB and firefly luciferase (*luc*) as a reporter gene. Cells were stimulated with the agonists PGD_2, DK-PGD_2 or BW245c for 3 h at 37 °C. The cells were lysed and the emitted light was measured as relative light units immediately. PGD_2 and the selective DP agonist BW245c induced CREB in HEK293 cells expressing DP or both, CRTH2 and DP. The activation of CREB was enhanced as soon as both receptors are expressed on the cell surface. **(A)** CREB is activated by PGD_2 in HEK-CRTH2+DP and in HEK-DP. **(B)** DK-PGD_2 fails to induce CREB. **(C)** BW245c mimics the effect of PGD_2 and activates CREB in HEK-CRTH2+DP and in HEK-DP cells. Data were normalized to vehicle and show means ± S.E.M. of n=3-4 individual experiments.

To further clarify how CREB activation is regulated I used specific antagonists to block either DP or CRTH2. Supporting the finding that mainly DP is involved in CREB regulation, the specific DP antagonist BWA868c completely blocked PGD_2-mediated CREB activation in HEK-CRTH2+DP cells, whereas the CRTH2 antagonist Cay10471 showed no inhibitory effect (Figure 7 A and B). Again, BW245c mimicked the actions of PGD_2 and its effect was reversed by the DP antagonist but not by the CRTH2 antagonist (Figure 7 C and D).

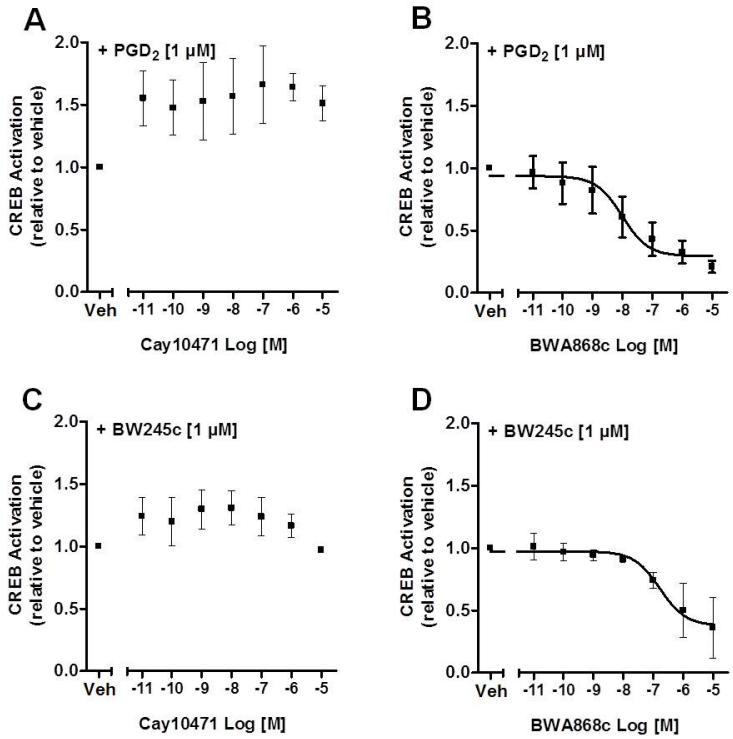


Figure 7. Agonist-induced CREB activation was inhibited by the selective DP antagonist BWA868c. Activation of CREB was measured in luciferase reporter gene assays in HEK-CRTH2+DP cells. HEK293 cells were transfected with plasmid DNA containing enhancer sites for CREB and firefly luciferase (*luc*) as a reporter gene. Cells were stimulated with the antagonists Cay10471 and BWA868c (20 min, 37 °C) prior to treatment with the agonists PGD_2 [1 µM] or BW245c [1 µM] for 3 h at 37 °C. The cells were lysed and the emitted light was measured immediately as relative light units. Pharmacological blockade of CRTH2 did not prevent CREB activation by PGD_2 or BW245c **(A, C)** whereas blocking of DP led to a significant inhibition of CREB activation **(B, D)**. Data were normalized to vehicle and show means ± S.E.M. of *n*=3-4 individual experiments.

4.1.2.2. DP is Essential for NFAT Activation

PGD_2-induced NFAT activation was 3- to 4-fold over vehicle in HEK-DP and 2- to 3-fold over vehicle in HEK-CRTH2+DP cells. In HEK-CRTH2+DP cells, PGD_2 was 10-fold more potent than in HEK-DP cells (Figure 4 A and see Table 4). Both PGD_2 and DK-PGD_2 failed to induce NFAT activation in HEK-CRTH2 cells. Notably, the selective CRTH2 agonist DK-PGD_2 provoked NFAT induction 3-fold over vehicle in HEK-CRTH2+DP cells (Figure 8 B) indicating that DP is essential for this signal transduction. BW245c-mediated NFAT induction was stronger in HEK-CRTH2+DP cells as compared to HEK-DP cells (Figure 8 C). Together, these data indicate that in case of NFAT regulation, DP seems to play an essential role but the co-expression of DP and CRTH2 receptors is important for a sufficient induction of NFAT. Remarkably, the co-expression of DP and CRTH2 itself increased NFAT activation even when signal transduction was activated with a selective DP agonist.

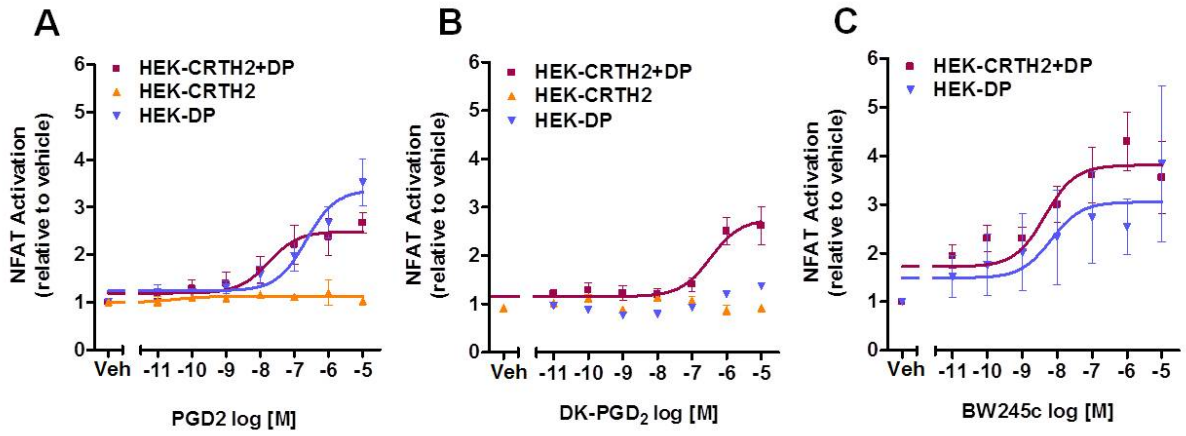


Figure 8. DP is essential for NFAT activation. Induction of NFAT in response to DP and/or CRTH2 agonists was measured in luciferase reporter gene assays in HEK-CRTH2+DP, HEK-CRTH2 and HEK-DP cells after transfection with 200 ng pNFAT-luc followed by stimulation with agonist for 3 h at 37 °C. NFAT activation was induced by PGD_2 in HEK-CRTH2 and HEK-DP cells, whereas HEK-CRTH2 showed no response **(A)**. The selective CRTH2 agonist DK-PGD_2 provoked NFAT activation only in HEK-CRTH2+DP cells **(B)** whereas the selective DP agonist BW245c activated NFAT in HEK-CRTH2+DP and HEK-DP cells **(C)**. Data show means ± S.E.M. of 3-4 individual experiments.

To further elucidate the specific roles of DP and CRTH2 in NFAT regulation, I tested the effect of specific antagonists. The cells were treated with increasing concentrations (≤10 µM) of the respective antagonist or vehicle previously to stimulation with 1 µM of PGD_2, DK-PGD_2 or BW245c. The DP antagonist MK0524 completely blocked PGD_2-mediated NFAT induction in HEK-CRTH2+DP cells, whereas the CRTH2 antagonist Cay10471 only partially reduced the PGD_2- and DK-PGD_2-induced responses. Simultaneous blockade of DP with MK0524 enhanced the Cay10471-mediated inhibition of DK-PGD_2-mediated NFAT activation (Figure 9 F). The DP antagonist BWA868c seems to have partial agonistic properties in case of NFAT induction in HEK-DP and HEK-CRTH2+DP cells (Figure 9 B, H). Consistently, it even provoked some NFAT activation itself supporting the previous observation made by our group and others that BWA868c has partial agonistic effects besides its antagonism for DP[75]. However, BWA868c decreased BW245c-induced NFAT activation (Figure 9 H) in HEK-CRTH2+DP cells. BWA868c had no effect on DK-PGD_2-mediated NFAT activation in HEK-CRTH2+DP (Figure 9 E) and Cay10471 did not inhibit BW245c-mediated NFAT activation (Figure 9 G) in HEK-CRTH2+DP cells.

Together these data indicate a close interaction of DP and CRTH2 receptors in the signal transduction leading to the translocation of NFAT into the nucleus. This gives further rise that the activation and function of DP and CRTH2 essentially depend on each other.

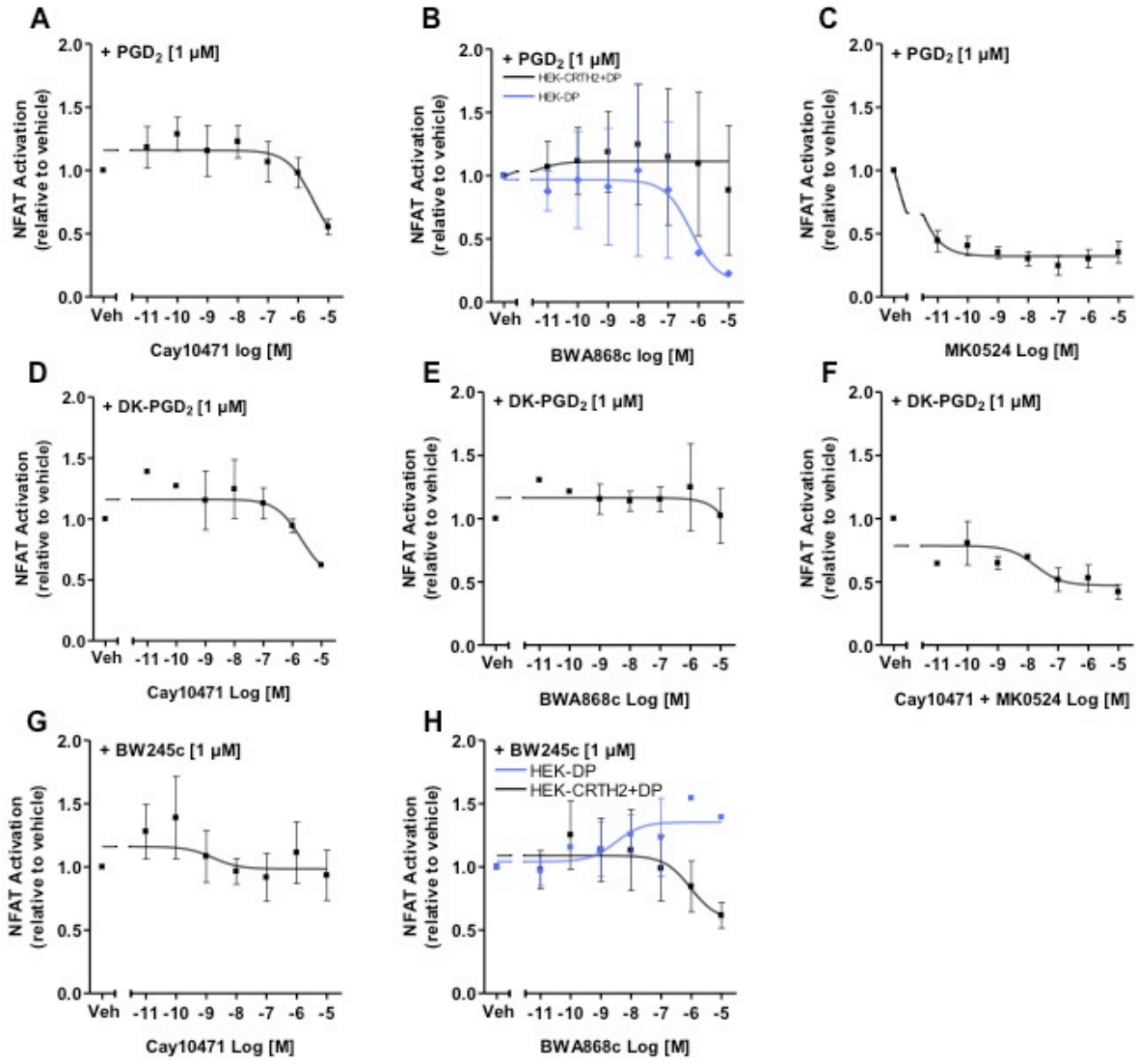


Figure 9. Blockade of either DP or CRTH2 lead to decreased NFAT activation in HEK-CRTH2+DP CELLS. NFAT activation in response to DP and/or CRTH2 agonists was measured in luciferase reporter gene assays in HEK-CRTH2+DP, HEK-CRTH2 and HEK-DP cells. Cells were transfected with 200 ng pNFAT-luc and treated with antagonists or vehicle for 20 min followed by treatment with agonists for 3h at 37 °C. MK0524 potently inhibited PGD_2 induced NFAT activation in HEK-CRTH2+DP cells **(C)** whereas Cay10471 slightly reduced DK-PGD_2- and PGD_2- mediated NFAT activation **(A, D)**. Simultaneous exposure to Cay10471 and MK0524 potentiated the inhibitory effect of Cay10471 on DK-PGD_2-induced NFAT activation **(F).** BWA868c exhibited partial agonism in case of NFAT activation. Data show means ± S.E.M. of 3-4 individual experiments.

4.1.2.3. Gαq, PI 3-kinase and ERK1/2 are Involved in DP-mediated NFAT Activation

It is known that DP couples to the Gαs-[14,75] and CRTH2 to the Gαi subunits[35] of heteromeric G-proteins and that DP and CRTH2 influence each other's signaling[41]. Therefore, I was interested in how and at which level this interaction occurs. To gain more insight into the downstream signaling network leading to NFAT activation I used pharmacological inhibitors. HEK-DP or HEK-CRTH2+DP cells were treated with the inhibitors for 20 min followed by stimulation with 10 nM of PGD_2 for 3 h at 37 °C. Inhibition of the Gαq subunit (MH-362-63-

8 [1 µM]), PI 3-kinase (Ly294002 [50 µM]) or ERK1/2 (PD-184161 [50 µM]) reduced NFAT activation to about 70 % in HEK-CRTH2+DP cells and to about 50 % in HEK-DP cells. This indicates that DP couples to the Gαq protein and liberates the Gβγ subunit which in turn activates PI 3-kinase and ERK1/2 via the small G-proteins Ras and Raf. Further on, Gαq proteins are known to activate phospholipase C that triggers Ca^{2+} release due to activation of the second messenger inositol (1,4,5) triphosphate (IP3). CRTH2 might contribute to NFAT induction by the release of Gβγ-subunit upon activation. RhoA is not involved in this particular pathway as indicated by unaffected NFAT activation following RhoA inhibition with Y27632 [50 µM] or C3 toxin [2 µM]. (Figure 10)

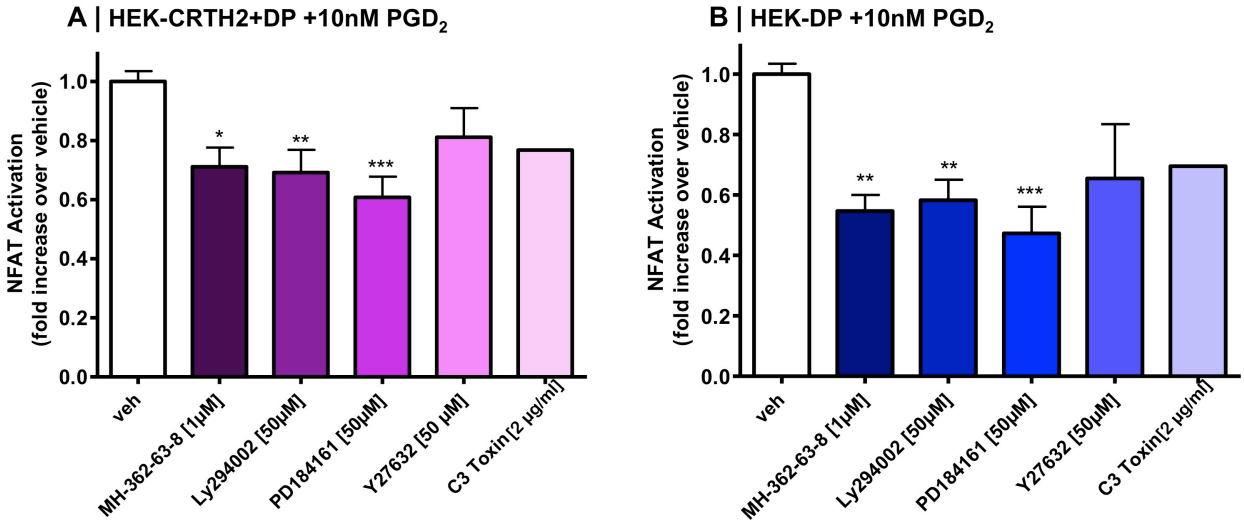


Figure 10. Gαq, PI 3-kinase and ERK1/2 are involved in DP-mediated NFAT activation. NFAT activation in response to PGD_2 was measured in luciferase reporter gene assays in HEK-CRTH2+DP and HEK-DP cells. Cells were treated with the respective inhibitor or vehicle for 20 min followed by stimulation with 10 nM of PGD_2 for 3 h at 37 °C. **(A)** In HEK-CRTH2+DP and **(B)** HEK-DP cells MH-362-63-8 (Gαq inhibitor) [1 µM], Ly294002 (PI 3-kinase inhibitor) [50 µM] and PD-184161 (ERK1/2 inhibitor) [50 µM] decreased NFAT activation whereas Y-27632 (ROCK inhibitor) [50 µM] was without effect. Data show means ± S.E.M. of at least 3 independent experiments. C3 toxin (Rho inhibitor) [2 µg/ml] was used to further confirm the effects of Y-27632 (these data are the means of one individual experiment).

The co-transfection of HEK-DP+CRTH2 cells with dominant negative (DN) RhoA (T19N) and pNFAT-luc showed no alterations in PGD_2-induced NFAT activation compared to co-transfection with pcDNA3.1. This confirms that RhoA is not implicated in this pathway (Figure 11A). As it was previously shown that LPI activated GPR55 induces NFAT via RhoA[71], the co-transfection of HEK GPR55 cells with DN RhoA and pNFAT-luc served as a positive control for the DN RhoA plasmid and the assay set-up.

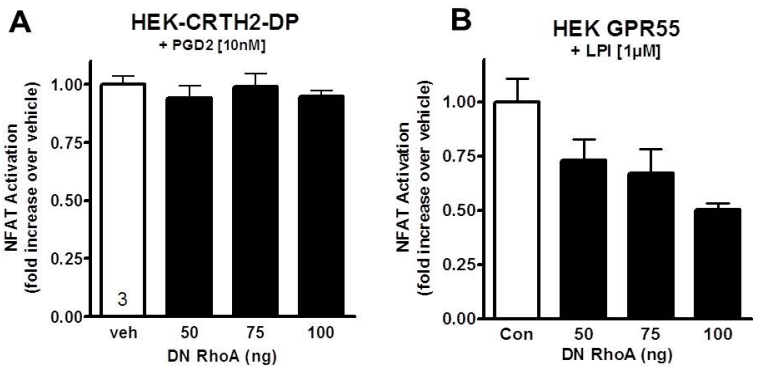


Figure 11. RhoA is not involved in CRTH2- and DP-mediated NFAT activation in HEK-CRTH2+DP cells. Cells were transfected with increasing concentrations of dominant negative (DN) RhoA or pcDNA 3.1 in combination with 100 ng of pNFAT-luc. NFAT activation was determined by luciferase reporter gene assay. NFAT activation was not altered by co-transfection of DN RhoA **(A)**. HEK-GPR55 cells were used as a positive control for DN RhoA **(B)**.

4.2. CRTH2-mediated SRE induction depends on a functional DP receptor

The serum response element (SRE) is induced by binding of the serum response factor (SRF) or the ternary complex factor (TCF) to the respective promoter element[61]. Our findings suggest that the DP receptor is essential for the SRE induction in our HEK cell model. PGD_2 potently induced SRE in HEK-CRTH2+DP and HEK-DP cells while it failed in HEK-CRTH2 cells (Figure 12 A). The finding that the selective DP agonist activates SRE in HEK-CRTH2+DP and HEK-DP cells shows that DP initially accounts for the SRE induction in this cell model. Remarkably, DP loses its potency to induce SRE in the presence of CRTH2 (Figure 12 C). The selective CRTH2 agonist DK-PGD_2 completely failed to induce SRE (Figure 12 B).

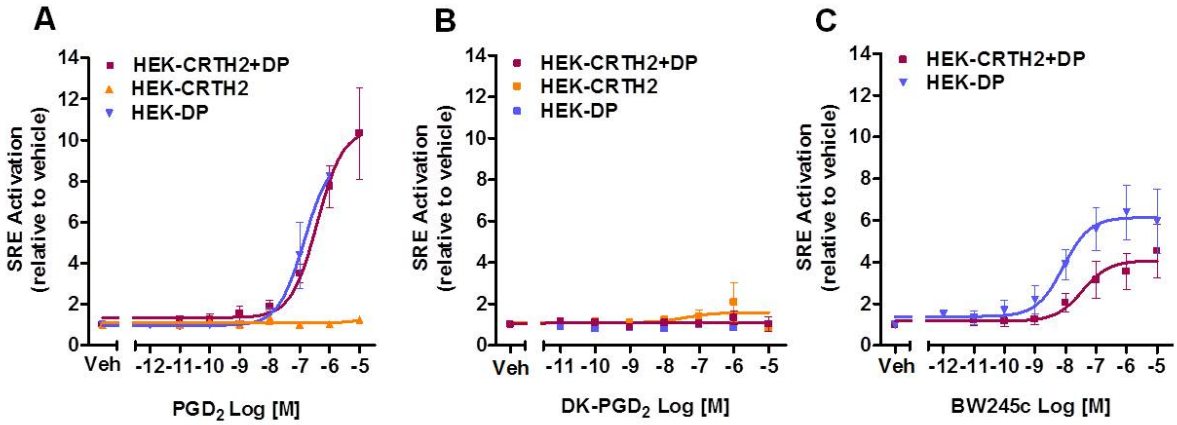


Figure 12. DP but not CRTH2 activates SRE. Induction of SRE in response to DP and/or CRTH2 agonists was measured in luciferase reporter gene assays in HEK-CRTH2+DP, HEK-CRTH2 and HEK-DP. The response to PGD_2 is similar in HEK-DP and HEK-CRTH2+DP **(A)**. DK-PGD_2 fails to induce SRE **(B)**. DP is able to induce SRE activation in response to BW245c but loses its activity in the presence of CRTH2 **(C)**. Data show mean ± S.E.M. of 3-6 independent experiments.

To further clarify the specific role of each receptor in SRE induction I pretreated the cells with specific antagonists for DP or CRTH2. Figure 13 A-C shows that the PGD_2-mediated SRE induction in HEK-CRTH2+DP cells could be successfully inhibited by the CRTH2 antagonist Cay10471 and the DP antagonists BWA868c and MK0524. The latter had the greatest potency to decrease the SRE induction. BW245c-mediated SRE induction could also be decreased by Cay10471 what suggests that CRTH2 contributes to the regulation of this pathway by influencing the DP-induced mechanisms (Figure 13 D-F).

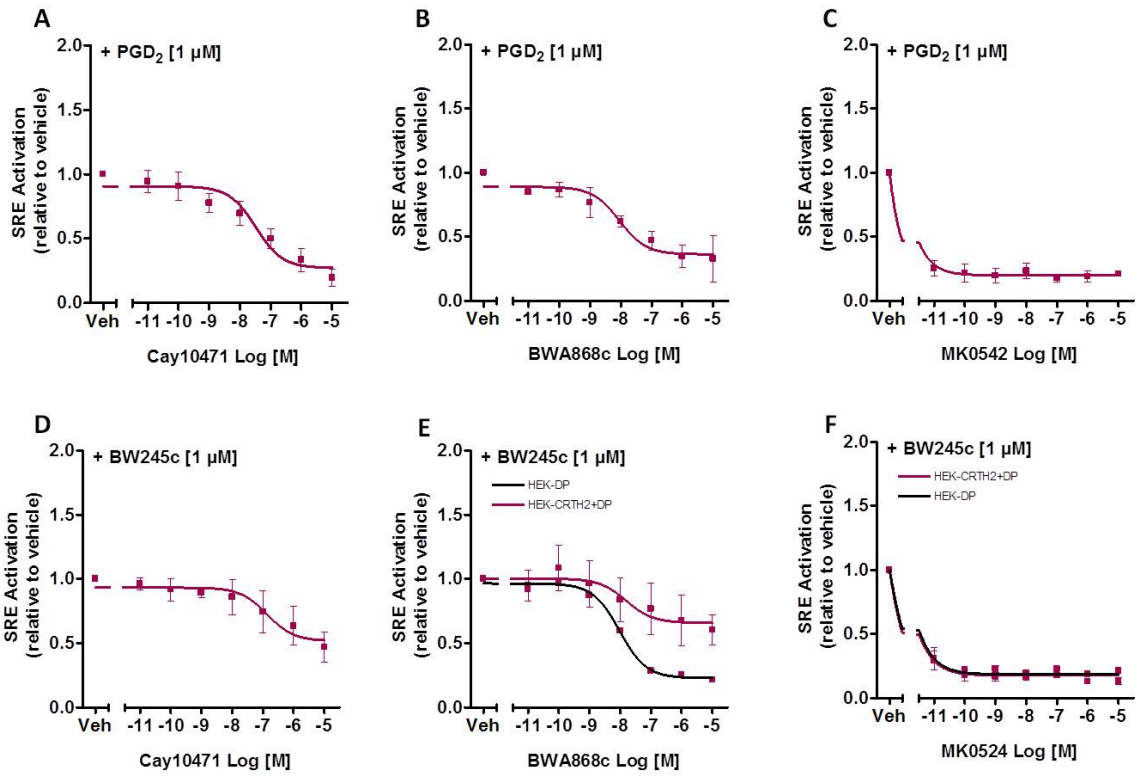


Figure 13. SRE induction in HEK-CRTH2+DP cells was inhibited by blocking either CRTH2 or DP. Induction of SRE was measured in HEK-CRTH2+DP and HEK-DP cells after treatment with specific antagonists or vehicle at increasing concentrations followed by agonist stimulation [1 µM] in luciferase reporter gene assays. PGD_2-mediated SRE induction was decreased by treatment with Cay10471 **(A)**, BWA868c **(B)**, and MK0524 **(C)**. SRE induction by BW245c was slightly reduced by Cay10741 **(D)**. BWA868c **(E)** and MK0524 **(F)** inhibited DP-mediated SRE induction in HEK-CRTH2+DP and HEK-DP cells. Data show means ± S.E.M. of at least 3 individual experiments.

CREB Activation - EC_{50} (nM)			
	CRTH2+DP	**CRTH2**	**DP**
PGD_2	0.13 ± 0.37	-	0.13 ± 0.65
DK-PGD_2	-	-	
BW245c	< 0.01	-	< 0.01

NFAT Activation - EC_{50} (nM)			
	CRTH2+DP	**CRTH2**	**DP**
PGD_2	18 ± 0.37	-	200 ± 0.22
DK-PGD_2	340 ± 0.26	-	-
BW245c	4.4 ± 0.44	-	6.6 ± 0.95

SRE Induction - EC_{50} (nM)			
	CRTH2+DP	**CRTH2**	**DP**
PGD_2	380 ± 0.21	-	140 ± 0.20
DK-PGD_2	-	-	-
BW245c	35 ± 0.46	-	8.4 ± 0.29

Table 4. EC_{50} values of transcription factor activation in HEK-DP+CRTH2, HEK-CRTH2 and HEK-DP cells induced by PGD_2, DK-PGD_2 and BW245c.

4.2.1.1. PI 3-kinase, ERK1/2 and RhoA are Involved in CRTH2- and DP-mediated SRE Induction

It is not clear which particular DP- and CRTH2-mediated pathways leads to the observed SRE induction. Therefore, I was interested in how SRE induction is regulated in detail and at which level the interaction between DP and CRTH2 may occurs. To this end, I used pharmacological inhibitors to interrupt the DP- and CRTH2-mediated signal transduction at different levels. The PI 3-kinase inhibitor Ly294002 as well as the ERK1/2 inhibitor PD-184161 almost abolished the SRE induction in HEK-DP and HEK-CRTH2+DP cells (Figure 14 A and B) indicating that PI 3-kinase and ERK1/2 are involved in this pathway. Importantly, the Rho inhibitor Y-27632 decreased SRE induction in HEK-DP cells to about 50 % as compared to vehicle (Figure 14 B), whereas in HEK-CRTH2+DP cells Y-27632 had only a slight inhibitory effect (Figure 14 A).

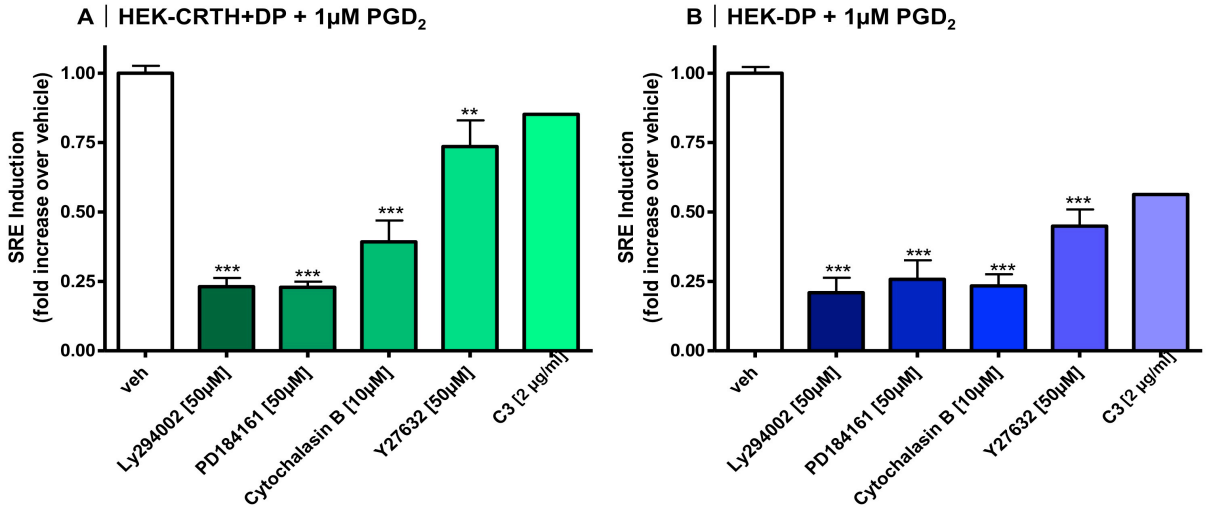


Figure 14. PI 3-kinase and ERK1/2 are involved in CRTH2- and DP-mediated SRE induction while DP additionally leads to RhoA-mediated SRE induction. Induction of SRE in response to PGD_2 was measured in luciferase reporter gene assays in HEK-CRTH2+DP and HEK-DP cells. Cells were treated with the respective inhibitor or vehicle for 20 min followed by stimulation with 1µM PGD_2 for 3 h at 37°C. Ly294002 (PI 3-kinase inhibitor), PD-184161 (ERK1/2) and cytochalasin B (inhibits microfilament formation) inhibited PGD_2-mediated SRE induction in HEK-CRTH2+DP cells **(A)**. Ly294002, PD-184161, cytochalasin B, Y-27632 (ROCK inhibitor) interfered with PGD_2-mediated SRE induction **(B)**. C3 toxin was used to further confirm the effects of Y-27632 – these data show the means of one individual experiment. In every other case data show means ± S.E.M. of at least 3 individual experiments.

Henece, these data show that CRTH2-mediated SRE induction is highly dependent on the expression and the functionality of DP. PGD_2 equally induced SRE in HEK-CRTH2+DP and HEK-DP cells, whereas sole DP activation induced a greater SRE induction in HEK-DP cells than in HEK-CRTH2+DP cells. Together with the observation that blockade of CRTH2 led to a more potent inhibition of SRE in PGD_2-treated HEK-CRTH2+DP cell than in those cells treated with BW245c it becomes obvious that CRTH2 plays a regulatory role in SRE induction. Still, CRTH2 transmits signaling only if functional DP receptors are co-expressed. The treatment with pharmacological inhibitors revealed that CRTH2 and DP activation involved pathways such as PI 3-kinase and ERK1/2. Moreover, DP induces SRE through an additional RhoA-dependent pathway.

The Luciferase reporter gene assays were partly conducted with Miriam Sedej during her PhD Thesis.

4.3. DP and CRTH2 Regulate Cellular Functions of Eosinophils

4.3.1. DP and CRTH2 Contribute to Actin Cytoskeleton Rearrangement

Leukocyte adhesion and transmigration are crucial steps in chronic and acute inflammation. Polymerization of actin monomers leads to formation of F-actin, which is a crucial step in these processes. To further clarify the roles of DP and CRTH2 in cytoskeletal rearrangement, isolated human eosinophils were incubated with specific antagonists of DP or CRTH2 or vehicle followed by treatment with 100 nM PGD_2, DK-PGD_2, BW245c or vehicle. F-actin polymers were visualized by staining with Texas-Red Phalloidin. I observed that both, DP and CRTH2 activation lead to changes in the actin cytoskeleton, whereas treatment with PGD_2 potentiated this effect. While vehicle-treated eosinophils had a completely round and compact shape, PGD_2 led to the formation of filopodia and lamellipodia, and stimulation with DK-PGD_2 or BW245c led to a scattered shape that might be due to the formation of lamellipodia but not filopodia. The stimulation of DP induced fewer morphological changes as compared to activation of CRTH2. The notion that both receptors are needed for a sufficient cellular response is further supported by the observation that simultaneous stimulation of eosinophils with DK-PGD_2 and BW245c resulted in a similar phenotype as the treatment of cells with PGD_2. A BW245c-induced desensitization of DP at low concentration [10 nM] did not influence the DK-PGD_2-mediated actin polymerization while pharmacologic blockade of DP with MK0524 [1 μM] reduced the F-actin formation mediated by BW245c as well as by DK-PGD_2. It was previously shown that Cay10471 abolishes PGD_2- and DK-PGD_2-induced actin remodeling[76].

I previously examined the induction of SRE by DP and CRTH2 in HEK-DP, HEK-CRTH2 and HEK-DP+CRTH2 cells and found that SRE is induced by DP- and CRTH2- mediated pathways that depend on the PI 3-kinase, ERK1/2 and RhoA (see 4.2). Therefore, I was interested whether the DP- and CRTH2- mediated actin cytoskeleton remodeling of eosinophils also depends on these key regulators. Inhibition of PI 3-kinase and ERK1/2 completely abolished PGD_2-mediated changes in the actin cytoskeleton of eosinophils. In contrast Y-27632, inhibitor of the Rho associated protein kinase did not alter the PGD_2-induced effect. (Figure 15)

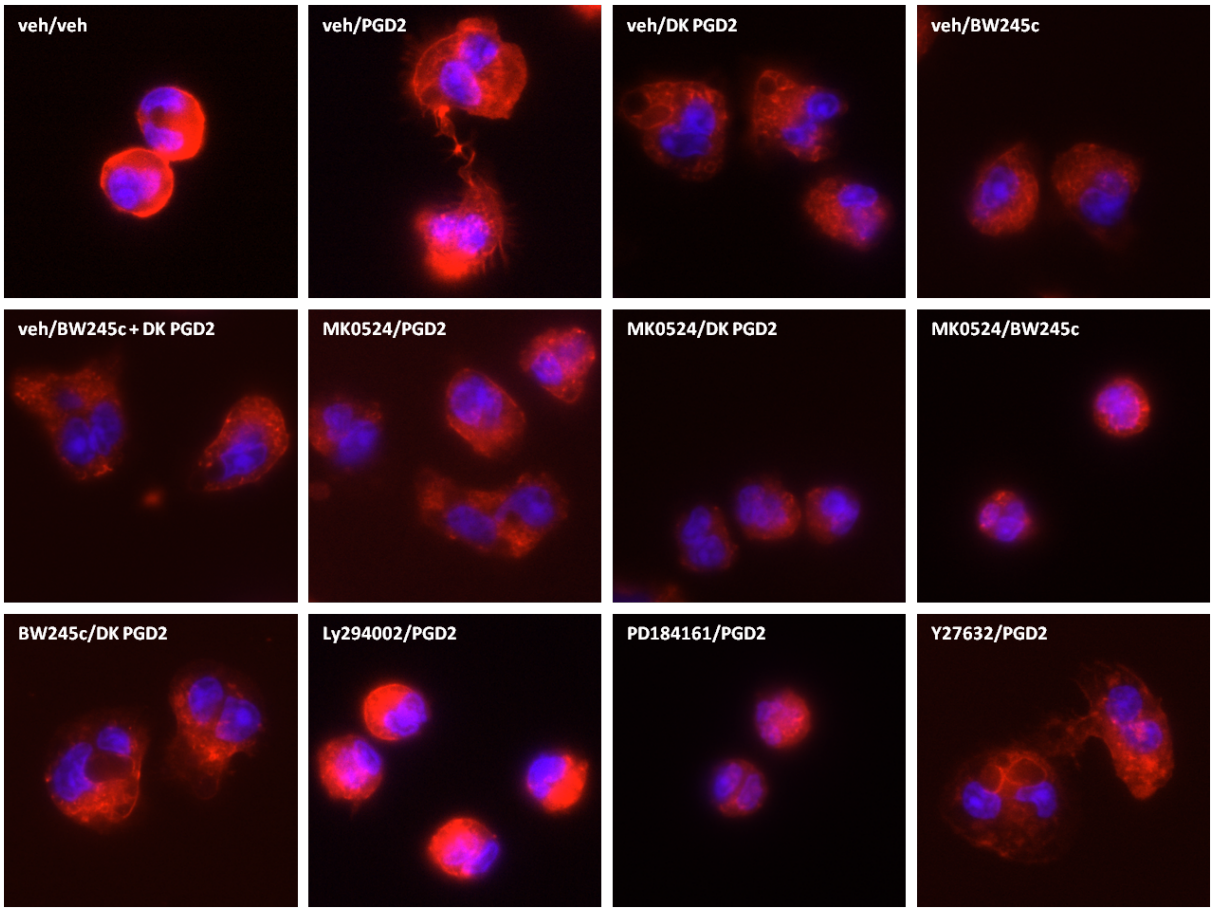


Figure 15. Stimulation of CRTH2 and DP induces actin polymerization in eosinophils. Human eosinophils were treated with antagonists (10 min), inhibitors (45 min) or vehicle prior to stimulation with 100 nM of agonist or vehicle for another 10 min. To visualize actin polymerization, cells were stained with Texas-Red phalloidin and analyzed with an Olympus IX70 fluorescence microscope and an Olympus DP50-CU digital camera. DK-PGD_2, BW245c and PGD_2 induced actin remodeling in human eosinophils. Simultaneous exposure of eosinophils to BW245c and DK-PGD_2 resulted in actin polymerization comparable to PGD_2-induced F-actin formation. DK-PGD_2- and BW245c-mediated effects were reduced by pre-treatment with MK0524. BW245c-induced desensitization of DP did not alter the DK-PGD_2-induced effect on actin polymerization. Pharmacologic inhibition of PI 3-kinase (Ly294002 [50 µM]) and ERK1/2 (PD-184161 [20 µM]) completely abolished actin remodeling while ROCK inhibition (Y-27632 [50 µM]) did not influence PGD_2-mediated changes in the actin cytoskeleton of human eosinophils. Representative images out of 3 independent experiments are shown.

4.3.2. Human Eosinophil Shape Change

To further elucidate the role of DP and CRTH2 in actin cytoskeleton-related cellular function I studied the particular involvement of each receptor in eosinophil shape change. To prove that CRTH2 plays a dominant role in actin remodeling in human eosinophils, I measured the PGD_2-, DK-PGD_2- and BW245c-induced shape change of isolated eosinophils by flow cytometry and specified the role of each receptor with the DP antagonists MK0524 and BWA868c and the CRTH2 antagonist Cay10471. Agonist-induced shape change of eosinophils was recorded as increase in the forward scatter.

PGD_2 and DK-PGD_2 induced the same maximum change in forward scatter. DK-PGD_2 treatment, however, was significantly less potent at 1-3 nM (P-value < 0.001) meaning that

about 2 times more DK-PGD_2 is necessary to fully activate shape change as compared to PGD_2. Eosinophils did not respond to the selective DP agonist BW245c (Figure 17 A). I observed that MK0524 had no effect on the PGD_2-mediated shape change, while BWA868c significantly reduced the potency of PGD_2. It can be speculated that this is due to a previously observed partial agonism of BWA868c[75]. Blockade of CRTH2 led to full inhibition of DK-PGD_2- and PGD_2-mediated shape change (Figure 17 B and C). Desensitization of DP with low concentration of BW245c did not alter DK-PGD_2-mediated changes in the forward scatter (Figure 17 D).

This set of data suggests that DP plays a potentiating role in CRTH2-mediated shape change whereas CRTH2 is the dominant receptor. DP activation failed to induce eosinophil shape change without the co-activation of CTRH2. DK-PGD_2 was capable to induce shape change but weather this demands the co-expression of DP remains to be elucidated.

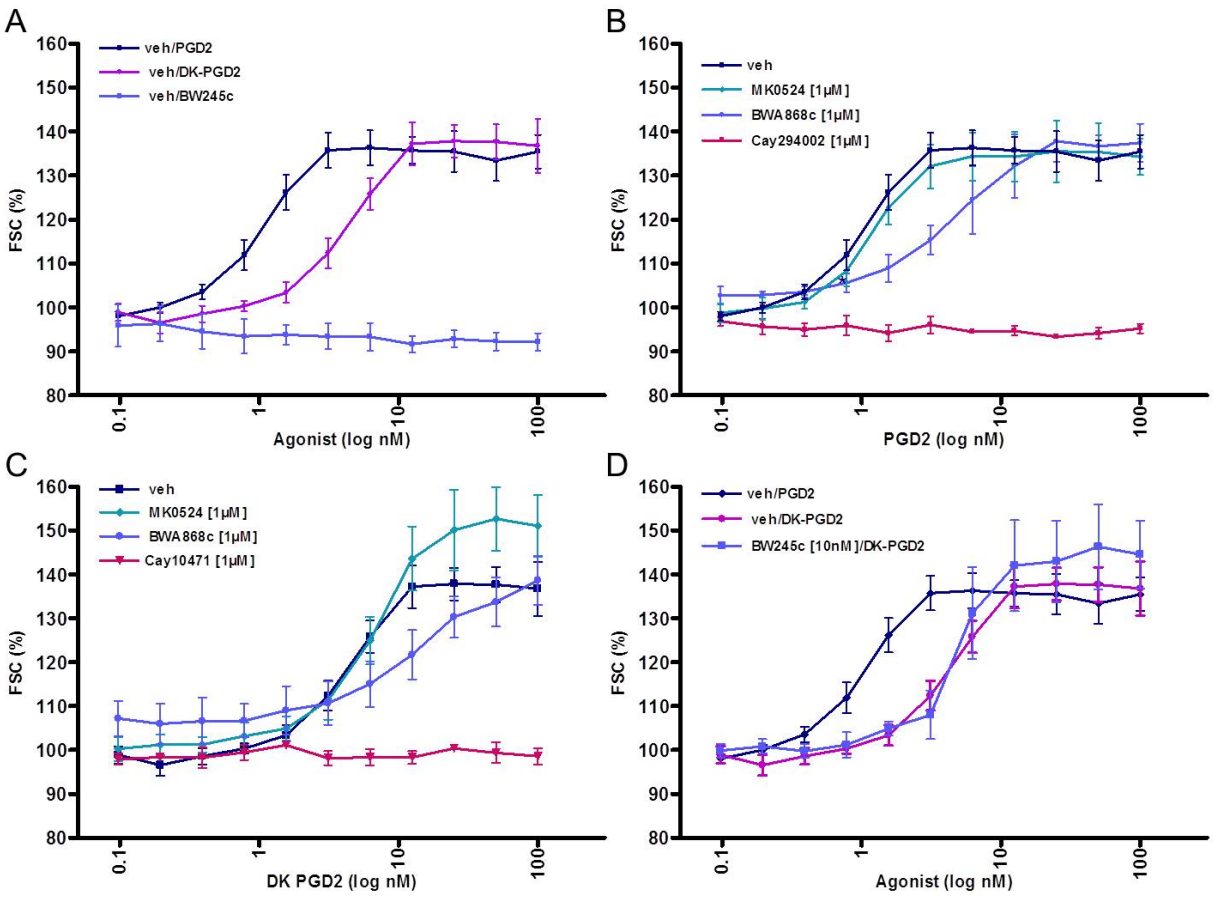


Figure 16. CRTH2 plays an essential role in PGD_2-induced eosinophil shape change. Human eosinophils were pre-treated with specific antagonists or vehicle followed by agonist stimulation for 4 min at 37 °C. Eosinophil shape change was determined by flow cytometry according to changes in the forward scatter. PGD_2 provoked eosinophil shape change more potently than DK-PGD_2; BW245c completely failed to induce shape change **(A)**. Cay10471 completely abolished shape change in response to PGD_2. MK0524 did not alter PGD_2-induced shape change while BWA868c slightly reduced the effect of PGD_2 **(B)**. DK-PGD_2 induced eosinophil shape change which was completely blocked by Cay10471 and reduced by BWA868c; MK0524 had no inhibitory effect **(C)**. BW245c-induced desensitization of DP did not affect CRTH2-mediated eosinophil shape change **(D)**. Data represent changes in forward scatter relative to vehicle and show means ± S.E.M of at least 3 independent experiments.

As I previously found implications of PI 3-kinase, ERK1/2 and RhoA in DP and CRTH2 mediated induction of SRE in HEK-CRTH2+DP and HEK-DP cells, I was interested in whether inhibition of these signal amplifiers alters PGD_2-mediated eosinophil shape change. Neither inhibition of PI 3-kinase nor ERK1/2 could prevent the increase in the forward scatter as compared to vehicle-treated cells. Inhibition of ROCK led to a lower base line signal as compared to vehicle-treated cells (Figure 17).

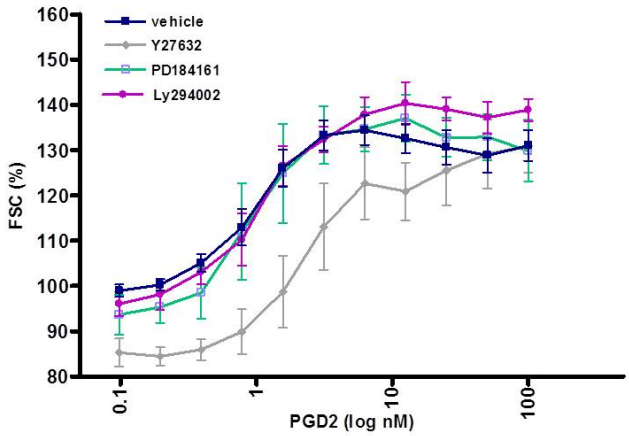


Figure 17. **The impact of inhibition of PI 3-kinase, ERK1/2 or ROCK in PGD_2-induced shape change of human eosinophils.** Cells were pre-treated with pharmacologic inhibitors or vehicle for 45 min followed by agonist stimulation for 4 min at 37 °C. Eosinophil shape change was determined by flow cytometry according to changes in the forward scatter. Y-27632 [50 μM] (ROCK inhibitor)-treated cells showed decreased forward scatter levels as compared to vehicle-treated cells. Inhibition of PI 3-kinase [50 μM] and ERK1/2 [20 μM] did not alter PGD_2-induced shape change of human eosinophils. Data represent changes in forward scatter relative to unstimulated cells and are shown as means ± S.E.M of at least 3 independent experiments.

4.4. Eosinophil Adhesion to Endothelial Cells under Shear Stress

I investigated the role of PGD_2 and its receptors DP and CRTH2 in eosinophil adhesion to endothelial cells in an assay that resembles the physiological conditions of a blood vessel. As it was previously shown that shear stress is very important for eosinophil transmigration[77], vascular endothelial cells (HMVEC-L) were grown on *in vitro* chambers and isolated eosinophils were perfused along the monolayer of HMVEC-L under physiological shear stress in thin channels mimicking small vessels. To activate the endothelial cells they were pre-treated with 10 pM of TNFα for 4 h at 37 °C.

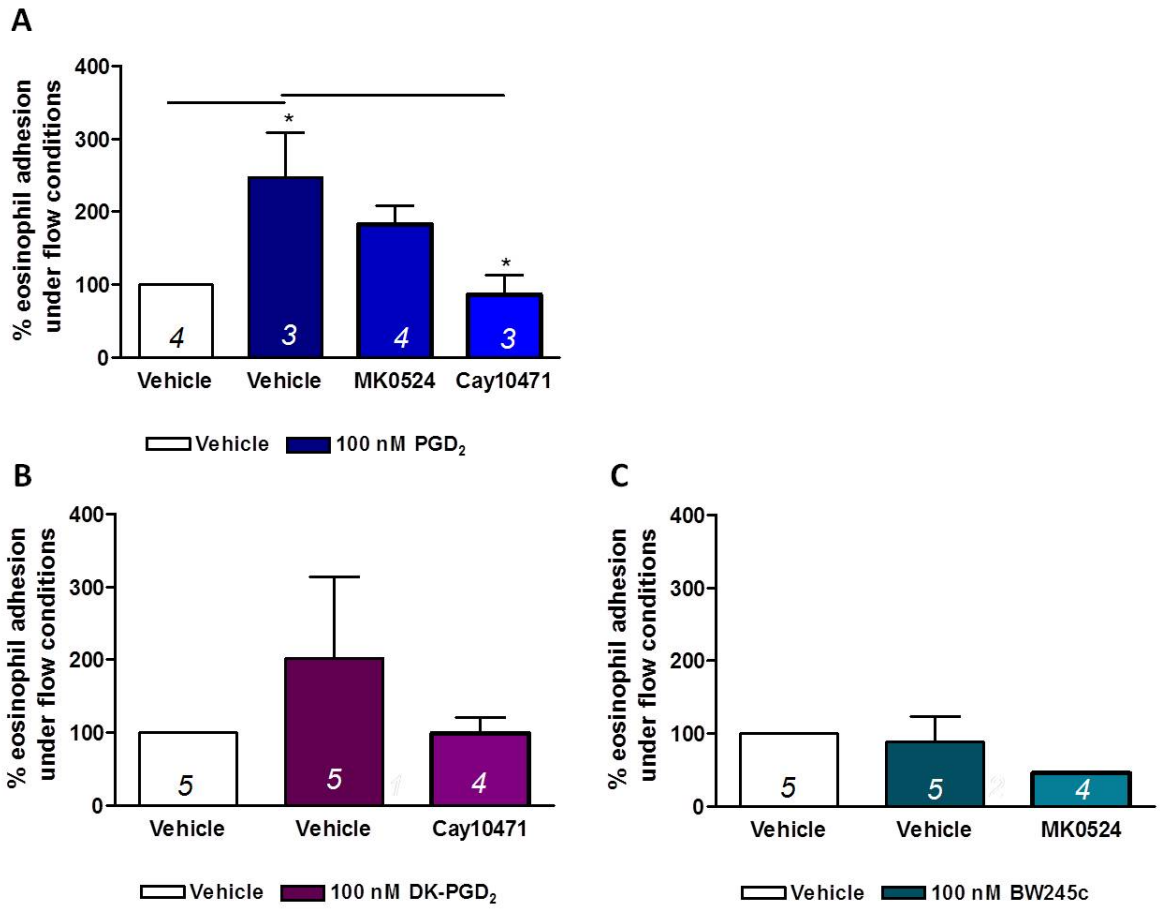


Figure 18. PGD_2 receptors are involved in eosinophil adhesion to HMVEC-L under flow conditions. Endothelial monolayers were grown in flow chambers and were activated with 10 pM of TNFα for 4 h at 37 °C. Freshly isolated eosinophils were pretreated with 1 µM of MK05324, Cay10471 or vehicle for 10 min at 37 °C. Eosinophils were then stimulated with 100 nM of **(A)** PGD_2, **(B)** DK-PGD_2, or **(C)** BW245c followed by perfusion across the endothelial monolayer for 2.5 min at 2 dyne/cm^2. Adhesion was recorded by microscope. Data show means + S.E.M. of 3-4 individual experiments.

I found that the adhesion of eosinophils to endothelial cells was about 2-3 times higher in cells treated with 100 nM of PGD_2 than in vehicle-treated cells. This effect was significantly reduced after treatment with 1 µM of Cay10471 (CRTH2 antagonist), while the DP antagonist MK0524 showed only a tendency towards inhibition (Figure 18A). The CRTH2 agonist DK-PGD_2 was less effective than PGD_2 in promoting eosinophil adhesion (Figure 18B) and the DP agonist BW245c completely failed (Figure 18C). Overall, CRTH2 seems to be the dominant receptor in adhesion of eosinophils to vascular endothelial cells.

5. Discussion

The aim of this thesis was to address the cooperative signaling of the two PGD_2 receptors, DP and CRTH2. These distinct G protein-coupled receptors account for contradictory cellular responses although both are bound to by PGD_2 with equal affinity. Thereby, PGD_2 seems to concurrently provoke pro- and anti-inflammatory responses that clearly need to be considered in approaches to treat allergic diseases like bronchial asthma[16]. Although, the signaling of PGD_2 receptors has drawn a lot of interest, the regulatory mechanisms behind have remained unclear up to now.

Recently the discovery that DP and CRTH2 form functional heteromeric signaling units has demonstrated the close interaction between the two opposing receptors[41]. As little is known about the downstream signaling pathways of DP and CRTH2 I hypothesized that

1. the transcription factors CREB, NFAT and SRE are activated by DP and/or CRTH2, since all of them are crucially involved in allergic responses
2. DP and CRTH2 downstream signaling is altered in consequence of heteromer formation

Hence, I set out to determine which particular DP- and CRTH2-mediated signaling cascades lead to the activation of the crucial regulators of PGD_2 signaling in inflammatory reactions, CREB, NFAT and SRE, in a HEK293 cell model previously generated in our lab.

The activated form of CREB, p-CREB, is enhanced in the epithelial and submucosal airways of asthmatic patients. The elevated p-CREB levels normalize upon glucocorticoid administration[78]. I found that DP mediates the activation of CREB. CRTH2 has a supportive role in the DP-mediated CREB activation indicated by a significant increase of CREB activation in cells co-expressing both receptors. These observations therefore allow the assumption that the activation of CREB results from DP-mediated Gαs-protein liberation as it is well established that CREB is activated by Gαs-proteins.

NFAT is implicated in inflammation by triggering cytokine expression[66] and apoptosis of immune cells correlated with eosinophil accumulation[69] and expression of eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN)[68]. The data obtained in this thesis indicate, that DP and CRTH2 closely interact in the activation of NFAT. It seems that DP is essential for this signaling cascade and CRTH2 has a modulatory role as activation or blockade of this receptor influenced the extent of NFAT activation. These data closely

correlate with the finding of Sedej et al. that the DP receptor enhances CRTH2-mediated Ca^{2+} signaling (upstream of NFAT) in HEK-CRTH2+DP cells as well as in eosinophils[41].
I could further show that DP-mediated NFAT activation requires coupling of DP to Gαq-proteins and that PI 3-kinase and ERK1/2 are key elements in this signaling cascade.

SRE regulates the remodeling of the actin cytoskeleton and thus is crucial for cellular functions like adhesion and migration[58]. The current experiments show that CRTH2-mediated SRE induction is dependent on the co-expression and the functionality of DP – which is therefore essential for SRE induction and can additionally induce SRE independently from CRTH2. Although CRTH2 is not able to induce SRE alone, its modulator role becomes obvious by the decreased SRE induction following pharmacological blockade of CRTH2. Moreover, pathway interruption with respective inhibitors revealed that both, DP and CRTH2, commit a pathway via PI 3-kinase and ERK1/2. DP additionally mediates the activation of RhoA and ROCK, which also contributes to SRE induction (Figure 19). This might be explained by DP-coupled Gα12/13-proteins or by Gβγ-subunit mediated activation of RhoA[79,80].
Taken together, the CRTH2-mediated signaling highly depends on a functional DP receptor.

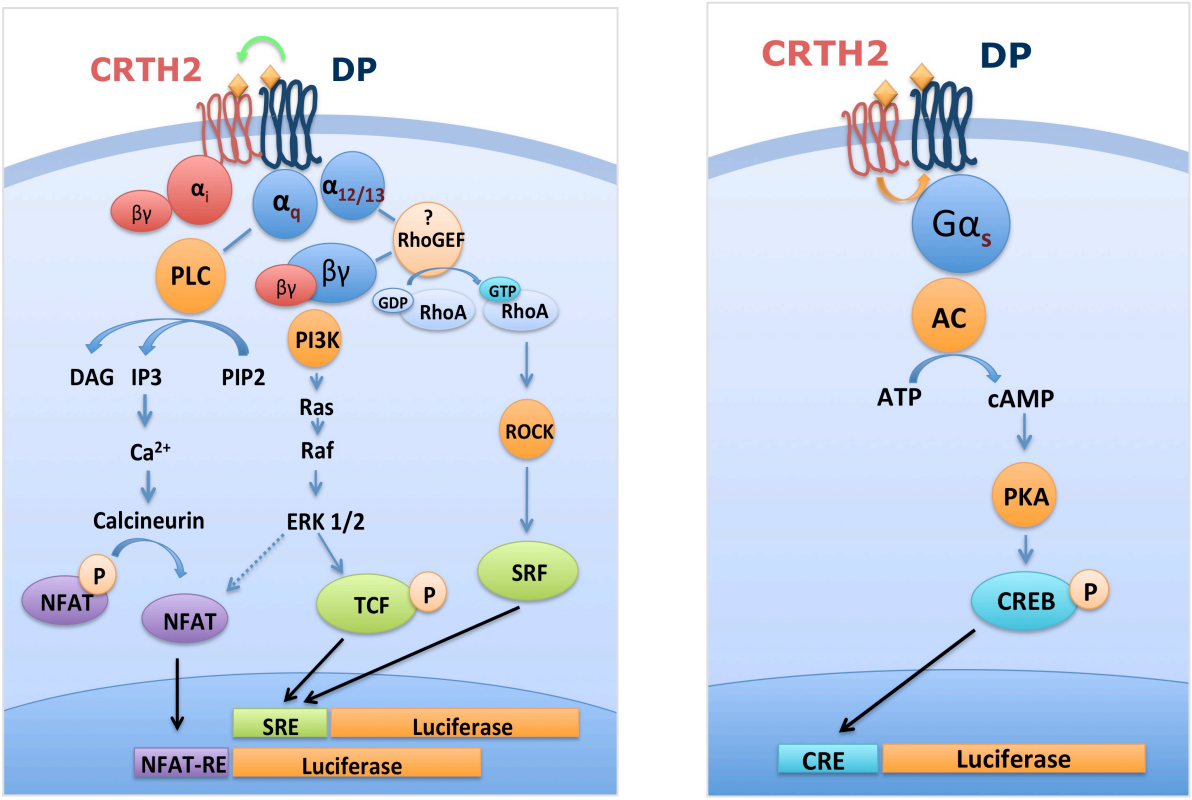


Figure 19. Proposed model of CRTH2- and DP- mediated signaling in HEK-DP+CRTH2 cells. DP is essential for signal transduction and CRTH2 relies on a functional DP receptor and becomes responsive in the presence of DP. DP is bound by Gαq or Gαs which activate divers pathways. DP might be additionally coupled to Gα12/13 in an SRE-inducing pathway.

In the ensuing experiments I aimed to find functional proofs for the observed cross-talk between DP and CRTH2 in the recombinant cell lines HEK-DP+CRTH2, HEK-DP and HEK-CRTH2 in primary cells. Due to the fact that DP and CRTH2 are co-expressed on eosinophils[62,81], which play a major role in allergic diseases, I performed experiments with peripheral blood eosinophils. Leukocyte rolling and adhesion are key events in the inflammatory response as these events are crucial for the infiltration of leukocytes to the site of inflammation and depend on actin cytoskeleton remodeling[82]. A role of DP and CRTH2 in these processes had not been investigated so far but is probably due to the observed DP- and CRTH2-mediated SRE induction.

Hence, I studied the impact of DP and CRTH2 on actin polymerization, adhesion to pulmonary endothelial cells and shape change of peripheral blood eosinophils.

I found that both, DP and CRTH2 contribute to the actin cytoskeleton remodeling as indicated by F-actin staining. Although CRTH2 activation by DK-PGD_2 induced more visible changes than DP activation by BW245c, the simultaneous activation of both receptors potentiated the actin cytoskeleton remodeling and led to the formation of filopodia and lamellopodia. This confirms that DP activity is beneficial for the signaling capacity of CRTH2. Inhibiting PI 3-kinase or ERK1/2 completely abolished the PGD_2-mediated changes in the actin cytoskeleton and the eosinophils remained in their inactive state, further supporting the observed SRE regulating pathways in the recombinant HEK293 cell model. In contrast, inhibition of RhoA did not interfere with PGD_2-mediated actin cytoskeleton remodeling. This may be due to the more dominant role of CRTH2 in eosinophils.

As DP- and CRTH2- obviously induced changes in the actin cytoskeleton I was interested in whether DP and CRTH2 might also regulate the adhesion of eosinophils to endothelial cells. Under *in vivo*-mimicking conditions PGD_2 significantly increased the adhesion of eosinophils to TNFα-activated endothelial cells. The stimulation of the eosinophils with specific agonist for DP or CRTH2 revealed that CRTH2 is the dominant receptor in the physiology of adhesion. However, it would be interesting to examine whether DP plays a promoting and supporting role also in this process. Evidence is given by the findings of Schratl, et al. [62]. The authors demonstrated that DP plays a supporting role in the mobilization and recruiting of eosinophils from the bone marrow. Pharmacological blockade of either DP or CRTH2 reduced PGD_2-mediated eosinophil release of the bone marrow what furthermore indicates the cooperative signaling among these receptors.

Additionally, I tested the impact of DP- and CRTH2-mediated signaling on the shape change of eosinophils. This assay records the actin cytoskeleton rearrangement by determining the

correlated increase of the cell volume/forward scatter. DK-PGD_2 effectively induced shape change of eosinophils whereas BW245c had no appreciable effect. Remarkably, although DK-PGD_2 stimulation reached the same maximum changes as PGD_2 did, the potency of DK-PGD_2 was significantly lower. This supports the finding that DP is needed to reach the maximum capacity of PGD_2. Pharmacological inhibition of PI 3-kinase, ERK1/2 or RhoA did not lead to recognizable alterations of eosinophil shape change, may be due to the short incubation time of 4 min with PGD_2. Additional shape change experiments using 10-min stimulation with PGD_2, as employed in the fluorescence microscopy experiments addressing F-actin phalloidin staining, will help to clarify this issue.

Taken together the obtained data demonstrate a close interaction of DP and CRTH2 in primary cells as well as in the recombinant cell model for DP and CRTH2 interaction. Powerful signaling of CRTH2 seems to rely on the functionality of DP although CRTH2 often dominates over DP. This might be worth to be considered in approaches to treat inflammatory diseases like bronchial asthma. The idea of developing drugs that incorporate heteromeric-receptor signaling units provides the possibility to target pathologies of the immune system more selectively and to prevent potentially harmful side effects.

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