Dissertation

The role of Prostaglandin D_2 receptors in inflammatory bowel diseases

submitted by

MA/PhD

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For the Academic Degree of

Doctor of Medical Science

(Dr. scient. med.)

at the

Medical University of Graz

Institute for Experimental and Clinical Pharmacology

under the supervision of

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2017

Declaration of authenticity

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I. Part of this thesis has been published in the following two articles:

1. Sturm EM, **Radnai B**, Jandl K, Stančić A, Parzmair GP, Högenauer C, Kump P, Wenzl H, Petritsch W, Pieber TR, Schuligoi R, Marsche G, Ferreirós N, Heinemann A, Schicho R.: Opposing roles of prostaglandin D2 receptors in ulcerative colitis. *J Immunol. 2014 Jul 15;193(2):827-39.* (1)

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2. **Radnai B**, Sturm EM, Stančić A, Jandl K, Labocha S, Ferreirós N, Grill M, Hasenoehrl C, Gorkiewicz G, Marsche G, Heinemann Á, Högenauer C, Schicho R.: Eosinophils Contribute to Intestinal Inflammation via Chemoattractant Receptor-homologous Molecule Expressed on Th2 Cells, CRTH2, in Experimental Crohn's Disease. *J Crohns Colitis. 2016 Sep;10(9):1087-95.* (2)

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VI. As a doctoral student, I have been affiliated with the Doctoral School ***General and Clinical Pathophysiology, PATHMED*** at the Medical University of Graz and received funding from the ***Austrian Science Fund Grants FWF P25633*** and ***FWF P22771*** of Rudolf Schicho***.***

Pécs (Hungary), May 2017

Acknowledgement

First of all I would like to thank ***Assoc. Prof. Dr. Rudolf Schicho***. As my scientific supervisor he taught me the basics of colitis research, offered attention and guidance to my work. He also introduced me into the world of scientific writing and brought a pleasant atmosphere in any types of scientific or friendly conversations. He performed the greatest contribution to my scientific development.

I would like to also thank to ***Ass. Prof. Dr. Eva Maria Sturm*** for her scientific cooperation (flow cytometric measurements) and for the interesting exchanges of research views, as well as for the nice talks as a kind colleague.

Thanks ***Ing. Veronika Pommer*** for giving me a guidance in our laboratory and for helping and teaching me in my experimental work.

I am very grateful to ***Prof. Dr. Ákos Heinemann,*** the head of the institute for giving me the opportunity to work in the Institute of Experimental and Clinical Pharmacology and for catalyzing the rapid integration of me and my family in Graz.

Special thanks to ***Prof. Dr. Christoph Högenauer*** and ***Prof. Dr. Gregor Gorkiewicz***, who collected human samples and made them us accessible. I am also indebted to all of the previously not mentioned coauthors of our publications on which this thesis was based, namely ***Angela Jačan PhD, Katharina Jandl PhD*** and ***Magdalena Grill PhD*** for their co-operation in the laboratory work, for ***Sandra Labocha Phd*** and ***Nerea Ferreirós PhD*** for performing LC/MS measurements***,*** and for ***Prof Dr. Günther Marsche*** for the scientific advices.

I also thank to all of the members of my thesis committee (***Rudolf Schicho, Ákos Heinemann, and Christoph Högenauer***) for giving me clear advices and help for accelerating my research.

Last, but not least I am really grateful to my family, to my wife ***Anett Radnai-Kiss***, and to my sons ***Gyula Radnai*** and ***Zétény Radnai*** for their love and support and for joining me in the great adventure in Graz.

This work is dedicated to my sons ***Gyula*** and ***Zétény***!

Always try to find the right way…

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I. Abbreviations

∆dbl-GATA:	∆dbl-GATA knockout
11-dh-TXB_2	11-dehydro-TXB_2
5-ASA:	5-aminosalicylic acid
AC:	Adenylyl cyclase
AOM:	Azoxymethane
ASA:	Acetylsalicylic acid
AZA:	Azathioprine
CAC:	Colitis associated cancer
CAI:	Clinical activity index
Cay:	Cay10595
CD:	Crohn's disease
CD14:	Cluster of differentiation-14
CLP:	Cecal ligation and puncture
COX:	Cyclooxygenase
CRTH2:	Chemoattractant receptor-homologous molecule expressed on T_H2 cell
CTRL:	Control
DK-PGD_2:	13,14-dihydro-15-keto PGD_2
DMSO:	Dimethyl sulfoxide
DP:	D-type prostanoid receptor
DSS:	Dextran sulfate sodium
Eot:	Eotaxin
EPO:	Eosinophil peroxidase

Erk:	Extracellular signal regulated kinase
GPCR:	G-protein coupled receptor
HBI:	Harvey-Bradshaw Index
HBSS:	Hanks' Balanced Salt Solution
HEPES:	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
H-PGDS:	Hematopoietic-type PGD synthase
HRP:	Horseradish-peroxidase
IBD:	Inflammatory bowel disease
IFN-γ:	Interferon-γ
Ig:	Immunoglobulin
IL:	Interleukin
IL-5tg:	IL-5 transgenic
ILC:	Innate lymphoid cells
INPP5D:	Inositol polyphosphate 5'-phosphatase D
JNK:	C-Jun N-terminal kinase
KO:	Knock out
LC-MS/MS:	Liquid chromatography-tandem mass-spectrometry
LOX:	Lipoxygenase
L-PGDS:	Lipocalin-type PGD synthase
MAPK:	Mitogen activated protein kinase
MCP-1:	Monocyte chemoattractant protein-1
MD2:	Myeloid differentiation factor 2
MEM:	Eagle's Minimum Essential Medium
MK:	MK0524 (Laropiprant)
MTS:	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MTX:	Methotrexate
MyD88:	Myeloid differentiation primary response gene 88
NAC:	N-acetylcysteine
NF-κB:	Nuclear factor-κ B
NSAID:	Nonsteroidal anti-inflammatory drugs
OC:	OC-459
OD:	Optical density
OVA:	Ovalbumin
PAMP:	Pathogen associated molecular pattern
PBS:	Phosphate-buffered saline
PG:	Prostaglandin
PGI_2:	Prostaglandin I_2, Prostacyclin
PI3K (PKB):	Phosphatidylinositol-3 kinase (protein kinase B)
PKA:	Protein kinase A
PLA_2:	Phospholipase A_2
PPAR γ:	Peroxisome proliferator-activated receptor γ
PS:	Penicillin/streptomycin
PUH:	Pulmonary arterial hypertension
ROS:	Reactive oxygen species
S.c.:	Subcutaneous
SDS:	Sodium dodecyl sulfate
SIRT:	Sirtuin
TBS:	Tris-buffered saline
TLR:	Toll-like receptor
TMB:	3,3´,5,5´-tetramethylbenzidine
TNBS:	2,4,6-trinitrobenzenesulfonic acid

TNFR:	TNF receptors
TNF-α:	Tumor necrosis factor-α
TP:	Thromboxane receptors
TRIS:	Tris-(hydroxymethyl)-aminomethane
TX:	Thromboxane
UC:	Ulcerative colitis
VEH:	Vehicle
WT:	Wild type

II. Summary / Zusammenfassung

Summary

Inflammatory bowel disease (IBD) is a chronic and recurring inflammation of the gastrointestinal tract. IBD affects approximately 5 million people in the world and meanwhile its prevalence and incidence (i.e. the number of newly identified patients) is continuously rising. IBD can be subdivided into two main forms, namely ulcerative colitis (UC) and Crohn's disease (CD). The etiology of IBD still remains unclear, however, it has been suggested to originate from a few sensitizing factors, including genetic background, environmental triggers, colonic microbiota and from inadequate immune responses against microbes. As a fatal consequence of long persisting IBD, colitis-associated cancer can develop. Current therapy involves anti-inflammatory medication, which intends to put IBD-patients into remission. PGD_2 receptors, i.e. chemoattractant receptor-homologues expressed in Th2 cells (CRTH2) and D-type prostanoid receptor-1 (DP) have been long known to be involved in many inflammatory disorders, but little is known about their role in IBD. Thus, we called into question, whether CRTH2 and DP could have an influence in the pathomechanism of UC and CD by exploring this topic in human samples, and in two experimental in vivo models of colitis, which share important features with human IBD.

We detected elevated concentrations of prostanoids in sera and higher amounts of CRTH2 receptor protein in the colon of UC and CD patients, as compared to their respective control subjects. Conversely, blood eosinophils isolated from UC-patients showed attenuated levels of membrane bound CRTH2. Eosinophils isolated from control subjects showed intense internalization of CRTH2 upon receptor activation. These findings suggested a crucial role of the PGD_2 - CRTH2 signaling axis in human IBD. To learn more about the role of PGD_2 receptors in colitis, we tested whether in vivo pharmacological inhibition of CRTH2 (with the antagonists Cay10595 and OC-459) and of DP (with MK0524 as a DP antagonist) would have an effect. We used dextran sulfate sodium (DSS) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) to induce UC-like and CD-like forms of experimental colitis, respectively, in mice. The CRTH2 antagonists Cay10595 and OC-459 improved the symptoms of colitis by inhibiting leukocyte recruitment; however, the two different inhibitors caused two different leukocyte profiles in the colon of DSS- and TNBS-colitic mice. MK0524 alone worsened the severity of colitis and in combination with OC-459, it eliminated the beneficial anti-inflammatory effects of CRTH2-antagonism. This phenomenon led us to

hypothesize that an interaction between CRTH2 and DP may exist. To investigate this mechanism, we activated CRTH2 and DP with DK-PGD_2 (a CRTH2 agonist) or PGD_2 (CRTH2 and DP agonist) in eosinophils and tested the effects of CRTH2 and DP-antagonists (OC-459 and MK0524, respectively) by measuring migration. Our findings suggested a clear pro-migratory activity for CRTH2 in eosinophils and in contrast to this, a pro- and/or anti-migratory activity of DP that depended on the receptor's ligand-binding state. Furthermore, we found that DP receptors modulate CRTH2 signaling, suggesting an interplay between the two receptors. *In vivo*, CRTH2-antagonism might have affected inhibition of eosinophil migration. Finally, we found that eosinophils played a clear pro-inflammatory role in the TNBS-model by using eosinophil-depleted Δdbl-GATA knockout mice and IL-5 transgene mice with eosinophilia.

In conclusion, based on our findings, CRTH2 antagonists (few of them are currently being tested in clinical trials as a future therapy in asthma) might be interesting as novel therapeutics for future IBD treatment.

Zusammenfassung

Chronisch entzündliche Darmerkrankungen (CED, *engl.: inflammatory bowel disease, IBD*) sind durch wiederholt auftretende oder persistierende Entzündungen des Magen-Darm-Trakts charakterisiert. CED betrifft nahezu 5 Millionen Menschen auf der Welt und die Prävalenz und Inzidenz der Krankheit steigt permanent. CED manifestiert sich in zwei Hauptformen, nämlich der Colitis Ulcerosa (CU, *engl.: ulcerative colitis, UC*) und dem Morbus Crohn (MC, *engl.: Crohn's disease, CD*). Als mögliche krankheitserregende Faktoren werden der genetische Hintergrund, Umweltfaktoren, Darmflora, und eine gegen die Darmflora überschießende Immunantwort angegeben. Die in der Klinik angewandte Therapie versucht die Krankheit in eine Remissionsphase zu bringen, vor allem unter Verwendung von Entzündungshemmern. Es ist seit langem bekannt, dass PGD_2 Rezeptoren, nämlich der „chemoattractant receptor-homologues expressed in Th2 cells" (CRTH2) und der „D-type prostanoid-receptor-1" (DP) eine Rolle in Entzündungsreaktionen haben. Trotzdem haben wir nur geringe Informationen über die Rolle, die sie in der CED spielen. Unser Ziel war es, die Rolle von CRTH2 und DP im Pathomechanismus der CU und MC anhand von humanen Darmbiopsien und zwei experimentell induzierten Kolitis-Modellen, die zahlreiche gemeinsame Charakteristika mit der humanen Form der Erkrankung teilen, zu untersuchen.

Im Vergleich mit der Kontrollgruppe detektierten wir höhere Prostanoid-Konzentrationen im Blutserum von CU- und MC-Patienten und höheren Mengen an CRTH2 Rezeptorproteine im Kolon. Die aus dem Blut von CU-Patienten isolierten Eosinophilen zeigten aber weniger membrangebundenes CRTH2 als in den Kontrollen. Übereinstimmend damit konnten wir in den aus Kontrollgruppen isolierten Eosinophilen eine starke CRTH2 Internalisierung nach Rezeptoraktivierung beobachten. Diese Ergebnisse weisen auf eine wichtige Rolle der PGD_2 - CRTH2 Signalachse in der CED hin. Um einen tieferen Einblick in die Rolle der PGD_2-Rezeptoren in der Kolitis zu gewinnen, haben wir eine pharmakologische Hemmung von CRTH2 (mittels Cay10595 und OC-459, als CRTH2 Antagonisten) und DP (mittels MK0524, als DP Antagonist) *in vivo* durchgeführt. Dazu wir benutzten die chemischen Agenzien Dextransulfat Sodium *(*DSS) und 2,4,6-Trinitrobenzolsulfonsäure (TNBS), um eine der humanen CU und MC entsprechende Kolitis in Mäusen zu induzieren. Cay10595 und OC-459 verbesserten die Symptome der Kolitis durch eine Hemmung der Leukozyten-Rekrutierung. In der DSS- und TNBS-Kolitis resultierten durch die CRTH2-Inhibitoren interessanterweise zwei unterschiedliche Leukozyten-Profile im Kolon der Mäuse. Verabreichung von MK0524 verschlimmerte den Schweregrad der Kolitis und wenn es mit OC-459 (kombiniert) gegeben wurde, hob es die protektive, antiinflammatorische Wirkung der CRTH2 Antagonisten auf. Dieses Phänomen

ließ darauf schließen, dass eine Interaktion zwischen CRTH2 und DP existieren könnte. Um diesen Mechanismus zu untersuchen, haben wir CRTH2 und DP in Eosinophilen mit DK-PGD_2 oder mit PGD_2 aktiviert und die Effekte des CRTH2 und DP-Antagonismus (durch OC-459 und MK0524) auf die Zellmigration gemessen. Unsere Ergebnisse zeigten eine klare promigratorische Rolle für CRTH2, hingegen eine pro- und/oder antimigratorische für DP, abhängig davon, ob der Rezeptor einen Liganden gebunden hatte oder nicht. Antagonismus von CRTH2 kann also eine Hemmung der Migration bewirken. Außerdem konnten wir die proinflammatorische Rolle von Eosinophilen in der TNBS-Kolitis mithilfe genetisch modifizierter Mäusen aufzeigen. Für diese Experimente haben wir Eosinophilen-depletierte Δdbl-GATA knockout Mäuse (welche eine Eosinopenie besitzen) und IL-5 transgenetische Mäuse (die eine Eosinophilie besitzen) verwendet.

Auf der Basis unserer Ergebnisse könnte man CRTH2-Antagonisten (einige von ihnen sind bereits in Klinischen Studien für Asthma bronchiale) als neue Pharmakotherapeutika der für die Behandlung von CED in Betracht ziehen.

III. Introduction

1. Inflammatory disorders of the gut

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is characterized by a chronic, relapsing and remitting inflammation that occurs in the intestinal tract (3). Its incidence and prevalence is permanently increasing worldwide (4) although in some regions, e.g. in Northern France, a plateau may have been reached (5). IBD affects about 5 million people in the world including 2.5 million only in Europe (6). The two major forms of IBD are **ulcerative colitis** (UC) and **Crohn's disease** (CD) (7). The etiology of the disease is still not completely known, but it is thought to be a result of interactions (3) between:

- **genetic factors,**
- **environmental triggers,**
- **microbiota and**
- **immune responses** (3)**.**

If IBD persists for a long period, it may lead to a malignant transformation of the affected tissues causing colitis-associated cancer (CAC) (8).

Although IBD probably has already reared its head in ancient communities, it seems to be a disorder of developed modern societies that practice the popular and trendy western life style and suffer from life-threatening **environmental factors**. Studies have reported marked differences in IBD-incidences between industrial and non-industrial countries in the world (9). By investigating the European population, it was suggested that an east-west gradient may exist, highlighting the higher exposure of West-European countries to environmental triggers compared to the East-European post-socialist states (10). Furthermore, in one of the wealthiest states of Europe, in Austria (11), incidences in IBD were shown to be more dominant in the largest urban areas than in rural areas of its investigated province Styria (12). The data underline the importance of life style, including

stress, smoking habits, diet, infections and use of antibiotics, oral contraceptives, or even breastfeeding in the development of IBD, next to environmental factors (13).

Besides life style and environmental factors, **genetic background** of the individual may play a priming role for the disease. For example, numerous genetic studies provide evidence for an involvement of single nucleotide polymorphisms, presence of susceptibility loci and gen variants in the onset of the disease, but surprisingly, each of these genetic factors contribute only by a small portion (20–25%) to the genetic risk (14). However, growing evidence supports the theory that epigenetic alterations, like DNA-methylation induced by environmental factors, might be implicated in the pathogenesis of IBD as well (14).

Since the most prominent histochemical sign of inflammation and active disease is the presence of leukocytes, such as neutrophils, macrophages, dendritic cells, T and B cells, in the intestinal mucosa (15), an **inappropriate immune response** has been regarded as a prime mechanism and possible inducer of IBD. For instance, recent studies raise the importance of T cells in IBD pathomechanism by showing altered effector T cell activation or T cell-mediated tolerance (15). But recently, also innate lymphoid cells and the whole innate immune system (16), including eosinophils, were described to be participants in the disease onset, offering new targets for researcher's interests.

The aberrant immune activation is often triggered by **colonic microbes**, which are part of the gut microbiome and maintain, but also may harm, intestinal homeostatic balance. The human colonic microbiome consists of approximately 200–300 species, 1800 genera, and 15,000-36,000 individual species that make up the total microbial load of the intestine (10^{13}–10^{14} cells). It includes predominantly physiologic bacteria, but sometimes also pathogenic microbes (17). Thus, it is a "hard job" for the immune system to find the right way to eliminate colitogenic bacteria, but to tolerate physiologic microorganisms at the same time. If this "job" is not well done, pathogenic bacteria can trigger the development of IBD by inducing a strong immune response. The efficacy of fecal microbiota transplantation as a novel therapy strongly supports the significance of diverse but balanced populations of colonic microorganisms (18).

In other words, because it is common that each of the abovementioned four IBD-triggering factors is only partially involved in the induction of the disease, the following expression is generally accepted: **IBD might be caused by an inappropriate immune reaction induced by environmental factors and/or colonic microbiota in genetically susceptible individuals** (16).

1.1.1. Ulcerative colitis

The first described form of IBD is **ulcerative colitis** (Colitis ulcerosa, UC). UC affects mainly the colon and rectum and causes severe symptoms, such as abdominal pain, diarrhea and rectal bleeding, which strongly influences the management and quality of life. It is often a chronic state with lifelong repeated periods, showing flare-ups and remission phases (19).

Its incidence amounts to 2–15/100,000 persons per year worldwide and shows geographic differences (4). Inflammatory processes that initiate UC are restricted to the mucosa and cause ulcers, edema and hemorrhages along the length of the large intestine. Macroscopic investigation shows aberrant and destroyed histological structures including crypt abscesses, alteration of the mucosal glands and depleted goblet cells (20). The sign that the disease is histologically active is marked by the presence of epithelial injury which is induced by immune cells, like macrophages or neutrophils. Neutrophils can be localized in crypt epithelium (cryptitis), crypt lumen and in surface epithelium and they damage the tissue after their infiltration (16). In the chronically active disease, even crypt shortening and branching can be observed (20).

On the one hand, therapy involves symptomatic treatment, such as suppression of inflammation and stimulation of mucosal healing by applying 5-aminosalicylates, steroids or immunosuppressants (21). Recently, biological treatments such as anti-tumor necrosis factor-(TNF)-α monoclonal antibodies (Infliximab, and more recently Golimumab) or anti-$\alpha_4\beta_7$-integrin monoclonal antibodies (Vedolizumab) are offered (19). On the other hand, despite the significant progresses in conservative drug therapy, about 20% of UC patients need surgery at one stage during their lifetime (22). In these cases, restorative proctocolectomy with ileal pouch-anal anastomosis are required for leading a normal, all-day life (22).

1.1.2. Crohn's disease

The second major entity of IBD is **Crohn's disease** (Morbus Crohn, CD), which was first described by Bernard Crohn and colleagues in 1932 as "regional ileitis" (23). Definition and description has changed in the past 90 years, but the name of the disease recalls one of the discoverers, namely Crohn, just because his name was the first in the alphabetical order of the authors (24).

Incidences in CD and their geographical alterations are notably similar to those of UC and amount to 3–15/100,000 persons per year. CD overall mimics the same tendency

as UC, and it is higher in countries “enjoying or suffering” from the modern way of life (4). One of the main differences to UC, however, is that in CD lesions can affect the entire gastrointestinal tract from the oral cavity to the rectum, but usually appear in the ileum and colon and cause the classical symptoms, like diarrhea, abdominal pain, weight loss and blood in the stool (25). Unlike in UC, CD is not only restricted to the mucosa, it emerges in all levels of the affected tissue and usually develops into a transmural inflammation. Furthermore, it can show extra-intestinal signs as well, most commonly arthritis, uveitis, stomatitis or rarely psoriasis or pyoderma gangrenosum (25). Physicians typically observe discontinuous inflamed islands in the normal tissue, a situation called “cobble-stoning”. The active phase of the disease may involve neutrophil and macrophage infiltration, causing aphthous ulcers, crypt anomalies and microabscesses (23). As CD becomes chronic, fibrostenosis or fistulae may develop. Structural distortions in the crypts are characteristic of CD (23).

Similar to UC, there is no curative therapy for CD patients. Thus, medication targets inflammation in order to push the active disease into remission and achieve mucosal healing (26). Medical treatments involve more or less the same drugs as those used in UC (e.g. 5-aminosalicylic acid [5-ASA], corticosteroids, and immunosuppressants, such as azathioprine) (27). However, as recently discussed by German authors, IBD-specialists tend to prescribe 5-ASA for monotherapy (10-36%) in CD (28) in Germany, despite the fact that the efficacy of 5-ASA in some cases of CD is only moderate and overall still not firmly established (29). This fact might raise the suspicion, that “authority based medicine” is still practiced in clinics instead of using the results of “evidence based medicine”.

Since (**I.**) no curative drug therapy is known to date for UC and CD and (**II.**) incidences are rising worldwide, (**III.**) and since the total costs of IBD treatment have amounted to $6.3 billion USD just in the United States between the years 2003-2004 (30), IBD has become one of the most investigated diseases!

1.2 Colitis-associated cancer (CAC)

In 1925, Crohn and Rosenberg first described **colitis-associated cancer** as a complication of UC (31). Today it is estimated that UC patients have a risk of 2%, or 8% and 18% for developing CAC, 10, 20 and 30 years after diagnosis, respectively. CD patients are probably equally exposed to a high risk of developing CAC (32).

Progression into CAC involves at least 4 biological requirements (33), namely:

- **genetic predisposition,**
- **chronic inflammation,**
- **altered gut microbiome,**
- **dysregulated wound healing and tissue regeneration** (33).

When these circumstances are present, inflammation of the colon, which could chronically drive a tumor promoting milieu, might develop. This is characterized by activated immune cells, high levels of inflammatory cytokines and reactive oxygen and nitrogen species (33). This means that inhibition of inflammation by any form during IBD may potentially protect from tumorigenesis.

2. Pathophysiology of IBD

2.1 Epithelial barrier

The question, whether an altered **epithelial barrier function** is a consequence or an initiating step of IBD is still unresolved, but from the experimental point of view, both scenarios are possible.

In the human body, the gastrointestinal tract forms the biggest surface that borders the external environment and has an estimated extension of 250 m^2 (34). To avoid uncontrolled invasion of environmental elements, such as bacteria or toxins into the gut tissue, the intestinal mucosa plays a defensive role by forming a physical and functional barrier (35). The physical barrier consists of epithelial cells in a monolayer, which are covered by a mucus coat (32). Disruption of the epithelial barrier integrity leads to enhanced permeability, which allows the invasion of luminal elements in the intestinal tissue (37). This means that luminal bacteria or viruses can enter the tissue through the disrupted barrier and directly activate intestinal immune cells resulting in gut inflammation, which further damages and harms the epithelial barrier. Accordingly, IBD patients show altered barrier function and increased gut permeability, which can influence the progression of the disease (37).

2.2 Immune activation

After disruption of the physical barrier, the second mechanism that comes into play, are cells of the **innate immune system** like macrophages or dendritic cells that recognize specific structural components of the invaders, so called pathogen-associated molecular patterns (PAMPs) that include e.g. lipoteichoic acid from Gram-positive (G(+)) bacteria (38) or lipopolysaccharides (LPS) of the Gram-negative (G(-)) bacterial cell wall (39).

Detection of G(-) bacteria such as *Escherichia coli* by innate immune cells is brought about by binding of LPS to the pattern recognition receptor, Toll-like receptor 4 (TLR4) on the surface of the immune cells like of macrophages (40). Thereby, besides other components, an active receptor complex with the cluster of differentiation 14 (CD14) and myeloid differentiation factor-2 (MD2) (41) is formed. The active LPS-TLR4-CD14-MD2 complex recruits adaptor proteins, like myeloid differentiation primary response gene 88 (MyD88), and activates a wide variety of proinflammatory signaling events involving e.g. the phosphorylation of mitogen activated protein kinases (MAPK): p38 MAP kinase (p38), extracellular signal regulated kinase (Erk) and the C-Jun N-terminal Kinase (JNK) (42). However, some anti-inflammatory pathways, like the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (PKB or Akt) pathway play a significant role in keeping the inflammation in balance by inhibiting MAPK activation (43).

The TLR4-activated signaling cascades induce the activation and nuclear translocation of transcription factors like nuclear-factor kappa B (NF-κB) (44). NF-κB is one of the most important proinflammatory transcription factors, and its constitutive activation has been involved in tumor occurrence and development (45) and in the resistance against cytotoxic therapy (46) as well. These phenomena underline the theory that inflammation and cancer share many common mechanisms (43, 44) and that inhibition of inflammation might prevent carcinogenesis.

In this above outlined scenario, a structural component of the G(-) bacteria activates TLR4 positive immune cells that produce high amounts of inflammatory mediators including:

- **inflammatory cytokines**, such as tumor necrosis factor-α (TNF-α) or interleukin-(IL)-1β and IL-6;
- **reactive oxygen species** (ROS), like superoxide ($\bullet O_2^-$) and most notably
- **lipid mediators**, like prostaglandins (PGs) (37), (40), (41), (42).

2.2.1 Cytokines

Cytokine production of activated immune cells is deeply involved in the initiation and prolongation of colonic inflammation and in intestinal injury. Cytokines are proteins (5-20 kDa) produced for cell-cell communications (49), and regulate cellular physiology, i.e. metabolic changes, differentiation, proliferation, survival or cell death. Therefore, they can affect tissue metabolism, embryonic growth, aging and the pathogenesis of different diseases (50).

Cytokines might have distinct proinflammatory or anti-inflammatory characteristics, but in many cases they exert both features and play key roles in the initiation, development and resolution of colitis (51). In cells and organs, inflammatory processes are mediated by a subset of different cytokines and not only by one single cytokine. In spite of that, exploring the role of each cytokines separately is very important to get a clear picture. The most important inflammatory cytokines, which are deeply involved in IBD pathology are **TNF-α, IL1-β** and **IL-6** that are outlined below.

2.2.1.1 TNF-α

Upregulation of **TNF-α** is known to be involved in the pathomechanism of numerous inflammatory diseases including IBD (52). In human IBD, polymorphisms of the TNF-α gene might produce colitogenic, as well as anti-colitogenic effects depending on the affected region and type of mutation. For example, polymorphism of the TNF-α-857 gene promoter region has been associated with CD in an Iranian Azeri Turkish population (53). In a very similar group of UC patients from the same nationality, the TNF-α-1031 T allele was found to play a colitogenic role, while on the other hand, the TNF-α-1031 C allele was suggested to maintain a rather protective characteristic (54). However, the molecular mechanisms these polymorphisms may trigger are not known until to date. Genetic ablation of TNF-α and the two known TNF-receptors (TNFR) in many animal models of IBD shed light on their function. TNF-$\alpha^{-/-}$ knockout mice were shown to develop a more moderate inflammation in TNBS-induced colitis than their wild type littermates (55). In contrast, TNFR1$^{-/-}$ knockout mice showed more severe signs of inflammation and higher mortality rate than the wild type animals (56). Interestingly, TNFR2$^{-/-}$ knockout mice were protected in DSS colitis compared to the control group (56). This means, TNFR1$^{-/-}$ knockout mice and TNFR2$^{-/-}$ knock out animals showed opposed effects in DSS-induced colitis.

These findings and the fact, that anti-TNF-α antibodies are effective (already available in IBD therapy (*see introduction 1.1.1.*)) clearly show the importance of the cytokine TNF-α in the pathology of IBD.

2.2.1.2 IL-1β

Next member of this cytokine triad is **IL-1β**. However, although its plasma level seems to be low, concentrations in colonic tissue are significantly increased in IBD, as measured in tissue cultures of lesions from UC and CD patients (57). Interestingly, IL-1α was indicated to be linked to disease activity in CD, whereas IL-1β and IL-1 receptor antagonist (IL-1Ra) were found to be associated rather with UC (58). In experimental colitis models, IL-1Ra protected the mice from *Clostridium difficile* toxin-induced inflammation (59) while IL-1β contributed to colitis by activating specific types of innate, IL-17A secreting, lymphoid cells and CD4(+) Th17 cells (60). Recently, it was shown that mice deficient of inositol polyphosphate 5'-phosphatase D (INPP5D or SHIP) developed spontaneous ileal inflammation by producing higher levels of IL1-β in comparison to wild type mice and this effect could be restored by administration of IL-1Ra (61). In addition, IL-10 deficient mice, which are used as a model for spontaneous chronic colitis, had increased concentrations of colonic IL-1β (62).

These facts highlight significant proinflammatory actions of IL-1β in colitis.

2.2.1.3 IL-6

In contrast, **IL-6** seems to play a "Janus-faced" role in development of IBD. This cytokine is suggested to own both proinflammatory and regenerative characteristics. Briefly, higher amounts of IL-6 were reported in CD patients suffering from a predominantly colon-affected type of the disease in comparison to control individuals (63). IL-6 was found to be crucially involved in the pathogenesis of Th1 cell-induced colitis in mice by using anti-IL-6R monoclonal antibodies (64). A decreased incidence of transmural colitis and a slight decrease in colitis severity was reported in a different model of T cell-induced colitis (65). In the experiments with this model, T cells ($CD4^{+}CD45RB^{high}$) isolated from $IL\text{-}6^{-/-}$ knockout mice induced a weaker, less pronounced colitis compared to T cells isolated from wild type animals (65). These findings draw attention to the importance of specifically T cell-derived IL-6. Interestingly, it was also demonstrated that IL-6, but not the IL-6 receptor (IL-6R), was involved in the inflammatory machinery. Mice, who received neutralizing IL-6 monoclonal antibodies showed less severe colitis compared to control in DSS-induced colitis, but

genetic ablation of IL-6R demonstrated no significant effects in the same model (66). This means that some additional targets of IL-6 must exist, emphasizing a lack of knowledge about this pleiotropic cytokine.

Besides the proinflammatory effects of IL-6, there are data available in the literature which indicate a "repair"-inducing role during long persisting colitis. It was shown, that IL-6 activates epithelial cell proliferation and wound healing, thereby promoting tissue restoration (67). But unfortunately, as a negative "side effect", activation of proliferation and wound healing is often accompanied by inhibition of apoptosis, which can directly lead to carcinogenesis (68). IL-6 can participate in these processes, e.g. by inhibiting the expression and activation of targets such as p53, the "guardian of the genome", among many others (69).

2.2.2 Reactive oxygen species (ROS)

Because ROS is involved in the generation of inflammation and also in prostaglandin synthesis (including PGD_2) (70), (71), (72), it is interesting to examine the relation between ROS and colitis. Based upon the "radical induction theory of ulcerative colitis" (73), ROS are essentially involved in UC not only as a "weapon" of the active immune cells at the end of the inflammatory cascade, but also as inducers of the whole inflammatory process at the level of epithelial cells. The theory suggests that an altered, abnormal metabolism of epithelial cells initiates ROS production. The **oxidative stress** destroys the epithelial barrier and allows the invasion of intestinal microbes into the lamina propria, where pattern recognition *(see above in 2.2)* happens to initiate inflammation in the intestine (73). This means that according to this theory, ROS could be the ultimate initiator.

The group of reactive oxygen species are composed of two subgroups namely,

the radicals (74), (75):

- **superoxide-radical ($^{\cdot}O_2^-$)**
- **hydroxyl-radical ($^{\cdot}O_2^{2-}$)** or
- **peroxyl-radical ($ROO^{\cdot}$)**

and **non-radicals** (74), (75):

- **hydrogen peroxide (H2O2)** or
- **hypochlorous acid (HOCl)**.

These highly reactive oxygen-containing substances are mainly produced by immune cells during inflammation as a part of the oxidative burst to protect the tissue against bacterial, fungal or viral invaders (76), (77), (78). Unlike cytokines, ROS do not bind to special receptors but they attack the structural components, enzymes and the genome of the invaders; they also largely oxidize them. But ROS are able to oxidize all types of macromolecules both in the invaders and in host-cells alike. In the latter, they may cause DNA damage (e.g. leading to mutations, strand breaks), lipid-peroxidation (e.g. leading to decreased membrane integrity and transmembrane signaling) and protein oxidation (e.g. leading to loss of enzyme function, altered metabolism) (79), (80), (81), (82). Therefore, as an integral part of the host's antimicrobial defense, ROS is largely responsible for the induction of inflammation as well. Generally, in cells, redox-sensitive signaling pathways (e.g. MAPK pathways) and transcription factors (e.g. NF-κB) exist (83), (84), (85), which respond to the high amounts of oxidizing agents to further increase the inflammation.

Very importantly, oxidative stress can induce the free radical-mediated oxidation of arachidonic acid, which results in a variety of different prostanoids, like isoprostanes, which are isomers of prostaglandins. These reactions are chemical reactions lacking any activity of prostaglandin producing enzymes (86).

Collectively, ROS production is deeply involved in the pathology of colitis. For example, N-acetylcysteine (NAC), a well-known radical scavenger, showed protecting effects in a model of acetic acid-induced colitis in pigs (87) and rats (88). Furthermore NAC prevented inflammation-induced cancer in a model of DSS and iron-enriched diet probably by decreasing nitrosative stress and cell proliferation (89). NAC showed protective effects on HEp-2 epithelial cells against oxidative stress induced by *Clostridium difficile* toxins (87). Even in a human study, NAC combined with mesalamine was found to decrease levels of MCP-1 and IL-8, which positively correlated with the improvement of clinical scores (91).

2.2.3 Lipid mediators: the eicosanoids

The third group of inflammatory agents active in IBD, besides cytokines and ROS, is the group of lipid mediators, such as the **eicosanoids**. "Eicosa" is a Greek word meaning "twenty", which indicates the presence of 20 carbon atoms in these molecules (92). Eicosanoids are synthesized by a multi-step process starting with the enzyme phospholipase A_2 (PLA_2). PLA_2 cleaves phospholipids localized in the membrane bilayer and produces arachidonic acid (polyunsaturated omega-6, 20:4) (93), which is the predominant precursor for the synthesis of eicosanoids (94).

Eicosanoids include the **leukotrienes** produced by the lipoxygenase (LOX) pathway and **prostanoids** synthesized by the cyclooxygenase (COX) pathway from arachidonic acid (93). Prostanoids can be further subdivided into prostacyclin (PGI_2), thromboxanes (TXs) and prostaglandins (PGs) (95). COX, or alternatively termed prostaglandin G/H synthase, exhibit two isoforms, that is COX-1, expressed constitutively for the overall maintenance of metabolic processes and COX-2, the inducible form (although in some cells of the gut also expressed constitutively), produced to mediate inflammation (96).

Thus, COX-2 is a central pharmacological target of anti-inflammatory therapy to regulate prostanoid synthesis. One of the most important non-steroidal anti-inflammatory drugs is aspirin (acetylsalicylic acid (ASA)). ASA and related products, such as ibuprofen inhibit prostanoid biosynthesis via blockade of COX isoforms (97). Unfortunately, ASA is not specific for the COX-2 isoform and inhibits COX-1 as well, which contributes to its side-effects by blocking not only the anti-inflammatory, but also the “housekeeping” prostanoid productions (97).

2.2.3.1 Thromboxanes

Upon the enzymatic reaction of COX, its product PGH_2 can be converted into thromboxane A_2 (TXA_2) by thromboxane A synthase (TXAS) and into PGI_2 by prostacyclin synthase (PGIS) (98). Both, TXA_2 and PGI_2 demonstrate a wide variety of pleiotropic effects. Since TXA_2 is highly unstable (99), it can only act via autocrine or paracrine ways by activating its thromboxane A2 receptors (TPs) (100). The “classical” effects of TXA_2 are the vasoconstrictor (101), platelet release-inducing and platelet aggregating effects (102). However, other effects have been described as well. TXA_2 has recently been shown to be involved in glucose homeostasis as demonstrated in TXAS deficient mice that had an altered insulin sensitivity (103). The serum levels of TXB_2 (degradation product of TXA_2) were found to be elevated in rheumatoid arthritis patients, and the antithrombogenic agent BM567 (Cayman Chemicals) inhibited NF-κB and COX-2 expression, yielding a clear pro-inflammatory effect for TXA_2 in the disease (104). In a bladder cancer cell model, it was found that the TXA_2 receptor regulated the transcription factor FOXO3 by two mechanisms, first, by phosphorylation via the Erk pathway and secondly, by deacetylation via induction of histone deacetylase SIRT1 (105), proposing a tumor promoting effect of TXA_2.

2.2.3.2 Prostacyclin

In contrast to TXA_2, prostacyclin (PGI_2) inhibits platelet activation and has anti-aggregatory effects, as already demonstrated in the 1970s-80s (106), (107). Because it is a potent vasodilator, prostacyclin analogs were widely used for the treatment of pulmonary arterial hypertension (PUH) (108). According to a network meta-analysis, the analog epoprostenol is suggested in the medication of PUH patients (109). It was also shown, that beraprost sodium (PGI_2 analog) prevents the decay of renal functions in chronic kidney disease patients (110). Furthermore, PGI_2 inhibited PGE_2-activated interferon-γ (IFN-γ) production in astrocytes suggesting a protective anti-inflammatory role in Alzheimer's disease (111). Unfortunately, the expression of prostacyclin-synthase (PGIS) correlates with a lower 10-year survival in breast cancer patients (112). In contrast, however, overexpression of PGIS decreased lung tumorigenesis in a lung cancer model of mice induced by tobacco smoke (113).

In general, TXA_2 and PGI_2 have opposing effects. Experiments demonstrate a proinflammatory role for TXA_2 and rather an anti-inflammatory role for PGI_2 although it should be taken into account that these mediators produce many pleiotropic effects.

2.2.3.3 Prostaglandins

The third group of prostanoids are the **prostaglandins**. Prostaglandins were first identified from the human sperm by Ulf Svante von Euler-Chelpin in 1935 (114). He hypothesized, that the compound is produced exclusively in the prostate gland and therefore named it prostaglandin.

To date, it has been clarified that prostaglandins are produced almost in the entire body, either constitutively or after induction. As it was previously summarized by others, prostaglandins are involved in the regulation of smooth muscle relaxation and contraction, neuronal activity, sensory fiber sensitization, fever and sleep, secretion and motility of the GI tract, ion and water transport in the kidney, apoptosis, cell differentiation, oncogenesis and blood platelet activity (115).

The first prostaglandin produced in the biosynthetic pathways is PGH_2. Other types of PGs predominantly are derivatives of PGH_2 (116). For example PGD_2, PGE_2 or $PGF_{2\alpha}$ are synthesized by their respective prostaglandin synthases, namely PGD- (116), (117), PGE- (116), (118) and PGF-synthase (116), (119) from their parent molecule PGH_2. Prostaglandins bind to their respective prostanoid receptors. Each of the receptors binds preferentially one of the prostanoids, which allows their classification. This means that e.g.

PGE receptors (EP receptors) predominantly bind PGE_2 while PGF receptors (FP receptors) recognize preferably $PGF_{2\alpha}$ (120).

In the following sections, we describe the structure, synthesis pathway and the receptors of PGD_2, as it was the target of our scientific interest.

2.2.3.3.1 Prostaglandin D_2

Prostaglandins are synthesized from PGH_2 by their respective synthases in the cell. PGD_2 is produced by two structurally different enzymes, namely the **lipocalin-type PGD synthase** (L-PGDS) and the **hematopoietic-type PGD synthase** (H-PGDS). L-PGDS was first isolated in 1985 from the rat and was originally called the “brain-type” prostaglandin D synthase (121) or GSH independent PGDS. H-PGDS was called the “spleen-type” or GSH dependent PGDS. The names used earlier recall the presumed expressing organ and the requirement of glutathione (GSH) as a cofactor during synthesis. The enzymes catalyze the production of PGD_2 from PGH_2 with (in the case of H-PGDS) or without (as in the case of L-PGDS) using sulfhydryl group of GSH or other H-atom donating molecules for cleaving the 9,11-endoperoxide portion of the cyclopentene ring to form a keto- and hydroxyl-groups (122).

L-PGDS is widely distributed in the human body (123), it is expressed e.g. in the central nervous system, heart (124), kidney (125) or the intestines (126) and it is also secreted into body fluids, such as the urine (127), (128) and the cerebrospinal fluid (129). L-PGDS has at least two important activities, i.e., the transport of small hydrophobic molecules (130), (131) and the well-known PGD_2 production. L-PGDS in the central nervous system was found to be correlated with visceral adiposity in humans (132). L-PGDS deficient mice are used as a model for glucose intolerance and insulin resistance (133), (134), which suggests a role in carbohydrate and lipid metabolism for this enzyme and therefore in diabetes as well. In addition, in a transient middle cerebral artery occlusion model, L-PGDS knockout mice were found to develop a higher infarct volume and brain edema as compared to the wild type animals (135). Similar effects were also demonstrated in permanent distal middle cerebral artery occlusion experiments, showing protective effects in focal cerebral ischemia (135).

L-PGDS seems to be involved in the development of colitis as well. It is present in the mucosa of active UC patients and correlates with disease activity (126). In the same study, it was also demonstrated, that L-PGDS deficient mice were less sensitive against dextran sodium sulfate (DSS)-induced colitis and showed only moderate signs of

inflammation as compared to wild type animals, which is suggestive of a proinflammatory role in this model. In contrast, enteric glial cells isolated from CD-patients showed decreased L-PGDS concentrations compared to control patients and this was found to account for the weaker intestinal barrier function (136), which suggests a protective and anti-inflammatory role for L-PGDS and L-PGDS-derived PGD_2.

H-PGDS, the other PGD_2 producing enzyme, is widely, but tissue-specifically, expressed in humans. In a study examining the distribution of H-PGDS in various tissues, high H-PGDS mRNA contents were found e.g. in adipose tissue and placenta, while moderate levels were measured in the intestines, lung or prostate (137). Furthermore, the same study highlighted that macrophages express high amounts of H-PGDS mRNA as compared to peripheral blood mononuclear cells, including lymphocytes and monocytes, suggesting a prominent physiological role for PGD_2 that originates from macrophages and that is produced by H-PGDS. Similar to L-PGDS, H-PGDS is involved in inflammation as well. For example, in a model of acute lung injury, genetic disruption of H-PGDS worsened the symptoms of lung inflammation. Interestingly, in this study, the biological effects of PGD_2 were strongly determined by its cellular source (e.g. endothelial or epithelial cell derived *vs* neutrophil derived PGD_2) (138). Similarly, H-PGDS knockout mice suffered from a more pronounced and severe colitis than their wild type littermates and they showed a higher number of tumors in an azoxymethane-(AOM)-DSS-induced colitis associated cancer model, underlining the importance of mast cell derived PGD_2 in inhibiting colitis and colitis-associated cancer (139). However, although the biological effects of a mediator can be determined by the site of its synthesis, it is also strongly influenced by its specific binding receptors, in this case by PGD_2 receptors.

3. Prostaglandin D_2 receptors: DP and CRTH2

3.1 Structure and distribution

PGD_2 binds to two known receptors, namely the **D-type prostanoid receptor 1** (DP1, previously DP) and **D-type prostanoid receptor 2** (DP2) or **chemoattractant receptor-homologous molecule expressed on TH2 cells** (CRTH2). Both PGD_2 receptors belong to the family of rhodopsin-like 7-transmembrane-spanning G protein-coupled receptors, or simply G-protein coupled receptors (GPCRs), but are otherwise structurally different. DP is a member of the prostanoid receptor subfamily, while CRTH2 is a chemokine receptor of the N-formyl-methionyl-leucyl-phenylalanine chemoattractant receptor subfamily (140).

The different PGD_2 receptors mediate different signaling pathways but can also mediate the same pathway in an opposing manner, such as the activation/inhibition of adenylyl cyclase (AC). Upon binding of a ligand, DP transduces its signal by binding G_s-type G protein and subsequent activation of AC and protein kinase A (PKA). On the other hand, CRTH2 signals via G_i-type protein and inhibits cAMP production, but at the same time enhances Ca^{2+} concentration in the cell (140). Interestingly, DP and CRTH2 form functional heteromers, and they are able to cooperate and influence each other's signaling activities. Thus, DP was shown to increase CRTH2 signaling (141).

Originally, **DP** was suggested to be expressed exclusively in human small intestine and retina (142), but today it is known that DP is widely expressed in many tissues (143), such as in the colon (144),(145), the corpus cavernosum smooth muscle (146) and very recently it was also found in nasal polyps (147) and hepatic stellate cells (148). DP is also present in immune cells (143), such as eosinophils (149), and others (150).

CRTH2 was first reported by Nagata et al. in 1999 (151). The laboratory intended to find molecules that expressed differentially in Th1 and Th2 cells, to allow the specification of the two Th-lymphocyte subsets. With CRTH2 they found a marker, which was selectively expressed in Th2 cells and which was very similar to chemoattractant receptors and was therefore termed "chemoattractant receptor-homologous molecule expressed on TH2 cells" (CRTH2) (151). Later it was demonstrated by the same group, that CRTH2 is the second PGD_2 receptor next to DP (152), the existence of which was long surmised. These findings led to the creation of the new, function-related, name for CRTH2, namely D prostanoid receptor 2. Today, it is known that, in contrast to DP, CRTH2 is mainly expressed in leukocytes (143), like eosinophils (153), (154), (155) and mast cells (156).

Consequently,

- The different structures of DP and CRTH2,
- the different activity of each of the receptors,
- the interactions between the receptors,
- the different signaling pathways, and
- the distinct expression and distribution patterns

can all strongly determine the biological roles of PGD_2 in different cells or tissues or in different pathological situations. Accordingly, although DP and CRTH2 are both involved in inflammation, they perform different biological activities.

3.2 DP in inflammation

The **PGD_2-DP axis** has been long implicated in inflammation but data are sometimes contradictory whether the receptor has pro- or anti-inflammatory functions (140).

For example, DP was suggested to mediate proinflammatory actions by inducing eosinophil infiltration into the lung in an ovalbumin-(OVA)-induced airway hyper-reactivity model causing asthma-like symptoms in mice (157). Interestingly, the results from this model (DP deficient mice were used), could not be verified in wild type animals, that received the DP agonist BW245C (158). In a LPS-induced inflammation model, activation of DP in macrophages enhanced neutrophil infiltration into the lung exacerbating inflammation (159). In addition, PGD_2 induced vascular endothelial growth factor production in nasal polyp fibroblasts via DP (160). Although DP can have proinflammatory characteristics, it is generally thought to rather mediate anti-inflammatory, protective actions. For example, PGD_2 plays an anti-inflammatory role via DP activation in collagen-induced arthritis in mice (161). Treatment with DP agonist BW245C significantly reduced joint inflammation, while the DP antagonist MK0524 showed the opposite, i.e. inflammation-promoting effects. PGD_2 showed both proinflammatory and anti-inflammatory effects in a croton oil-induced dermatitis model in H-PGD synthase transgenic mice (162). It is possible that the anti-inflammatory action in the early inflammatory phase was due to DP activation, which could have enhanced the vascular barrier function. This effect could be neutralized by using a DP antagonist (BW A868C). DP agonist BW245C, but not the CRTH2 agonist DK-PGD_2 protected against aluminum overload-induced neuronal damage (163).

Although there is vast literature on the action of DP in inflammation, little is known about the role of the DP receptor in human colitis. Since colonic PGD_2 production was reported to be increased in UC patients in remission as compared to patients with active UC (and also the DP expression was found to be elevated), it was suggested that the PGD_2 – DP axis might play an anti-inflammatory, pro-resolution role in IBD (164). In addition, PGD_2 production during colitis could be involved in the development of preneoplastic lesions via DP, emphasizing the importance of the receptor in the development of colorectal cancer (165). In an animal model of TNBS-induced colitis, DP showed anti-inflammatory characteristics via inhibition of granulocyte recruitment into the colon (166).

To summarize, DP seems to possess protective, anti-inflammatory, immune-suppressive effects as compared to CRTH2, which seems to play a proinflammatory role, thereby providing an explanation for the sometimes diverse effects of PGD_2 described in the literature.

3.3 CRTH2 in inflammation

As detailed above, **CRTH2** is mostly expressed in immune cells (143) and has been described as a chemoattractant receptor mediating chemotactic signals. Therefore, CRTH2 is also involved in inflammation and induces migration of leukocytes upon activation. Only one study demonstrates protective anti-inflammatory effects for CRTH2, namely in adjuvant-induced joint inflammation in mice (167), otherwise CRTH2 has been linked with proinflammatory effects.

Thus, CRTH2 has been described to promote lung inflammation (168). In a helminth-induced pulmonary type 2 lung inflammation model in mice, it was recently found that CRTH2-positive group 2 innate lymphoid cells (ILC2s) can migrate into the lung and participate in the induction of inflammation. The study further showed that genetic ablation of CRTH2 significantly decreased the number of ILC2s in lung tissue and improved the disease severity (168). Interestingly, CRTH2-positive ILC2 cells were reported to be involved in the pathogenesis of human asthma as well (169). As previously outlined, PGD_2 demonstrated biphasic, both pro- and anti-inflammatory effects in a croton oil-induced dermatitis model in H-PGD synthase transgenic mice (162). The role of PGD_2 receptors was dependent on the phase of the disease. Namely, DP was shown to be protective in the early phase, while CRTH2 induced inflammation in the late phase by mediating neutrophil migration (162). CRTH2 activity has been also associated with human eosinophilic esophagitis, a disease during which the PGD_2-CRTH2 axis seems to promote the migration of eosinophils to the esophageal mucosa (170). To underscore the proinflammatory action

of CRTH2, genetic depletion of CRTH2 significantly protected mice from cecal ligation and puncture (CLP)-induced death and reduced sepsis by modulating neutrophil migration (171). Furthermore, CRTH2 has been associated with chronic allergic inflammation of the skin (172) and with eosinophilic airway inflammation (173).

Concerning CRTH2 in the literature, surprisingly only a small amount of data deals with a potential role in colitis, but the few studies that are available from this field indicate a proinflammatory role. CRTH2-positive immune cells were shown by immunohistochemical analysis in the mucosa of UC patients, mainly in slightly inflamed regions and at the border of inflamed regions (174). In contrast, another study demonstrates only a tendency, but not statistically significant increase in CRTH2 mRNA in colonic biopsies of UC patients compared to healthy individuals (164). This findings let us hypothesize that CRTH2 is probably not produced per se (or only in small amount) in the colon during colitis, but it can appear on the surface of active immune cells that infiltrate the colon. The more leukocytes are recruited to the colon during inflammation, the higher is the amount of CRTH2 that can be detected in the mucosa. In addition, an earlier study already revealed a critical role for CRTH2 in DSS-induced experimental colitis in mice by showing decreased signs of inflammation and an improvement in the symptoms of the disease in the CRTH2-antagonist-treated group compared to vehicle-treated animals (175). But the exact role and mechanism of CRTH2's proinflammatory actions has remained unknown.

4. Aims of the study

We aimed to clarify the role of the PGD_2 receptors in the pathogenesis of IBD and focused mainly on the role of CRTH2 by using a broad spectrum of approaches, including *in vivo* models of experimental IBD, *in vitro* experiments with eosinophils (that highly express CRTH2), analysis of human blood samples and colon biopsies from UC and CD patients as well as from healthy individuals.

IV. Materials and methods

1. Patients

IBD patients with diagnosed and confirmed active disease (from mild to severe forms of the disease) and healthy subjects were recruited by physicians of the Department of Internal Medicine, Medical University of Graz. Precisely, UC patients (n = 18), ***(Table 1.)*** and healthy (CTRL) subjects (n = 17) or CD patients (n = 36), ***(Table 2.)*** and CTRL subjects (n = 21) were asked for donating blood samples or colonic biopsies. The patient's distribution by age and sex in the UC and respective CTRL group, or in the CD and respective CTRL group was similar (1), (2).

UC and CD (active or remission) was diagnosed by a gastroenterologist using standard procedures and according to standard diagnostic criteria (176), (177). Colonoscopy was performed during the routine clinical, diagnostic interventions in IBD patients. CTRL subjects either participated in the colon cancer screening program or they had been hospitalized upon gastrointestinal bleeding with unknown origin and underwent colonoscopy. Excluding criteria were:

(**I.**) comorbid disorders, (**II.**) recognized infections, (**III**.) gravidity, or (**IV**.) use of NSAIDs including acetylsalicylic acid (1), (2).

For determination of prostaglandins, blood samples were taken from UC patients (n = 13) ***(Table 1.)***, the respective CTRL subjects (n = 12), from CD patients (n = 31) ***(Table 2.)*** and the respective CTRL subjects (n = 15). Blood was collected in Vacuette ® serum tubes obtained from Greiner-Bio-One (Kremsmünster, Austria). Serum samples were frozen in liquid N_2 and kept at -80°C. Prostaglandins from blood samples were determined by mass spectrometry in collaboration with the Institute of Clinical Pharmacology, Goethe University, Frankfurt/Main in Germany (1), (2).

Colonic biopsies have been also taken during colonoscopy from UC patients (n = 5) ***(Table 1.)*** and from their respective CTRLs (n = 5) and from CD patients (n = 5) and from their respective CTRLs (n = 6) ***(Table 2.)***. Tissue biopsies were taken from the affected, inflamed regions of the colon. After excision, they were either fixed in 10% phosphate-buffered formalin to be processed for immunohistochemical staining or they were immediately frozen (-80°C) and investigated later by immunoblot technique (1), (2).

UC patients	Mayo score	CAI	Treatment
1	moderate	4	5-ASA, Corticosteroids
2	mild	2	AZA
3	mild	2	5-ASA
4	mild	1	5-ASA, AZA
5	mild	2	—
6	moderate	8	5-ASA; TNFα antibodies
7	moderate	4	Corticosteroids
8	moderate	5	5-ASA, AZA
9	moderate	8	5-ASA
10	moderate	11	5-ASA
11	moderate	6	5-ASA
12	moderate	12	5-ASA
13	moderate	6	5-ASA
14*	severe	9	5-ASA, Corticosteroids
15*	moderate	5	5-ASA, AZA
16*	moderate	4	Corticosteroids
17*	moderate	6	5-ASA, Corticosteroids
18*	mild	3	5-ASA

Table 1. **Characteristics of ulcerative colitis patients**
(**CAI**, Clinical activity index (*Rachmilewitz index*); **5-ASA**, 5-aminosalicylic-acid; **AZA**, azathioprine) Patients ***Nr. 1-13*** donated blood samples, *Patients ***Nr. 14-18*** donated colon biopsies. **Mayo score:** mild (3-5), moderate (6-10), severe (11-12). *[Reproduced from Sturm EM, Radnai B et al. J Immunol, 2014, (1) with permission of publisher The American Association of Immunologists, Inc. Copyright 2014., modified by the author.]*

CD patients	HBI	Treatment	CD patients	HBI	Treatment
1	1	Adalimumab	19	9	AZA
2	7	Infliximab, AZA	20	12	Infliximab, AZA
3	5	AZA, Steroids	21	17	AZA
4	11	Adalimumab	22	0	AZA, 5-ASA
5	0	Infliximab	23	0	Infliximab
6	10	Adalimumab, AZA	24	20	Infliximab, AZA
7	0	5-ASA	25	3	AZA, Adalimumab
8	0	Infliximab, AZA	26	10	AZA, Infliximab
9	10	Infliximab	27	13	Infliximab
10	0	AZA, Infliximab	28	0	Steroids, 5-ASA
11	0	none	29	2	Infliximab
12	0	AZA	30	0	Infliximab
13	0	AZA	31	1	Adalimumab
14	2	none	32*	8	Adalimumab, MTX
15	3	Adalimumab, AZA	33*	12	AZA
16	16	none	34*	10	AZA, Infliximab
17	2	Steroids, Adalimumab	35*	3	none
18	9	Steroids, 5-ASA	36*	3	Sulfasalazine

Table 2. **Characteristics of Crohn patients**
(**HBI**, Harvey-Bradshaw Index; **AZA**, azathioprine; **5-ASA**, 5-aminosalicylic-acid; **MTX**, methotrexate). Patients ***Nr. 1-31*** donated blood samples, *Patients ***Nr. 32-36*** donated colon biopsies. *[Reproduced from Radnai B, Sturm EM et al. J Crohns Colitis, 2016 (2) with permission of publisher Oxford University Press, Copyright 2017, modified by the author].*

The human studies were planned and performed with the agreement of the Ethics Committee of the Medical University of Graz (protocol numbers: 24-281 ex 11/12 for CD study; and protocol numbers: 23-002 ex 10/11 and 24-281 ex 11/12 for UC study). All participating individuals completed a written agreement (1), (2).

2. Animal models

In our experimental colitis models, we used wild type mice of two different strains, namely C57BL/6 and CD1, both of which were obtained from Charles River (Sulzfeld, Germany) (1), (2).

Genetically modified ∆dbl-GATA knockout and IL-5 transgenic mice (both on Balb/C background) were a kind gift from Dr. Helene Rosenberg (National Institute of Health, Bethesda, Maryland, USA) and were bred in our institutional animal unit. In the experiments investigating ∆dbl-GATA knockout and IL-5 transgenic mice, we used Balb/C wild types as a control for the genetic modification (2).

All of the investigated animals were kept under the same conditions (22 °C; 12 hours light / 12 hours dark cycle) in plastic cages with sawdust bedding material and all received standard chow and tap water freely, without any restrictions (1), (2).

Investigations were only performed with sex-, age- and body weight-matched animals. Mice were kept in our unit for at least 2 weeks before the experiments were started (1), (2).

2.1. Dextran sulfate sodium-(DSS)-induced colitis

DSS-colitis was induced in C57BL/6 mice (males, 5–9 week old, 20–26 g of body weight) by supplementing drinking water with 3 % (w/v) dextran sulfate sodium (DSS salt; 36–50,000 Da; MP Biomedicals Europe). Mice consumed the DSS-solution over a time period of seven days ***(Fig 1.)***. Control animals received pure tap water only. Mice were treated with pharmacological inhibitors of PGD_2 receptors once a day, namely with Cay10595 (CRTH2 antagonist; Cayman Chemicals, Ann Arbor, Michigan, USA) at 5 mg/kg (161), subcutaneously or with MK0524 (DP antagonist; Cayman Chemicals) at 1 mg/kg (178), subcutaneously or in combination with Cay10595. Control (CTRL) mice received vehicle only (1).

Body weights and the general condition of the animals were monitored daily. 7 days after the start of the experiments, mice were anesthetized and blood samples were taken by cardiac puncture for prostaglandin measurements. Mice were euthanized by cervical

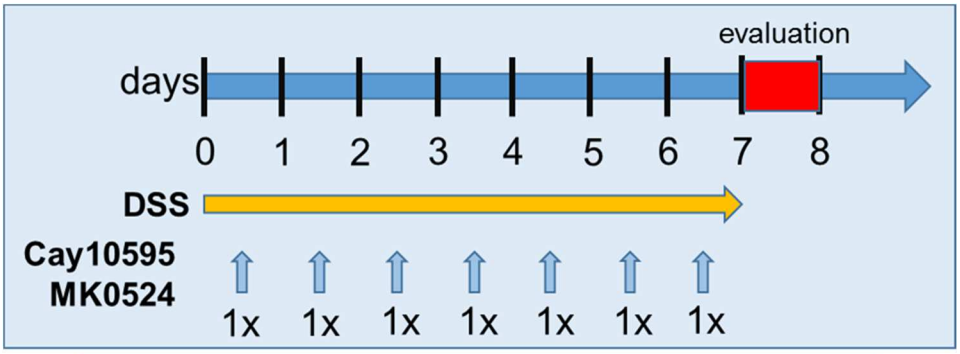

Fig. 1. **DSS-induced colitis: the experimental set up**
(**DSS**, dextran sulfate sodium; **Cay10595**, CRTH2 antagonist; **MK0524**, DP antagonist)
[The figure was created by the author]

dislocation and the colon was removed. Subsequently, the colons were evaluated by using a macroscopic inflammation scoring system, which was adapted from *Kimbal ES. et al.* (179) and performed in a blinded fashion ***(Table 3.)*** (1.)

Ulcers	Stool	Colon damage	Colon weight	Colon length
0 point = no ulcers	0 point = normal (well-formed faecal pellets)	0 point = no inflammation	0 point = <5%	0 point = <5%
1 point = 1-2 ulcers	1 point = loosely shaped moist pellets	1 point = 1 reddening, mild inflammation	1 point = 5-14%	1 point = 5-14%
2 points = 3 ulcers	2 points = amorphous, moist, sticky pellets	2 points = moderate inflammation (widely distributed)	2 points = 15-24%	2 points = 15-24%
3 points = 4-5 ulcers	3 points = diarrhoea	3 points = severe inflammation (extensively distributed)	3 points = 25-35%	3 points = 25-35%
4 points = >5 ulcers	Presence of blood in stool +1 point		4 points = >35%	4 points = >35%

Table 3. **Macroscopic scoring for the evaluation of DSS-induced colitis**
(semiquantitative scoring system adapted from *Kimbal ES., 2004*)
[The table was created by the author]

Thereafter, a part of the colon was immersed in 10% PBS buffered formalin for immunohistochemical analysis. Other parts were frozen immediately in liquid nitrogen for further biochemical evaluations, like enzyme-linked immunosorbent assays (ELISA) for the measurement of cytokines and immunoblots for the detection of receptor proteins. Determination of leukocyte recruitment required the whole colon and was made with freshly prepared colon tissue from a separate group of mice (1).

In this experimental setting, we performed experiments in 4 groups of mice, namely a Cay10595-treated group (abbreviated as **Cay**), MK0524-treated group **(MK**), a group treated with both Cay10595- and MK0524 (**Cay+MK**) and respective vehicle-treated groups (**VEH**) (1).

Experiments in mice were planned and performed with the agreement of the Austrian Federal Ministry of Science and Research (protocol number: BMWF-66.010/0146-II/3b/2012) and conducted according to the "Animal Research: Reporting In Vivo Experiments" (***ARRIVE***) guidelines (1).

2.2. 2,4,6-trinitrobenzenesulfonic acid-(TNBS)-induced colitis

Because of their higher susceptibility to TNBS, CD1 mice (male, 10-19 week old, 25-30 g of body weight) were used for TNBS-colitis model. Colitis was induced by

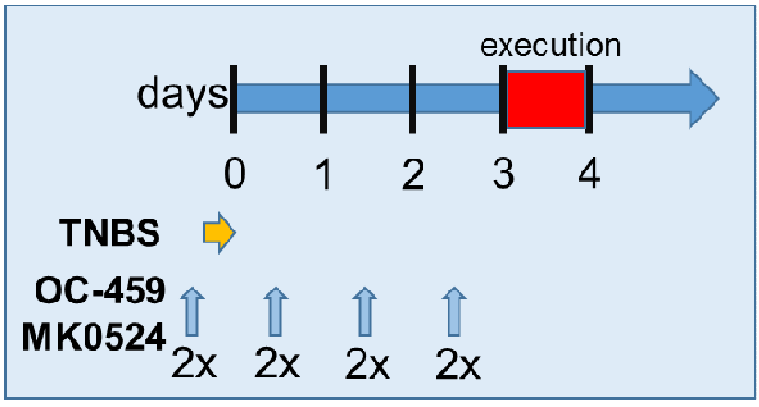

Fig. 2. **TNBS-induced colitis: the experimental set up**
(**TNBS**, trinitrobenzenesulfonic acid; **OC-459**, CRTH2 antagonist; **MK0524**, DP antagonist) *[The figure was created by the author]*

challenging mice with one bolus of TNBS (4 mg in 100µl 30% ethanol; Sigma-aldrich) intrarectally by use of a blunt ended gavage needle ***(Fig. 2.)***. To this end, animals were anesthetized with isoflurane (Combi-vet® Anesthesia vaporizer) directly before TNBS administration. Control (CTRL) animals received TNBS vehicle only (100µl of 30% ethanol) under the same conditions. In search of a more potent CRTH2 agonist we discovered in preliminary that OC-495 inhibited inflammation in DSS colitis thus we used OC for the TNBS colitis experiments. For the inhibition of PGD_2 receptors, we subcutaneously applied either OC-459 (CRTH2 antagonist; 0.1 mg/kg or respective vehicle; Cayman Chemicals, Ann Arbor, Michigan, USA) (180) or MK0524 (DP antagonist; 1 mg/kg or respective vehicle; Cayman Chemicals) alone, or both in combination, twice a day. Additionally, in the TNBS model, the inhibitors were also applied as a pretreatment one day before TNBS challenge ***(Fig. 2.)*** (2).

Body weights and the general appearance of treated animals were examined daily. On day 3, mice were anesthetized by isoflurane, blood samples were taken by cardiac puncture and colons were removed. Colon was prepared for inflammatory scoring, then processed for immunohistochemical staining or frozen for further evaluation as detailed before in the *materials and methods (2.2.1.)* section. Macroscopic scoring was performed as described by *Schicho R. et al*. (181) in a blinded fashion ***(Table 4.)*** (2).

ulcer	adhesion	Colon shortening (based on the mean length of CTRL)	Wall thicknes	+ 1 point (each)
0.5 points for each 0.5 cm	0 point = absent	0 point = <15%	Points refer the measured thickness in mm.	hemorrhage
	1 point = 1 adhesion	1 point = >15%		fecal blood
	2 points = 2 adhesion or more	2 points = >25%		diarrhea

Table 4. **Macroscopic scoring for the evaluation of TNBS-induced colitis**
(semiquantitative scoring system described by *Schicho et al., 2011*)
[The table was created by the author]

To evaluate the effect of pharmacological inhibition of PGD_2 receptors in the experimental settings of TNBS colitis, our treatment groups were divided into an OC-459-(abbreviated as **OC**), a MK0524-(**MK**)-treated group, a group that received OC-459 and MK0524 in combination (**OC+MK**), and a vehicle group (**VEH**). Control animals (**CTRL;** no TNBS) received vehicle. In addition, we used ∆dbl-GATA knockout (∆**dbl-GATA**) and IL-5 transgenic (**Il-5tg**) mice to explore the role of eosinophils in the colitis model. Genetically modified animals did not receive any treatments except the induction of colitis (**TNBS**) or vehicle only as a control (**CTRL**) (2).

The described experiments in mice were planned and performed with the agreement of the Austrian Federal Ministry of Science and Research (protocol number 66.010/0018/-WF/V/3b/2015). Moreover, experimental procedures were carried out according to the ***ARRIVE*** guidelines for reporting experiments involving animals (2).

3. Liquid chromatography/mass spectrometry

For the determination of eicosanoids, namely of PGD_2, Δ^{12}-PGJ_2, PGE_2, $PGF_{2\alpha}$, TXB_2 and 11-dehydro-(dh)-TXB_2, liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed (1), (2).

The measurements were performed by our collaboration partners, namely by ***Sandra Labocha Phd*** and ***Nerea Ferreirós PhD*** from the Institute of Clinical Pharmacology, Goethe University, Frankfurt/Main in Germany. Mice were treated and processed by us in our lab of the Institute of Experimental and Clinical Pharmacology, Medical University of Graz, as described in the *animal models (IV.2.)* section of the dissertation. Serum samples were frozen in liquid nitrogen immediately upon removal and transported on dry ice to Germany, where the LC-MS/MS measurements were accomplished (1), (2). A brief summary on the LC-MS/MS method is given below (1):

Briefly, the liquid chromatograph separated the serum constituents for further MS determination. The tandem mass spectrometric equipment (electrospray ionization MS/MS operated in negative ion mode) ionized and separated the molecules twice by their mass/charge (M/Z) ratio and detected them. The system involved a hybrid triple quadrupole–ion trap MS (QTrap 5500; AB Sciex, Darmstadt, Germany) with a Turbo-V-source working in negative electrospray ionization mode, a binary pump and degasser (Agilent 1200; Agilent, Waldbron, Germany), and an autosampler (HTC Pal; Chromtech, Idstein, Germany) (1).

In the procedure, the human serum samples derived from both UC and CD patients were spiked with internal standards (isotopically labeled) and extracted with ethyl acetate. The ethyl acetate extraction was repeated for a total amount of two extractions. After that, the organic extracts were evaporated and resolved immediately in the mobile phase of the liquid chromatograph. Separation of serum compounds was completed in a Synergi Hydro-RP column (150x2 mm I.D., 4 µm; Phenomenex, Aschaffenburg, Germany) under gradient conditions (300 µl/min). The mobile phases were water/formic acid solution (100:0.0025, v/v) and acetonitrile/formic acid solution (100:0.0025, v/v). 45 µl of samples were injected, which were running for 16 min (1).

For quantitation, Analyst Software V1.5 (Applied Biosystems, Darmstadt, Germany) was used (1).

4. Cytokine measurement

For cytokine measurements, we removed colon tissue, which was then frozen in liquid nitrogen and kept at -80 °C. Samples were then thawed and transferred into extraction buffer (50 mM TRIS, 10 mM EDTA, 1% Triton-X, including protease- and phosphatase-inhibitor cocktails [Roche Diagnostics, Vienna, Austria]) (2).

Protein concentrations were first normalized to the tissue wet weights (20 mg / 50 µl), thereafter samples were homogenized mechanically by using a potter homogenizer and an ultra-turrax. Furthermore, the homogenates were sonicated for 10 seconds in ice. Protein concentrations were measured as an additional (second) normalization step. Only samples with normalized protein concentrations were used for cytokine determination (2).

For the detection of TNF-α, IL-1β and IL-6, mouse Readyset&go ELISA kits (eBioscience Inc., San Diego, CA, USA) were used. In our measurements, we applied the protocol of the manufacturer delivered with the kit without any modifications (2):

In brief, we coated Corning Costar 96-well plates with capture antibody diluted in coating buffer. Capture antibodies specifically recognize the target proteins (TNF-α, IL-1β or IL-6). Then, we sealed and incubated the plate overnight at 4 °C under gentle shaking. Next day, wells were washed three-times with wash buffer (1xPBS with 0.05% Tween-20) and blocked by a protein solution (ELISA diluent; recommended by the manufacturer) for 1 hr. After washing the wells three-times, we added 2-fold serial dilutions of standard (recombinant target protein) and colon extracts to perform the standard curve and to determine cytokine concentrations, respectively. Plates were incubated overnight at 4 °C. After 5 washes, detection antibody (biotinylated) was added and the plate was incubated at

room temperature for 1 hr. The plate was then washed again five-times, avidin-horseradish-peroxidase antibody (avidin-HRP) was added and the plate was incubated for 30 min. Avidin binds to the biotinylated antibody, thereby marking the target protein. Thereafter, 7 washes were performed (to reach the most effective and specific identification) and 3,3´,5,5´-tetramethylbenzidine (TMB) solution was added. TMB is a substrate for the enzyme HRP and after its oxidation it turns blue allowing the determination of cytokine concentrations by using spectrophotometric methods. After 15 minutes of incubation at room temperature, we stopped the reaction and changed the color of the dye to yellow by adding stop solution (2N H_2SO_4).

Optical density (OD) of the yellow dye was measured at 450 nm by using a plate-reader system from BioRad (Hercules, California, USA) (2).

5. Protein concentration measurements

For the determination of protein concentration in our samples, we used the Bradford method (2).

Briefly, first we diluted our samples (1:100) with extraction buffer and added 100 µl of each into wells of a 96-well plate. We always measured the protein concentrations in triplicates. To perform a standard curve, we used 5 different bovine serum albumin solutions at concentrations of 64, 48, 32, 16, and 8 µg/ml. As a blank we used 100-fold diluted extraction buffer, exactly the same which were previously used by the protein extraction. We added 100 µl from the diluted standards and from the blank into the wells of a 96-well plate, as well. A total of five concentrations including the 0 mg/ml, represented the 5 points of the calibration curve. For the spectrophotometrical analysis we added 100 µl BioRad reagent (BioRad Protein Assay Kit II, Biorad).

Optical densities (OD_{595}) of the standards and samples were measured with a Microplate-Spectrophotometer (BioRad Hercules, California, USA) (2).

6. Immunoblot

In order to determine the amount of CRTH2 protein in colonic tissues, whole cell extracts were made from the colonic biopsies of UC- and CD-patients and CTRLs, and from colonic segments of C57BL/6 and CD1 mice. Samples were normalized first to the wet weight (20 mg / 50 µl), homogenized in the same extraction buffer as used for cytokine measurements (*see above*), and sonicated. Thereafter, we added equal amounts of 2x

sample buffer, (15% glycerol, 5% SDS, 5% 2-mercaptoethanol, and bromphenol blue in TRIS-HCl [pH 6.8]) and kept the samples at 95 °C for 5 minutes (1), (2).

We used SDS-PAGE technique (Life Technologies, Invitrogen, Vienna, Austria) for the separation of proteins and for the detection of receptor proteins. After separation on acrylamide gels, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). To avoid unspecific binding of detecting antibodies, we blocked the membrane by using TBS-based (1 mM $CaCl_2$, 136mM NaCl, 2.5 mM KCl, 25 mM TRIS-HCl) blocking buffer (TBS with 5% low fat milk powder and 0.1% Tween-20). After that, the membrane was incubated overnight at 4 °C with rabbit anti-CRTH2 antibody (1:1000; Acris Antibodies, Herford, Germany) or mouse anti-β-actin antibodies (1:7500; Sigma, St. Louis, Missouri, USA). After washing the membrane with wash buffer (TBS-Tween), the membrane was incubated with HRP-conjugated anti-rabbit antibodies (1:7500; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) at room temperature for 1 hour. The wash process was repeated once again and finally we applied ECL Western blotting substrate onto the membrane for visualization (1), (2).

Quantification of the bands was performed by using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). As a further normalization step, we normalized protein expression of CRTH2 to the respective actin level (1), (2).

7. Immunohistochemistry and histochemical staining

For the immunohistological detection of PGD_2 receptors in tissue sections (5 µm), we used paraffin-embedded sections of human colon from UC- and CD-patients as well as from CTRL subjects. In addition, colon sections of DSS- or TNBS-colitic animals treated with CRTH2 antagonist (or vehicle) were used. As a staining procedure, we performed the ABC method (Vectastain ABC kit; Vector Laboratories, Burlingname, Calofornia, USA) and followed the instructions of the manufacturer's, which were only slightly modified (1), (2).

Briefly, paraffin-embedded sections were deparaffinized (10 minutes in xylene), hydrated through an alcohol series (100%-50%, 5-10 minutes each step), washed (PBS; 5 min) and microwaved (2x5 min) in citrate buffer (pH 6; 10 mM). After soaking the sections again (PBS, 10 min), quenching of peroxidases was performed by using 0.3% H_2O_2 diluted in PBS (30 min). The washing process was repeated and sections were incubated with blocking buffer (PBS containing 0.3% Triton X, 1% goat serum, 1% bovine serum albumin, 1-2 hours). After 1 hour of incubation with the rabbit primary antibody anti-CRTH2 (1:200,

Acris Antibodies, Herford, Germany) or antibody/control peptide in combination (1:10, Acris Antibodies, Herford, Germany) slides were washed again and a biotinylated secondary anti-rabbit antibody solution (from the ABC kit; Vector Laboratories, Burlingname, Calofornia, USA) was added. For visualization, we used 3-3´-diaminobenzidine (DAB) and then counterstained the colonic sections with hematoxylin (1), (2).

To identify eosinophils in the colonic sections, we applied Sirius Red (Direct Red 80®, Sigma) staining (2).

Images were shot with an Olympus DP 50 camera (Olympus, Vienna, Austria) and analyzed by Cell^A imaging software (Olympus). In the immunohistochemical images of colonic sections only contrast and brightness were modified (1), (2).

8. Eosinophil chemotaxis assay

To assess migration of eosinophil granulocytes upon activation and/or inhibition of the PGD_2 receptors, we performed *in vitro* experiments with eosinophils in chemotaxis chambers using isolated and purified human blood eosinophils (2).

Summarily, following pretreatment with OC-459 (CRTH2 antagonist, 1 µM) or MK0524 (DP antagonist, 1 nM) or in combination, eosinophils (10^5 cells) were transferred into the upper wells of a microBoyden chamber. Upper wells were divided from the lower wells by a polycarbonate filter (5 µm pore-size, polyvinylpyrrolidone-free), which was permeable for the migrating eosinophils. PGD_2 (which acts as an agonist for both CRTH2 and DP, 30nM) or 13,14-dihydro-15-keto-PGD_2 (DK-PGD_2, specific CRTH2 agonist, 30 nM) or assay buffer were added into the lower wells. In this experimental setting, eosinophils migrated through the filter toward the agonist gradient into the bottom wells. To exclude basic, random movements of eosinophils from the measurements, we determined the baseline migration by using assay buffer without any agonist treatment. Following incubation of cells in a humidified incubator (1 hr at 37 °C), the filter was removed and the number of migrated cells in the lower wells were determined by flow cytometry (FACSCalibur, Becton-Dickinson, Mountain View, California, USA) (2).

All of the flow cytometric measurements were performed under guidance of ***Ass. Prof.*** **Dr.** ***Eva Maria Sturm and Dr. Katharina Jandl***, Institute of Experimental and Clinical Pharmacology, Medical University of Graz.

9. Leukocyte recruitment assay

To determine the type of immune cells responsible for the inflammation, colon was removed at the end of the colitis experiments, leukocytes were isolated from colon tissue and determined by flow cytometry (1), (2).

First, the colon was soaked in Hank's Buffered Salt Solution (HBSS). Afterwards, they were weighed and cut into approximately 0,5 cm pieces. The pieces were incubated (37 °C, 10 min) and washed (4-times) with 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid and penicillin/streptomycin containing HBSS (HEPES+PS-HBSS) buffer. After four wash cycles, samples were transferred into complete RPMI 1640 medium and rinsed for 5 minutes. To digest the tissue, samples were incubated in the same RPMI with collagenase type II (100 U/ml, Life technologies) for 1 hour at 37 °C. After this step, we isolated the leukocytes by using a 40 µm cell strainer. The leukocytes that passed through the strainer were washed in PBS (2-times), fixed by fixative organic solutions, and kept at 4 °C (1), (2).

For identification of the leukocytes, we used a FACSCalibur flow cytometer. Data were normalized to colon weight and presented as percentage of total living cells (1), (2).

10. CRTH2 detection in human eosinophils by flow cytometry

In these experiments, whole blood taken from UC patients and healthy subjects was used. To have an erythrocyte-free sample, citrated blood was lysed by using BD FACS lysing solution (1x) and then washed in PBS. Next, fixative solution was added to the samples and they were kept at 4 °C for 10 minutes. The wash process was repeated and we blocked the cells with Ultra-V Block (Lonza, Allendale, NJ) for 30 minutes at 4 ˚C. To visualize the CRTH2 receptor, we applied Alexa Fluor 647–conjugated anti-human CRTH2-antibody (rat) or Alexa Fluor 647–conjugated IgG2a (rat) isotype control (10 µg/ml; BD Biosciences) for 30 minutes at 4 ˚C. Cells were washed again in PBS, fixed by fixative solution and kept on ice (1).

For the measurement of CRTH2 on eosinophils, we applied flow cytometry (FACSCalibur). The eosinophil population was detected by determining the forward and side scatter parameters and autofluorescence in unstained samples (1).

11. CRTH2 receptor internalization

In these experiments, whole blood cells (citrated) were used, which were incubated with CRTH2 agonists (PGD_2, DK-PGD_2, 11-dh-TXB_2), to induce receptor internalization, or with eotaxin. Following incubation (1 hr at 37 ˚C), the internalization process was stopped by cooling the cells off in ice (10 min). To eliminate erythrocytes, we used 1x BD FACS lysing solution and washed the cells with PBS and subsequently incubated in antibody-diluent (Dako). For cell staining, we applied Alexa Fluor 647-conjugated rat anti-human CRTH2 antibody or Alexa Fluor 647-conjugated rat IgG2a isotype control (10 mg/ml, BD Bioscience) and incubated cells for 30 minutes at 4 ˚C. After that, cells were washed again in PBS and fixed in organic fixative solutions (1).

Flow cytometric measurements were performed by using FACSCalibur flow cytometer. CD16-negative cells appearing in higher side scatter regions were considered as eosinophils (1).

12. CRTH2 detection in Caco-2 colonic epithelial cells

Caco-2 human epithelial colorectal adenocarcinoma cells (#HTB-37 from ATCC, Manassas, VA, USA) were used to evaluate the role of CRTH2 in intestinal epithelial cells. CRTH2 receptor was visualized by using flow cytometry and Western blot.

First, we washed Caco-2 cells in PBS and stained them with Alexa Fluor 647-conjugated rat anti-human CRTH2 Ab. As an isotype control, we applied Alexa Fluor 647-conjugated rat IgG2a (10 µg/ml; BD Biosciences) for 30 minutes at 4°C. Subsequently, samples were washed with PBS and fixative solution was added. Until the flow cytometric evaluation with FACSCalibur samples were kept on ice.

CRTH2 detection by immunoblot was performed as detailed in the *materials and methods* (*IV/6) section* of the dissertation. Briefly, Caco-2 cells were seeded in 6-well plate and incubated until reaching ~80% confluence. Then they were washed with PBS, harvested by a cell scraper and sonicated in extraction buffer for 15 seconds on ice. Thereafter, we added equal amounts of 2x sample buffer and processed the samples as detailed above.

13. Survival of Caco-2 cells with MTS test

Caco-2 cells were seeded in 96-well plates in a density of $2.5x10^3$ cells/well in Eagle's Minimum Essential Medium (MEM) containing 10% FBS and 1% Penicillin-Streptomycin (PS) and incubated overnight at standard culture conditions (at 37 °C in a humidified incubator with 5% CO_2 atmosphere).

To test the effect of PGD_2 on the survival of Caco-2 cells, medium was changed on the next day for fresh one at a final volume of 200 µl/well, which contained 10-50 µM PGD_2 dissolved in DMSO. To exclude DMSO-induced cytotoxicity in our measurements, control cells (VEH) received the same volume of DMSO as it was given as a vehicle with the different PGD_2 treatments.

As a CRTH2 antagonist, Cay10595 (Cayman Chemicals) was used. Under the same conditions as detailed above, we pre-treated our cells with 30 nM Cay10595 for 30 minutes and induced cell death by using 10-50 µM PGD_2 (in a final volume of 200 µl). Because Cay10595 and PGD_2 were both dissolved in DMSO, control cells received the same amount of DMSO, as it was added with the 50 µM PGD_2 treatment (highest PGD_2 concentration) plus with the Cay10595 treatment as a vehicle.

To evaluate the action of Cay10595 on oxidative stress-induced cell death, we applied H_2O_2 (300 µM) after a 30 min pretreatment with Cay10595 (30 nM). Control and H_2O_2-induced cells received DMSO as a vehicle in the same volume that was used for the Cay10595 treatment.

After 48 hours of incubation, an additional 20 µl/well of MTS Reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was added and cells were incubated for another 4 hours. Absorbance of the oxidized formazan dye was measured at 490 nm, which was proportional to the number of living cells.

14. Morphologic changes of Caco-2 cells

Caco-2 cells were seeded in 6-well plates and cultured until reaching about 80% of confluence. Next day, medium (MEM; 10% FBS, and 1% PS) was replaced for fresh one, and 30 and 50 µM of PGD_2 was added. Control cells received the same volume of DMSO as given for the highest (50 µM) PGD_2 treatment to exclude cytotoxicity by DMSO in our treatments.

24 and 48 hours after PGD_2 induction, pictures were taken by using Olympus DP 50 camera (Olympus, Vienna, Austria).

15. Data analysis

Statistical analysis was performed by applying Prism 4.03 (GraphPad Software, USA). We performed either one-way ANOVA with Tukey's post hoc test or Student's *t* test. $p<0,05$ values were defined as significant (1), (2).

V. Figures

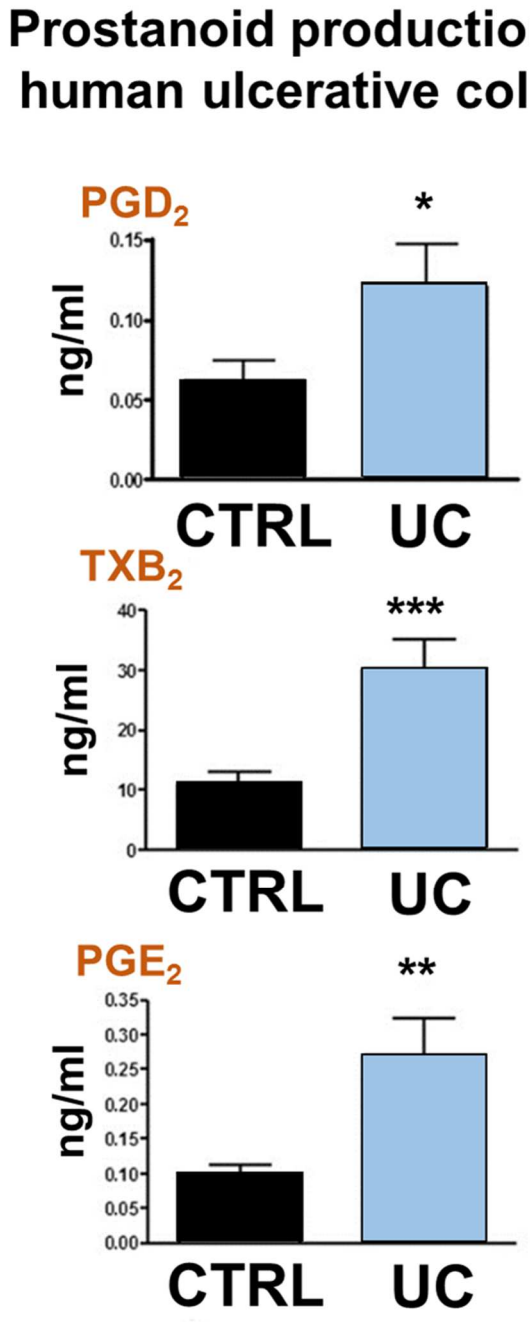

Figure. 3. **Mass spectrometric analysis of blood prostanoid concentrations in ulcerative colitis.** Sera of ulcerative colitis patients (n = 13) and control subjects (n = 12) were collected in serum tubes and analyzed by LC-MS/MS. Concentrations of PGD_2, TXB_2, and PGE_2 were measured (ng/ml). Data are presented as means±SD; Student's *t* test. P values < 0.05 were considered significant. * p<0.05; ** p<0.01; *** p<0.001. *Abbr.:* **CTRL** = control (healthy individuals); **UC** = ulcerative colitis patients . *[Reproduced from Sturm EM, Radnai B et al. J Immunol, 2014, (1) with permission of publisher The American Association of Immunologists, Inc. Copyright 2014., modified by the author.]*

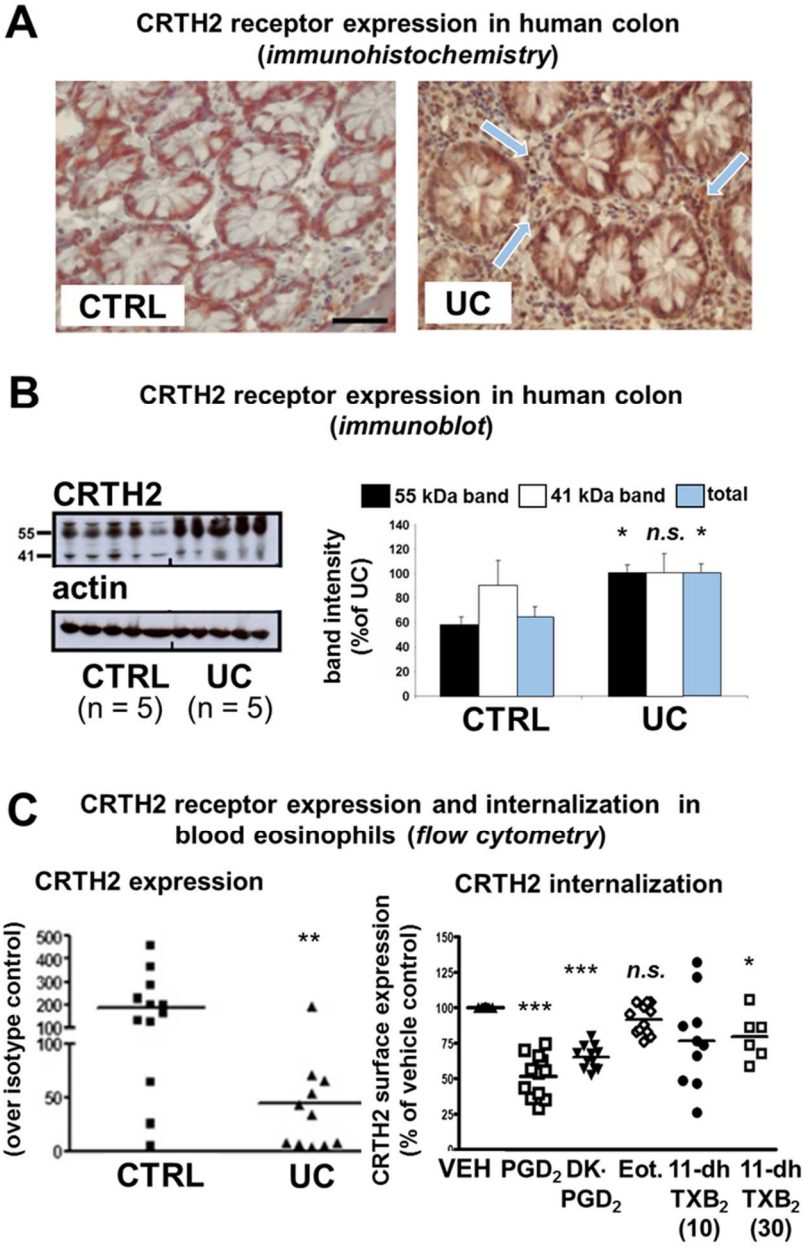

Figure. 4. **CRTH2 receptor expression in human UC.** The expression of CRTH2 was assessed in UC patients and in healthy individuals. CRTH2 was detected in the colon by **(A)** immunohistochemistry (*blue arrows* show CRTH2 positive leukocytes; representative images; scale bar, 50 μm) and by **(B)** immunoblot (UC values represent 100%; n = 5; means±SD, UC band intensities were set at 100%; Student's *t* test). **(C)** CRTH2 expression, as well as receptor internalization were measured in eosinophils by flow cytometry. Data are expressed as fold increase of fluorescence over isotype control (means±SD; n = 8 -13; Student *t* test) or as percentage of vehicle control (means±SD; n = 6-12; ANOVA). The p values < 0.05 were considered significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; *n.s.* = non-significant. *Abbr.:* **CTRL** = control; **UC** = ulcerative colitis; **VEH** = vehicle; **PGD_2** = prostaglandin D_2 (1 μM); **DK-PGD_2** = 13,14-dihydro-15-keto-PGD_2 (1 μM); **Eot**. = eotaxin (10 nM); **11-dhTXB_2** = 11-dehydrothromboxane B_2 (10 μM, 30 μM). *[Reproduced from Sturm EM, Radnai B et al. J Immunol, 2014, (1) with permission of publisher The American Association of Immunologists, Inc. Copyright 2014., modified by the author.]*

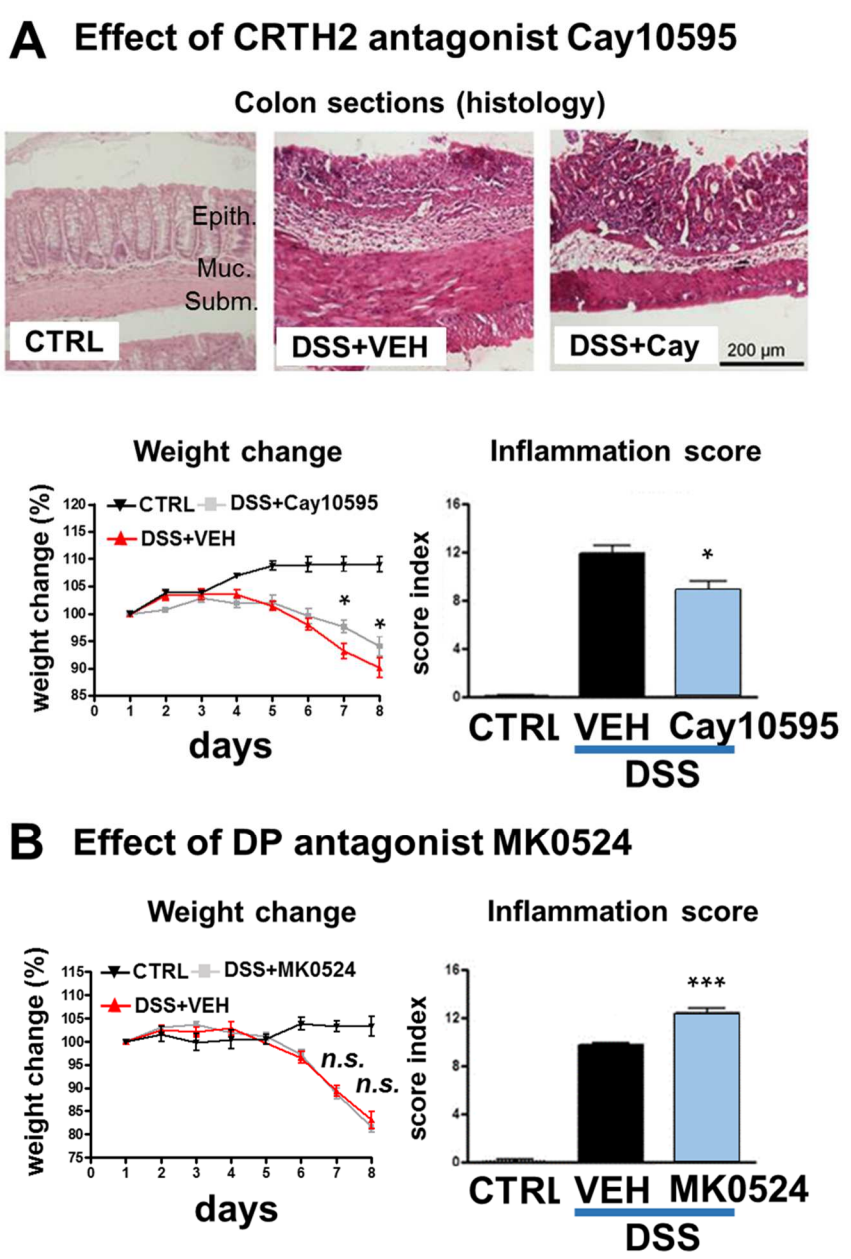

Figure. 5. **CRTH2 and DP antagonism in experimental colitis induced by dextran sulfate sodium (DSS) in mice.** The effect of CRTH2 antagonist Cay10595 and DP antagonist MK0524 was evaluated **(A)** Effects of Cay10595 were determined by histology (representative images of colon sections, 5 µm; stained with Hematoxylin&Eosin; scale bar, 200 µm), by measuring body weight (n = 8-12; expressed as percentage of control group; means±SEM; ANOVA, Tukey's post hoc), and by macroscopic scoring of inflammation (n = 8-12; means±SEM; ANOVA, Tukey's post hoc). **(B)** The effects of MK0524 were analyzed by measuring weight change (n = 6-8; expressed as percentage of control group; means±SEM; ANOVA, Tukey's post hoc) and by calculating inflammation scores (n = 6-8; means±SEM; ANOVA, Tukey's post hoc). p values < 0.05 were considered significant. * $p<0.05$; *** $p<0.001$; *n.s.* = non-significant. *Abbr.:* **CTRL** = control, **VEH** = vehicle, **DSS** = dextran sulfate sodium; **Cay** = Cay10595. *[Reproduced from Sturm EM, Radnai B et al. J Immunol, 2014, (1) with permission of publisher The American Association of Immunologists, Inc. Copyright 2014., modified by the author.]*

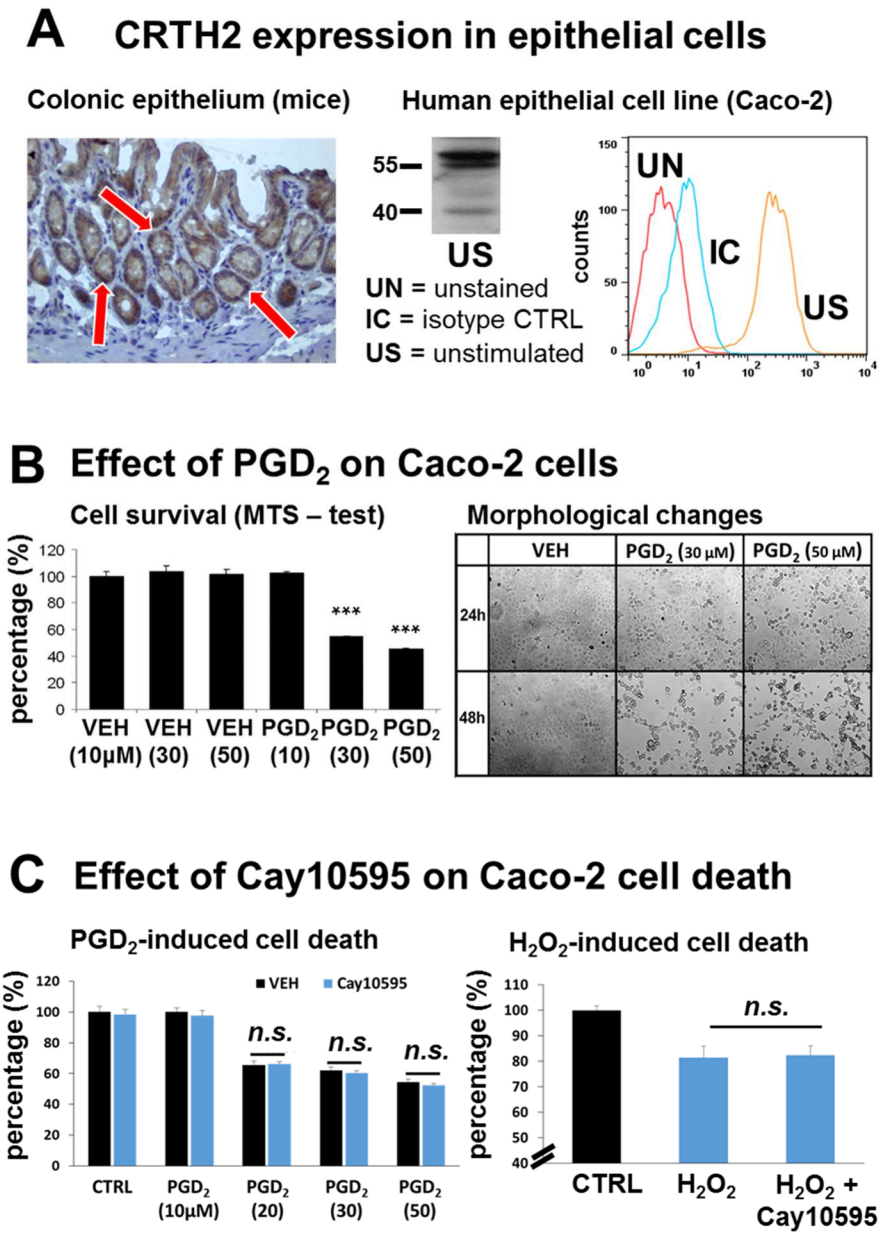

Figure. 6. **Effect of CRTH2 antagonism on PGD_2-induced cell death in Caco-2 human epithelial cell line. (A)** The presence of CRTH2 in the colonic epithelium of mice (representative images of a colonic section (immunohistochemistry*; red arrows* point at epithelial cells; n = 3) and in Caco-2 human epithelial cell line (immunoblot and flow cytometry; n = 6). **(B)** Effect of PGD_2 (10-50 µM) on cell survival (MTS test) and cell morphology (n = 3-6; vehicle treated cells received the same amount of DMSO as in the 10-50 µM PGD_2 treatment groups, respectively; means±SD; Student *t* test). **(C)** Effect of CRTH2 antagonist (Cay10595) on PGD_2-induced cell death (n = 6; control cells received the same amount of DMSO as the 50 µM treatment group; means±SD; Student's *t* test) and on H_2O_2-induced cell death (n = 6; control and H_2O_2-treated cells received the same amount of DMSO as the Cay10595 treatment group; means±SD; Student's *t* test. P values < 0.05 were considered significant. *** p < 0.001; *n.s.* = non-significant. *Abbr.:* **CTRL** = control, **VEH** = vehicle, **PGD_2** = Prostaglandin D_2. [*unpublished results]*

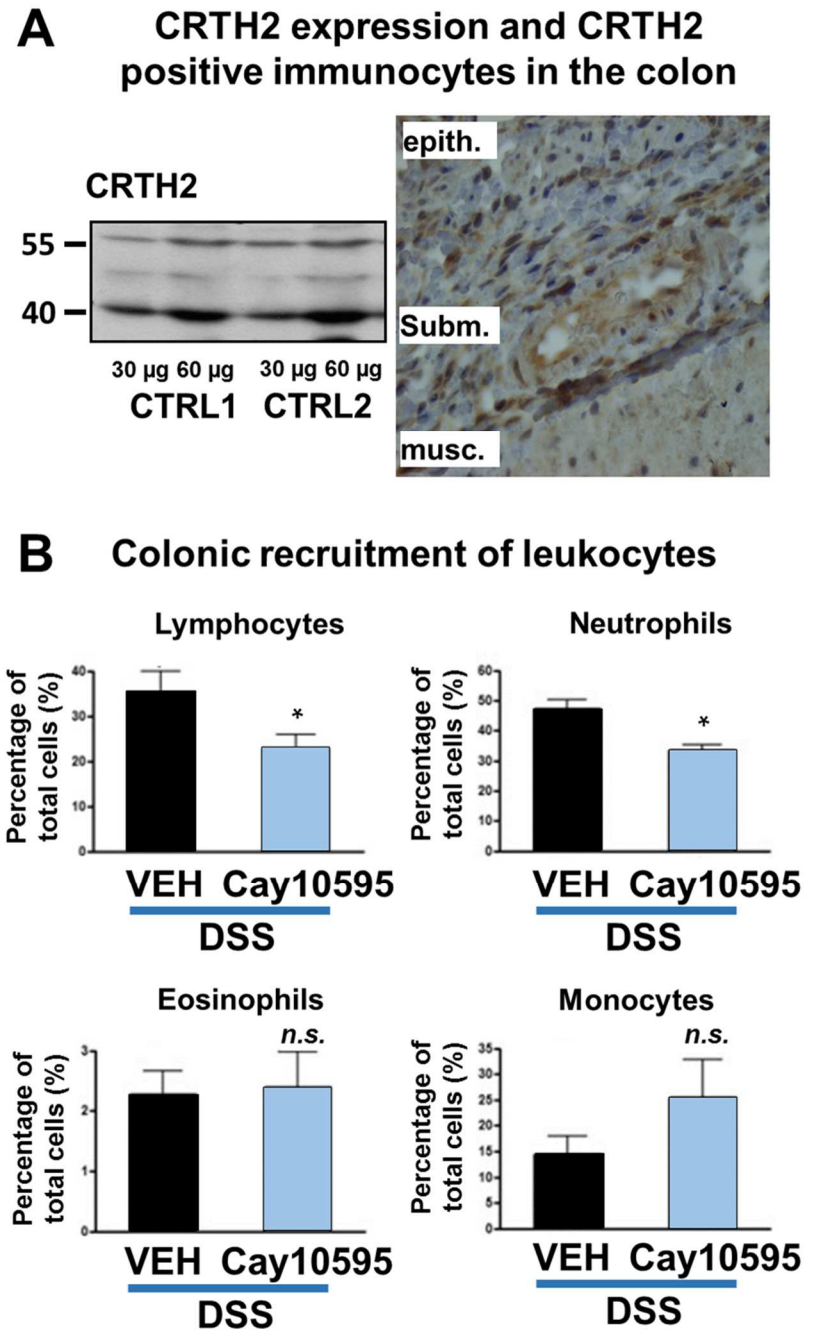

Figure. 7. **Presence of CRTH2 positive cells in the colon of mice and colonic recruitment of leukocytes upon CRTH2 antagonism in DSS colitis. (A)** The presence of CRTH2 receptors and CRTH2 positive lamina propria cells was evaluated. CRTH2 was detected by immunoblot in CTRL mice (n = 2; 30 and 60 µg proteins per lane) and by immunohistochemistry in DSS mice (representative image of colon sections; 5 µm; n = 3). **(B)** Colonic recruitment of leukocytes in vehicle (DMSO-treated) and Cay10595-treated DSS-induced groups (n = 4; means±SD; Student's *t* test). The p values < 0.05 were considered significant. * $p < 0.05$; *n.s.* = non-significant. Abbr.: **CTRL** = control, **VEH** = vehicle, **DSS** = Dextran sulfate sodium. *[Reproduced from Sturm EM, Radnai B et al. J Immunol, 2014, (1) with permission of publisher The American Association of Immunologists, Inc. Copyright 2014., modified by the author; the figure contains unpublished results.]*

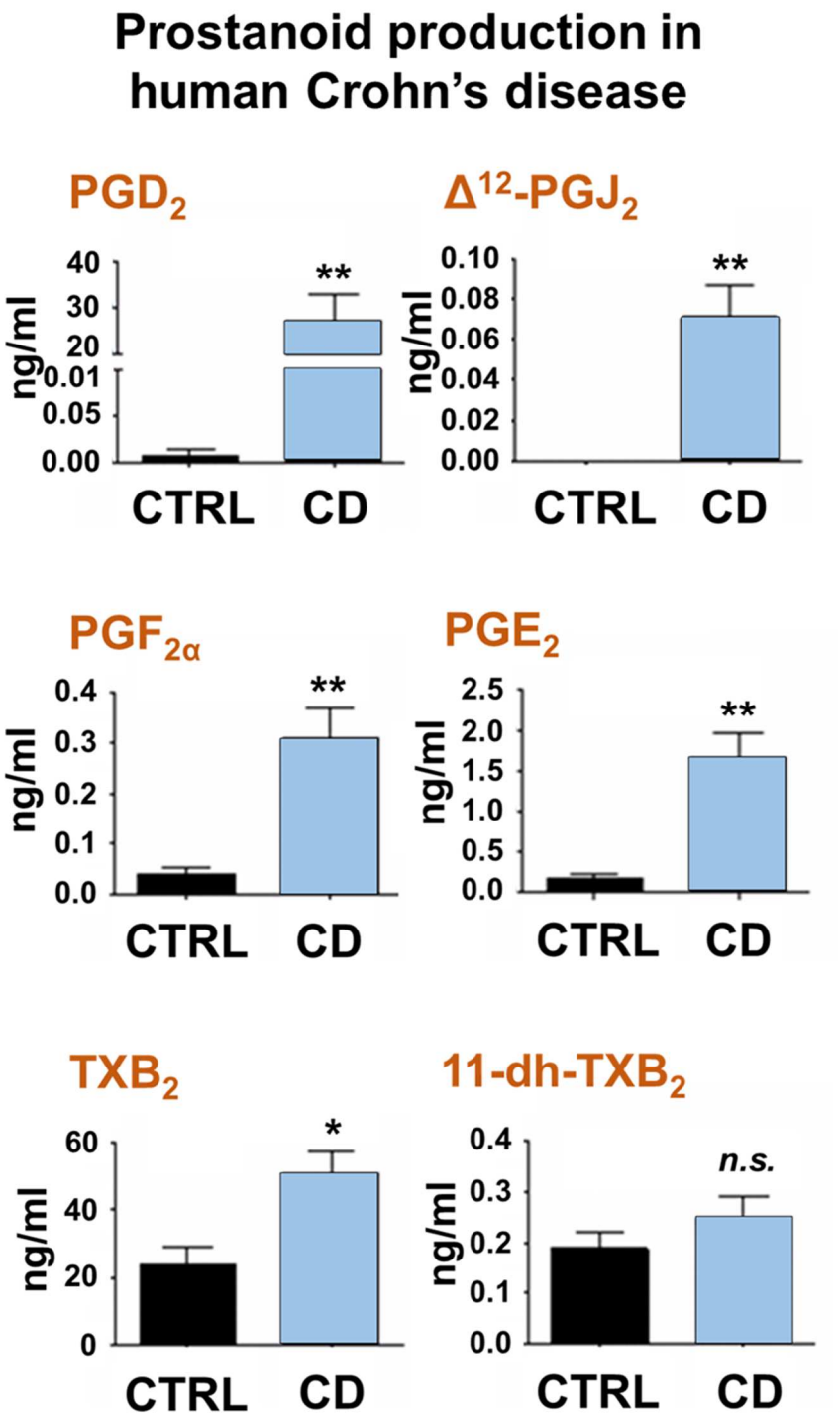

Figure. 8. **Mass spectrometric analysis of prostanoid concentrations in Crohn's disease.** Sera of Crohn's disease patients (CD; n = 31) and control subjects (CTRL; n = 15) were collected in serum tubes and analyzed by LC-MS/MS. Concentrations (ng/ml) of PGD_2, Δ^{12}-PGJ_2; $PGF_{2\alpha}$, PGE_2, TXB_2, and 11-dh-TXB_2 were measured in CD patients and in CTRLs (healthy individuals). Data are presented as means±SD; Student's *t* test; p values < 0.05 were considered significant. * $p<0.05$; ** $p<0.01$; *n.s.* = non-significant. *Abbr.:* **CTRL** = control, **CD** = Crohn's disease. *[Reproduced from Radnai B, Sturm EM et al. J Crohns Colitis, 2016 (2) with permission of publisher Oxford University Press, Copyright 2017, modified by the author]*

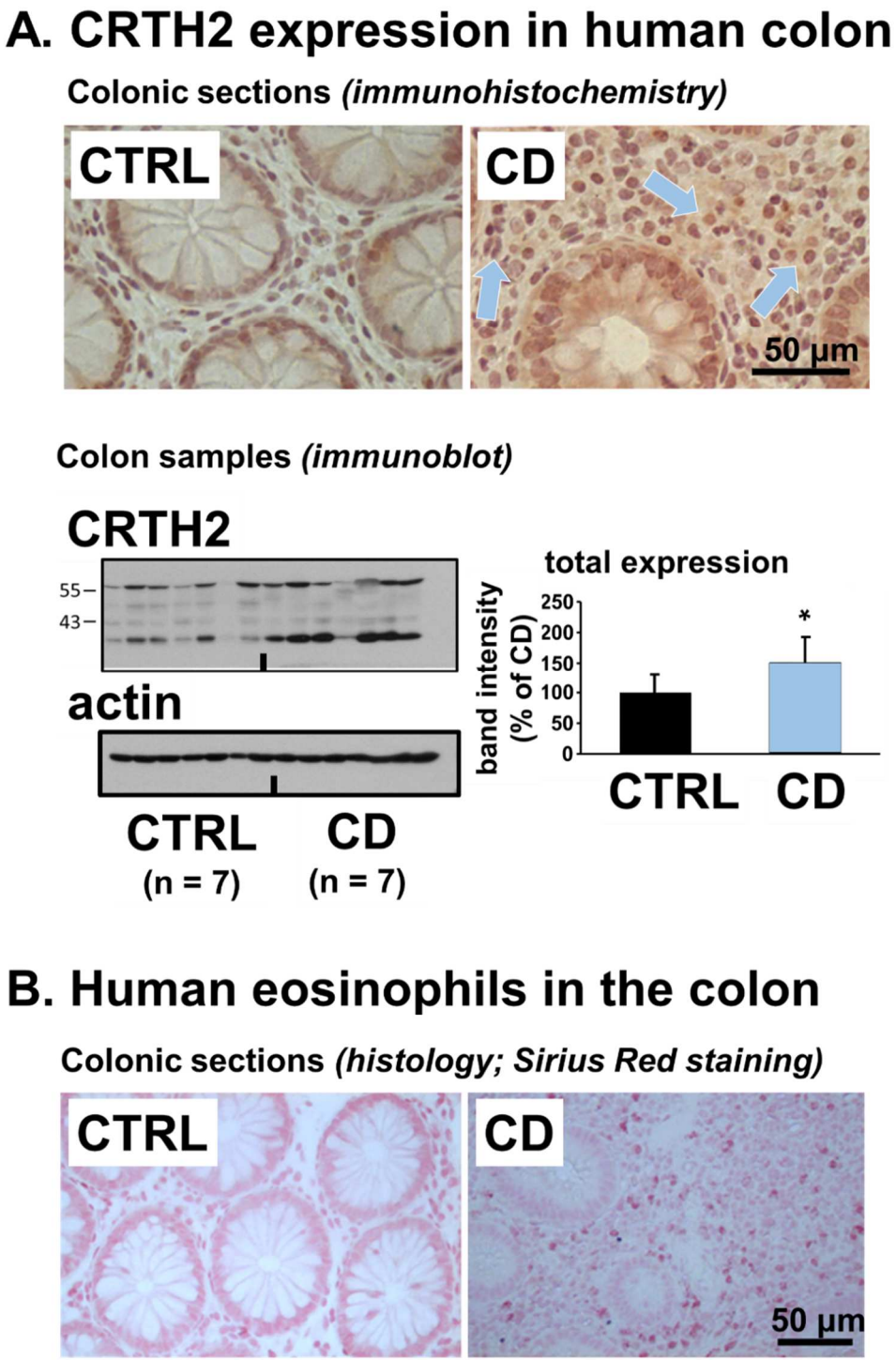

Figure. 9. **CRTH2 receptor amounts, CRTH2 positive cells and eosinophils in Crohn's disease. (A)** Representative histological images (n = 5; colon sections; 5 µm) show CRTH2 positive leukocytes (examples are indicated by *blue arrows*). Total amount of CRTH2 protein was measured by immunoblot (n = 7; means±SD of band intensities (percentage of CD), Student's *t* test). **(B)** The presence of eosinophils in colonic sections (representative images with Sirius Red staining, n = 3; calibration bar represents 50 µm). P values < 0.05 were considered significant. * p<0.05. *Abbr.:* **CTRL** = control, **CD** = Crohn's disease. *[Reproduced from Radnai B, Sturm EM et al. J Crohns Colitis, 2016 (2) with permission of publisher Oxford University Press, Copyright 2017, modified by the author]*

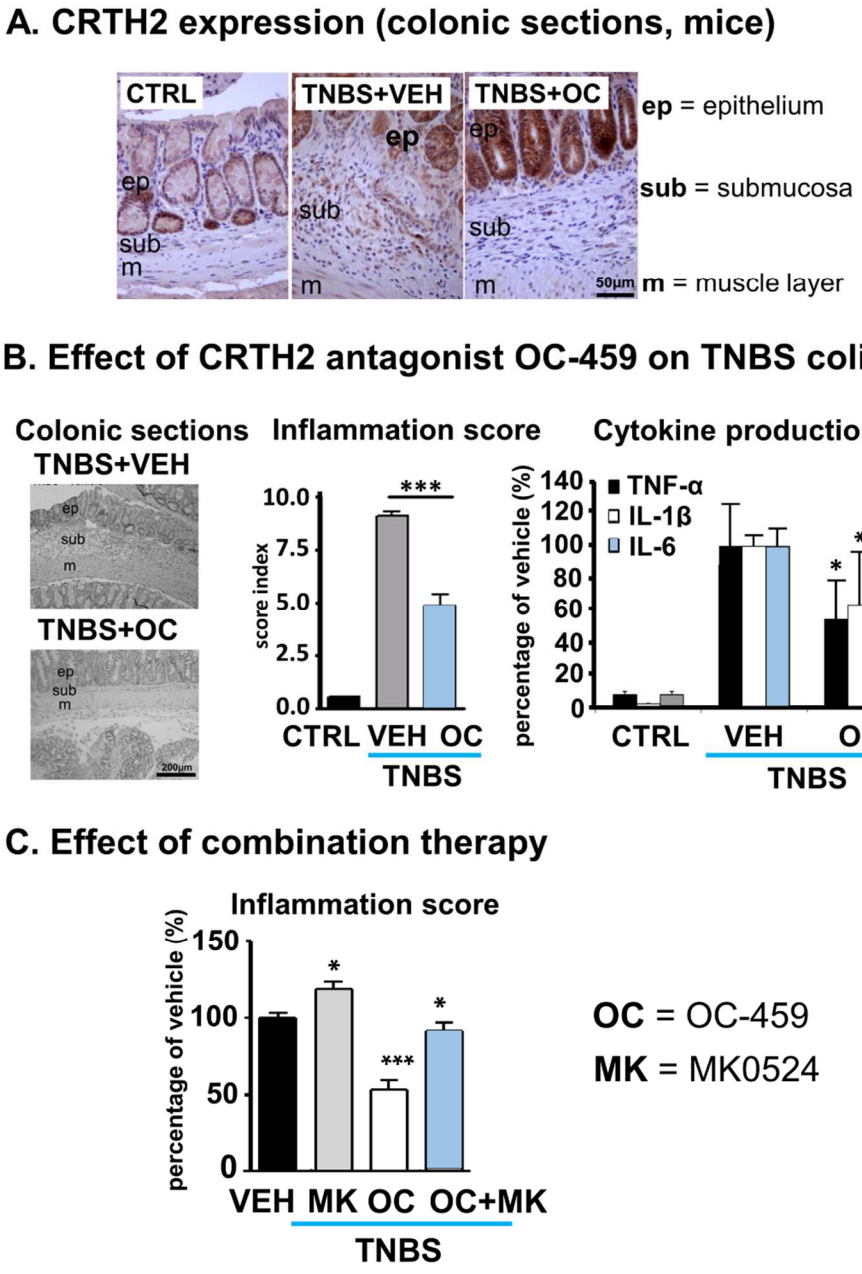

Figure. 10. **Effect of CRTH2 and DP antagonism on TNBS-induced experimental colitis in mice. (A)** CRTH2 positive cells were detected by immunohistochemistry (representative images of n = 6). **(B)** The effect of CRTH2 antagonist OC-459 was assessed by using histology (representative images of colonic sections, n = 6), by evaluating the macroscopical signs of inflammation (inflammation scores) and by measuring TNF-α, IL-1β and IL-6 production (n = 6-10; means±SEM, score index, percentage of vehicle; Student's *t* test). **(C)** The effect of the DP antagonist (MK0524) alone or in combination with OC-459 (OC-459+MK0524) was evaluated by macroscopical scoring (n = 6-10; means±SEM, percentage of vehicle; one-way ANOVA). P values < 0.05 were considered significant. * $p < 0.05$; *** $p < 0.001$; *n.s.* = non-significant. *Abbr.:* **CTRL** = control, **VEH** = vehicle, **OC** = OC-459; **MK** = MK0524. *[Reproduced from Radnai B, Sturm EM et al. J Crohns Colitis, 2016 (2) with permission of publisher Oxford University Press, Copyright 2017, modified by the author]*

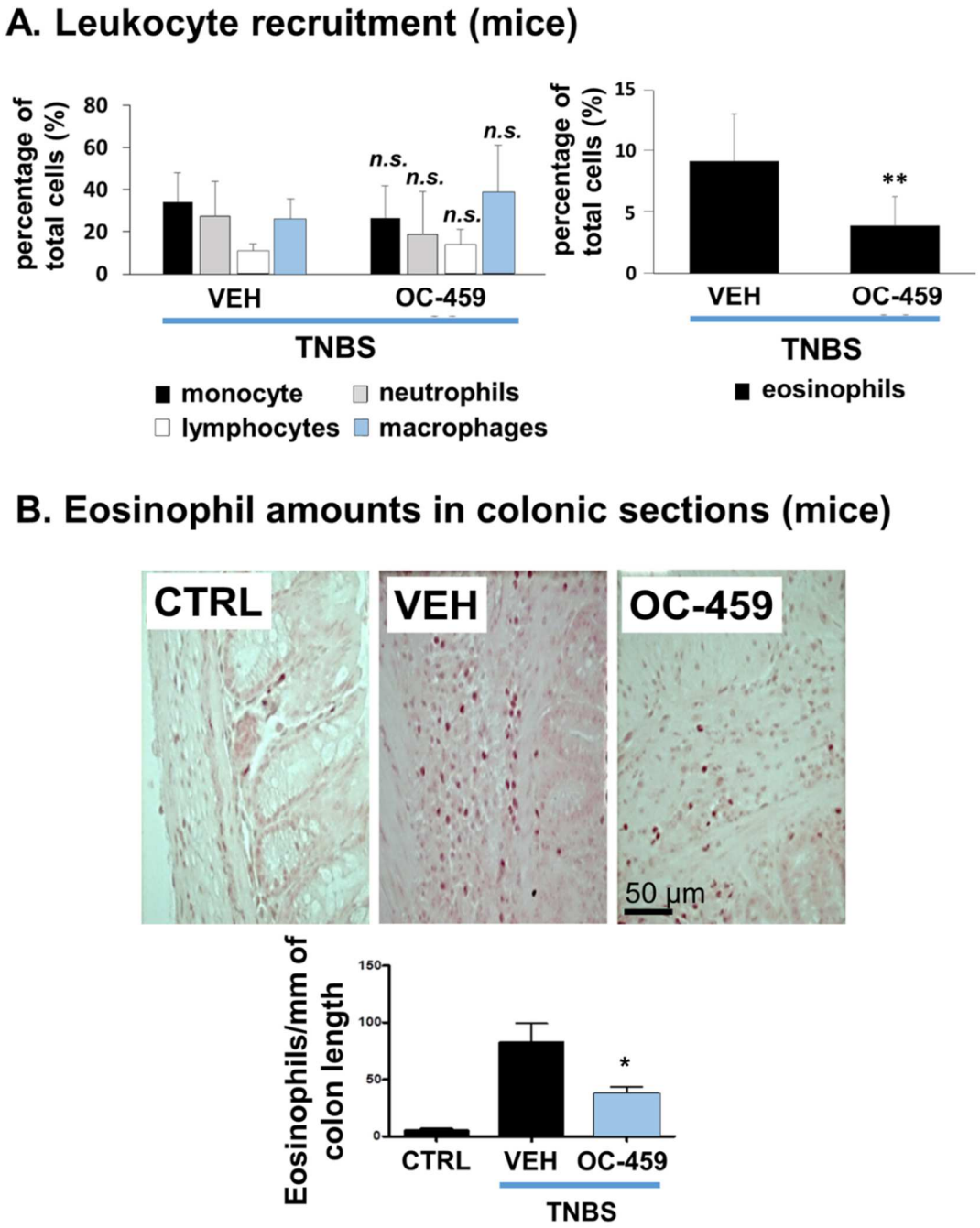

Figure. 11. **Effect of CRTH2 antagonism on colonic migration of eosinophils. (A)** The effect of OC-459 (CRTH2 antagonist) on colonic recruitment of leukocytes were measured in TNBS colitis (n = 9; means±SD, presented as percentage of total cells; Student's *t* test). **(B)** Colonic recruitment of eosinophils were further confirmed by evaluating colonic sections (whole colon; 5 µm) stained with Sirius Red (specific staining for eosinophils). Sirius Red positive eosinophils were counted (representative images of n = 6-13; means±SD, presented as eosinophils / mm of colon length, Student's *t* test). The p values < 0.05 were considered significant. * p<0.05; *n.s.* = non-significant. *Abbr.:* **CTRL** = control, **VEH** = vehicle, **OC** = OC-459; **MK** = MK0524. *[Reproduced from Radnai B, Sturm EM et al. J Crohns Colitis, 2016 (2) with permission of publisher Oxford University Press, Copyright 2017, modified by the author]*

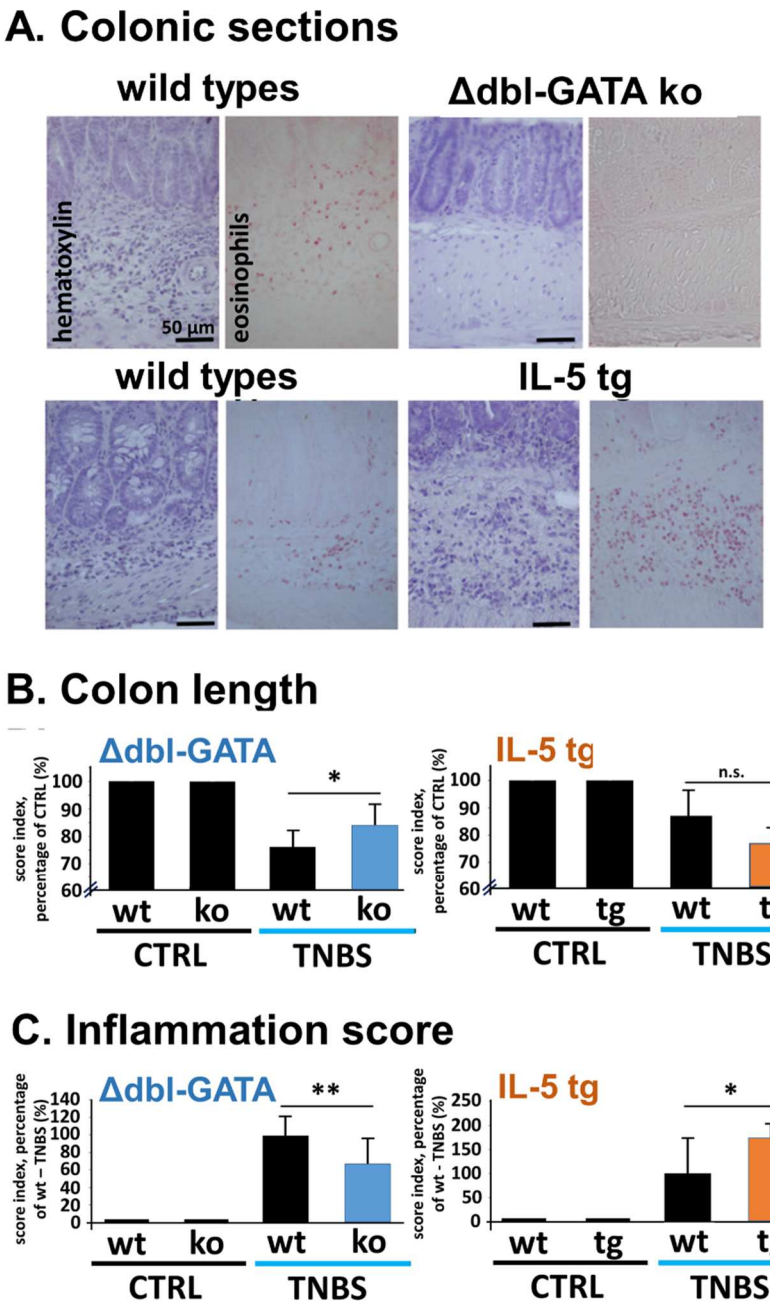

Figure. 12. **Role of eosinophils in TNBS colitis in mice.** TNBS colitis was induced in eosinophil-depleted Δdbl-GATA mice and in IL-5 transgenic mice, which display strong eosinophilia. **(A)** Confirmation of different phenotypes in genetically modified mice (eosinopenia in Δdbl-GATA and eosinophilia in IL-6 transgenic mice) compared to respective wild types by histology (colonic sections 5 µm, stained with hematoxylin or with Sirius Red a specific staining for detecting eosinophils, n = 7-12). The severity of the colitis was evaluated by **(C)** inflammation scoring, which involves the measurement of **(B)** colon length among other macroscopic parameters detailed in *mat&meth* section (n = 7-12; means±SD, expressed in percentage, Student's *t* test). The p values < 0.05 were considered significant. * p<0.05; ** p<0.01; *n.s.* = non-significant. *Abbr.:* **wt** = wild types; **ko** = knockout, genetic depletion; **tg** = transgenic; **CTRL** = control. *[Reproduced from Radnai B, Sturm EM et al. J Crohns Colitis, 2016 (2) with permission of publisher Oxford University Press, Copyright 2017, modified by the author]*

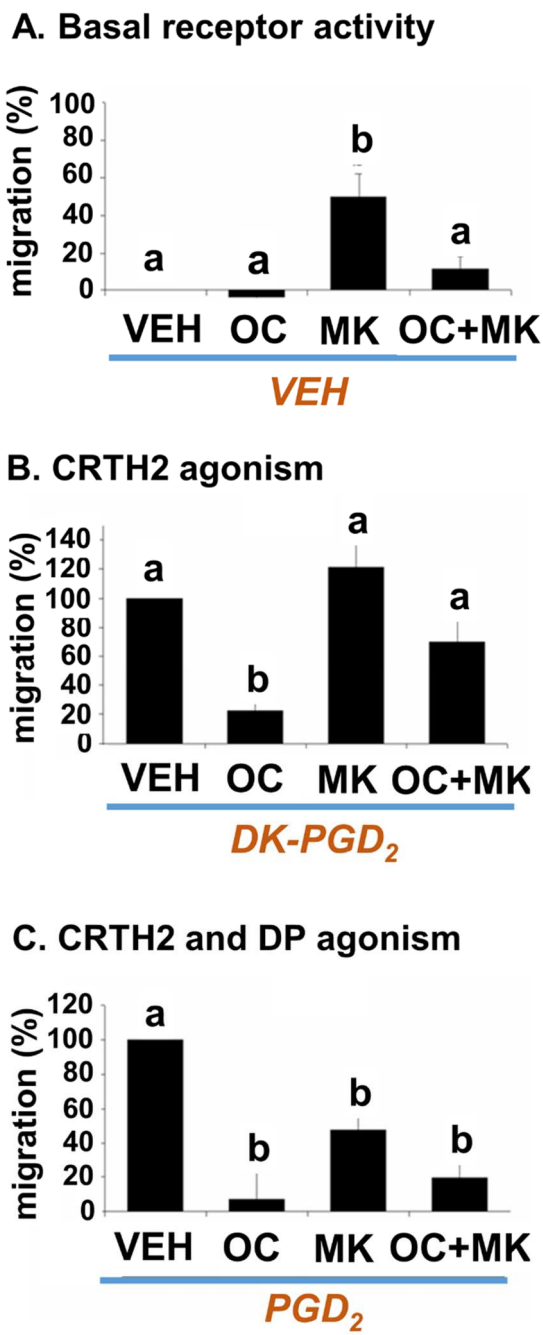

Figure. 13. **Eosinophil migration after incubation with CRTH2 and DP antagonists (applied alone or in combination).** Migration of isolated blood eosinophils was induced via PGD_2 receptor agonism, and the effects of OC-459 (CRTH2 antagonist), MK0524 (DP antagonist) and OC-459+MK0524, applied in combination, was evaluated. **(A)** Basal activity without any receptor stimulation (only vehicle; vehicle were set at 0%). **(B)** CRTH2 activation with DK-PGD_2 (specific CRTH2 agonist), vehicle were set at 100%. **(C)** Activation by PGD_2 (agonist for both CRTH2 and DP), vehicle were set at 100%. Data are expressed as means±SD (n = 7, ANOVA). p values < 0.05 were considered significant. Means without a common letter differ. *Abbr.:* **VEH** = vehicle, **OC** = OC-459; **MK** = MK0524. *[Reproduced from Radnai B, Sturm EM et al. J Crohns Colitis, 2016 (2) with permission of publisher Oxford University Press, Copyright 2017, modified by the author. The figure contains unpublished results.]*

VI. Results

1. Prostanoid levels are enhanced during ulcerative colitis

To assess the involvement of prostanoids (some have CRTH2 agonist activity but all of the prostanoids measured have a role in the pathomechanism of ulcerative colitis), we measured the concentrations of PGD_2, TXB_2, and PGE_2 in sera of UC patients and healthy individuals (CTRL) ***(Fig. 3.)*** (1). We found an increase in the concentration of all three measured prostanoid types (PGD_2, TXB_2, and PGE_2) in the UC group as compared to the CTRL cohort. Importantly, the levels of PGD_2 and TXB_2 (PGD_2 and a derivative of TXB_2, namely 11-dh-TXB_2 [180] are endogenous CRTH2 ligands) were increased approximately 2-fold and 3-fold, respectively. Furthermore, with the concentration of at about ~30 ng/ml, TXB_2 represents the highest serum level of the measured prostanoids in our experiments. However, it is important to note, that the basal TXB_2 level (serum concentration in CTRL subjects) was found to be approximately 10-20x higher than e.g. that of basal PGD_2. In addition, PGE_2 and PGD_2 showed comparable concentrations in the CTRL cohort (1).

These findings are suggestive of a strong involvement of CRTH2 and DP receptors during human UC, because the most important activating ligand, PGD_2, is present in high amounts in the sera of UC patients.

2. Higher levels of CRTH2 are found in the colon of UC patients, but expression of CRTH2 in blood eosinophils is decreased and accompanied by receptor internalization

After the detection of increased prostanoid concentrations in UC, including those of PGD_2, we intended to investigate the CRTH2 receptor as well. First, we measured CRTH2 protein expression in the colon of UC patients and in CTRL subjects ***(Fig. 4.)*** (1). Images of colonic sections (representative images) showed an increased number of CRTH2 immunopositive cells accumulated in the colonic submucosa of UC patients as compared to CTRLs ***(Fig. 4. A.)***, however, we could observe a low amount of CRTH2-positive cells in the CTRL cohort as well. We hypothesized, that the CRTH2-positive cells between the crypts were largely leukocytes (1).

To directly quantify the increase in receptor protein, we measured CRTH2 expression by immunoblot technique using a specific rabbit anti-CRTH2 antibody ***(Fig. 4. B.)***. CRTH2 is a GPCR that can be N-glycosylated at many sites and it presents many divers glycosylated forms (41 kDa and 55 kDa bands) (151); however, the reason for the presence of different glycosylated forms and their biochemical roles remain unknown. After evaluating the band densities, we found an about 1,5-fold increase in the amount of CRTH2 receptor proteins (total) in UC, which originates mainly from the enhanced expression of the 55 kDa band, whereas the 41 kDa band was not significantly changed (1).

In addition, we measured CRTH2 expression by flow cytometry in human peripheral blood eosinophils in UC and CTRL groups. After measuring CRTH2 (localized in the cell membrane), we found lower levels of CRTH2 on eosinophils isolated from UC patients in comparison with eosinophils isolated from the CTRL cohort subjects ***(Fig. 4. C.)***. Since the anti-CRTH2 antibody used in the measurements only detected CRTH2 on the cell surface (cells were not permeabilized), we could not measure internalized CRTH2 proteins. But since desensitization of GPCRs also occurs in cells, namely in the plasma, CRTH2 ought to be internalized into the cell. Therefore, we assessed CRTH2 expression after application of CRTH2 agonists in eosinophils isolated from healthy individuals. The internalization measurements showed that CRTH2 agonists (PGD_2, DK-PGD_2, 11-dh-TXB_2 [30 nM]) led to the loss of receptors on the surface of eosinophils suggesting that CRTH2 may have been indeed internalized. Eotaxin, which induces eosinophil migration identically to PGD_2, but with no effect on CRTH2 (151), did not influence CRTH2 receptor internalization (1).

In conclusion, we measured elevated concentrations of CRTH2 proteins and higher numbers of CRTH2-expressing leukocytes in colonic biopsies of UC patients, but decreased amounts of CRTH2 in the membrane of blood eosinophils of these patients, probably because of an active receptor-internalization.

3. CRTH2 antagonism improves, but DP antagonism exacerbates DSS-induced colitis

In order to clarify the role of PGD_2 receptors in experimental colitis in mice, we applied pharmacological inhibitors for CRTH2 and DP. The antagonists Cay10595 (CRTH2 antagonist) and MK0524 (DP antagonist) were tested in DSS-induced colitis ***(Fig. 5.)*** (1).

In short, Cay10595 showed anti-inflammatory effects. We detected an improvement of colonic tissue histopathology (H&E staining) after Cay10595 treatment (5 mg/kg, s.c., once a day), which prevented erosion and degeneration of epithelium and inhibited swelling

of mucosa and submucosa compared to DSS-mice receiving vehicle. In addition, CRTH2 antagonist Cay10595 reduced weight loss of DSS-colitic animals and diminished macroscopical signs of inflammation, exhibiting significantly lower inflammation scores ***(Fig. 5. A.)***. Contrastingly, MK0524 (1 mg/kg, s.c., once a day) exhibited proinflammatory effects. Although we could not detect any significant changes in the body weight of MK0524-treated DSS animals, we found a more pronounced and stronger inflammation than in the DSS+VEH group. Thus, MK0524 treatment resulted in higher inflammation scores ***(Fig. 5. B.)*** (1).

4. CRTH2 antagonism does not affect experimentally-induced epithelial cell death

In vivo, CRTH2 antagonist Cay10595 showed anti-inflammatory effects in DSS colitis (1), so the question emerged, whether Cay10595 could be protective via the colonic epithelial barrier, or more precisely, if it affects the epithelium directly or indirectly through the inhibition of leukocyte activation. Therefore, we evaluated the effect of Cay10595 at the level of the colonic epithelium in mice and assessed PGD_2 receptor expression. In addition, we evaluated the effect of CRTH2 antagonism in PGD_2- and in H_2O_2-induced epithelial cell death ***(Fig. 6.)***.

First, we determined the presence of CRTH2 receptors in the colonic epithelium in mice by immunohistochemistry ***(Fig. 6. A.)***. Next, as shown before, we stained CRTH2 in biopsies of the human colonic mucosa ***(Fig. 4. A. and B.)*** (1) as well as in the Caco-2 human epithelial colorectal cell line ***(Fig. 6. A.)***. After the presence of the receptor was confirmed, we treated Caco-2 cells with PGD_2 (10-50 µM) for 24 or 48 hours and measured cell viability and morphological changes of the cells. PGD_2 dose-dependently killed Caco-2 cells and caused morphologic changes that led to lower cell numbers, and to shrinking and probably necrotization of the cells ***(Fig. 6. B.)***.

A substantial cell death and the most pronounced morphologic alteration was measured 48 hrs after PGD_2-treatment. Surprisingly, the PGD_2-reduced cell viability of Caco-2 cells seemed to be totally independent of CRTH2, since Cay10595 could not influence the survival of the Caco-2 cells ***(Fig. 6. C.)***. We also tested the effect of CRTH2 antagonism by giving Cay10595 on the second day of the experiment freshly again (*data not shown*) to avoid the decline in inhibitor concentration and the loss of the antagonizing effect under the long incubation period. Cay10595 was found to be ineffective under these circumstances as well.

Secondly, we tested CRTH2 antagonism in oxidative stress-induced cell death. Cay10595 did not show any beneficial effects in H_2O_2-induced (300µM) cell death measured by the MTS-test ***(Fig. 6. C.)***.

In the *in vitro* model of Caco-2 epithelial cells, we could not detect any protective effect of CRTH2 antagonism, therefore, we intended to explore whether Cay10595 may have an immunomodulatory effect and measured the recruitment of leukocytes into the colon.

5. CRTH2-positive leukocytes are present in the colon and CRTH2 antagonism reduces colonic recruitment of lymphocytes and neutrophils in DSS-colitis

We hypothesized that immune cells could represent important candidates as biological targets of CRTH2 antagonism. Because one of CRTH2's most prominent effect is the induction of chemotaxis in some immunocytes, such as eosinophils and TH2 cells, we measured the recruitment of leukocytes to the colon in the model of DSS-induced colitis (1).

First, we confirmed the presence of CRTH2 receptors by Western blotting and detected CRTH2 protein in the colonic tissue of control (untreated) mice ***(Fig. 7. A.,*** *(left, immunoblot))*. These experiments revealed, that the CRTH2 receptor is present in the colon under physiologic circumstances.

Cay10595 treatment reduced the migration of lymphocytes and neutrophils into the colon, but did not influence the recruitment of monocytes and eosinophils (which were only present in very low amounts); however, monocytes showed a tendency, however not significant, towards more migration ***(Fig. 7. B.)*** (1).

To conclude, in our model of DSS-induced experimental colitis, CRTH2 activation might be mainly responsible for effects on immunocytes, precisely for the migration of lymphocytes and neutrophils, but probably not for monocytes and eosinophils.

6. Levels of prostanoids are higher in Crohn's disease patients than in healthy subjects

Since prostanoid production was found to be enhanced in UC, we also intended to measure the concentrations of prostanoids, such as of PGD_2, Δ^{12}-PGJ_2, $PGF_{2\alpha}$, PGE_2, TXB_2, and 11-dh-TXB_2 in the sera of CD patients and in healthy individuals (CTRL) ***(Fig. 8.)***

(2). Levels of PGD_2, Δ^{12}-PGJ_2, $PGF_{2\alpha}$, PGE_2, TXB_2, but not of 11-dh-TXB_2 were increased in the CD group compared to the CTRL cohort. Similar to our findings in UC patients, the levels of the endogenous CRTH2 agonists PGD_2, Δ^{12}-PGJ_2 (also a CRTH2 agonist (183)) were found to be increased in CD. TXB_2 showed the highest concentrations among the measured prostanoids in UC as well as in CD ***(Fig. 8.)***. Most interestingly, absolute concentrations of PGD_2 in CD patients (~ 25 ng/ml) (2) are dramatically higher (~ 200x) than in UC patients (~ 0,12 ng/ml) (1) ***(Fig. 3 and 8.)***.

These findings suggest active PGD_2 receptor signaling in CD and probably an important role of PGD_2 in the CD pathomechanism.

7. CRTH2 protein and numbers of eosinophils and other CRTH2 positive leukocytes are higher in Crohn's disease than in healthy subjects

In CD patients, we detected higher levels of PGD_2 and Δ^{12}-PGJ_2 (known to act as endogenous CRTH2 agonists) than in control subjects. Next, we analyzed the amount of CRTH2 receptor protein and the number of CRTH2 positive cells including eosinophils in the colon of CD patients and compared them to the data collected from the colon of control subjects ***(Fig. 9.)*** (2).

Representative images of colonic sections showed high amounts of CRTH2 positive leukocytes in CD ***(Fig. 9. A.)***. Thus, we quantified CRTH2 by immunoblot and found an about 1.5-fold increase in the total amount of CRTH2 receptor protein in CD *vs* healthy subjects ***(Fig. 9. A.)***. As a consequence, we concluded, that higher levels of endogenous CRTH2 agonists (i.e. of the prostanoids PGD_2 and Δ^{12}-PGJ_2) and of CRTH2 receptors are present in the colon of CD patients, highlighting the importance of the PGD_2 – CRTH2 axis not only in UC, but also in CD (2).

Since the role of eosinophils in CD has not yet been clarified (eosinophils are CRTH2 positive (*see above in introduction section [3.1.]*) and as their actions are influenced by CRTH2 under many pathophysiological conditions, like in allergy (184), we determined the amount of eosinophils in the colon of CD and control patients. We found an increase in Sirius Red-stained eosinophils in CD patients in comparison to CTRL subjects ***(Fig. 9. B.)*** (2).

Our data indicated that CRTH2 and perhaps CRTH2-positive eosinophils could be involved in the pathology of CD.

8. CRTH2 antagonism protects against TNBS-induced experimental Crohn's disease

CRTH2 was found to be highly present in the colon of human CD ***(Fig. 9. A.)***. Accordingly, we wanted to detect it in the colon of our TNBS mouse model as well. Indeed, the number of CRTH2 positive cells was found to be increased in the lamina propria of TNBS-colitic animals (***Fig. 10. A.;*** *representative images*). Immunohistochemical staining of CRTH2 suggested similar expression in the epithelium of OC-459-treated TNBS mice and the VEH-treated group while there seemed less CRTH2 immunoreactive cells in the lamina propria. ***(Fig. 10. A.)*** (2).

Histology of colonic sections showed improved tissue structures and less pronounced signs of inflammation upon OC-459 treatment ***(Fig. 10. B.)***. These findings reveals an overall anti-inflammatory action by CRTH2 antagonism. Therefore, we further evaluated the effect of OC-459 by macroscopical scoring of colonic tissue and observed significantly decreased inflammation scores ***(Fig. 10. B.)*** (2).

Because inflammation is characterized by elevated inflammatory cytokine expression, we measured the production of TNF-α, IL-1β, and IL-6 in vehicle- and OC-459-treated TNBS-induced colitic mice. We found lower concentrations of all three cytokines after the CRTH2 antagonist treatment which further supported the hypothesis that CRTH2 inhibition generally has anti-inflammatory effects ***(Fig. 10. B.)***. Thus, CRTH2 seems to play a proinflammatory role in the TNBS-induced colitis model (2).

The next aim was to clarify the role of DP in the same model and to show whether an interplay existed between the two known PGD_2 receptors. Therefore, we treated our animals with MK0524 or OC-459 alone or in combination and induced colitis with TNBS. By evaluating inflammatory damage of the colon, we demonstrated higher inflammation scores in the MK0524 treatment group as compared to vehicle-treated mice. This suggested an anti-inflammatory role for DP in this model. Given in combination with OC-459, MK0524 diminished the anti-inflammatory effect of OC-459 ***(Fig. 10. C.)*** (2).

In conclusion, CRTH2 showed pro-inflammatory, while DP showed anti-inflammatory behavior in the TNBS colitis model similar to what we demonstrated in the DSS-induced experimental UC model.

9. Recruitment of eosinophils to the colon is inhibited by CRTH2 antagonism in TNBS-induced experimental colitis

After demonstrating anti-inflammatory effects of OC-459 in TNBS colitis, we intended to explore its biological targets. Because the CRTH2 antagonist failed to show any effects on the PGD_2-induced cell death in epithelial cells ***(Fig. 6. B-C.)***, we started with measuring the recruitment of leukocytes that prominently express PGD_2 receptors, to the colon ***(Fig. 11.)*** (2).

The flow cytometric measurements revealed significantly decreased numbers of eosinophils in OC-459-treated mice as compared to the vehicle group, however, other leukocytes, like neutrophils showed only a slight tendency of decrease ***(Fig. 11. A.)***. To underline and verify these findings, we made sections of the inflamed colon from mice that underwent the same treatments and stained the eosinophils with Sirius Red. By counting the Sirius Red positive cells, we found a lower density (cells / mm of colon length) of eosinophils in the OC-459 group compared to vehicle-treated TNBS mice ***(Fig. 11. B.)*** (2).

These measurements raised the possibility, that the reduction of eosinophils might be essentially involved in the actions of OC-459, as its direct biological target. But do eosinophils play a role in inducing TNBS colitis at all?

10. Eosinophils contribute to inflammation in TNBS-colitis

To answer the question whether eosinophils play a proinflammatory role in colitis we applied our TNBS colitis model to genetically modified mice that either suffered from eosinopenia or from eosinophilia ***(Fig. 12.)***. Whereas Δdbl-GATA knockout mice lack eosinophils, IL-5 transgenic mice (IL-5tg) display elevated eosinophil numbers. Colitis was induced by TNBS and we compared the genetically modified mice (Balb/c background) with their respective wild types (2).

Upon completion of the experiments, we first confirmed the presence/absence of eosinophils by evaluating colonic sections of mice from the different genetic background by Sirius Red staining. As it was expected, Δdbl-GATA mice were found to lack eosinophils, while IL-5 transgenic mice showed higher amounts of Sirius Red positive cells as compared to wild type animals ***(Fig. 12. A.)*** (2).

Macroscopic evaluation of colitis was performed, that included evaluation of inflammation scores and the colon length as a subscore. Δdbl-GATA mice were protected

against TNBS-induced colitis and demonstrated lower inflammation scores and inhibited colon shortening (less inflamed, longer colons) ***(Fig. 12. B, C.)***. In contrast to this, IL-5tg mice were found to be more affected by colitis and showed higher inflammation scores and (however not statistically significant) an increase in colon shortening (more inflamed, shorter colon) ***(Fig. 12. B, C.)*** (2).

These results indicate a clear proinflammatory role for eosinophils in our model of TNBS-induced colitis.

11. Migration of eosinophils is induced by CRTH2 activity and is differentially regulated by DP, depending on the ligand binding state

In our TNBS colitis experiments, we provided evidence for an anti-inflammatory effect of CRTH2 inhibition ***(Fig. 10.)*** and showed that eosinophils contribute to this effect as potential biological targets of CRTH2 antagonism ***(Fig. 11)***. To elucidate whether CRTH2 and DP could influence each other's signaling in eosinophils, we isolated human blood eosinophil granulocytes and induced their migration by using either DK-PGD_2 (specific CRTH2 agonist) or PGD_2 (CRTH2 and DP agonist). We then analyzed the effects of CRTH2 (2) and DP inhibitors, i.e. of OC-459 and MK0524, respectively, alone or in combination, on eosinophil migration ***(Fig. 13.)***.

First we measured the basal migratory activity of eosinophils without any receptor agonists. Subsequently, we investigated migration at the presence of OC-459 (2) or MK0524 alone or in combination. Basal migration of cells (no agonist activation, no treatments) were set at zero. In this experimental setting, OC-459 failed to activate or inhibit eosinophil migration (2), however MK0524 alone could induce it. A combination of OC-459 and MK0524 did not induce significant changes in basal migration as compared to vehicle treated cells, however a small increase could be observed ***(Fig. 13. A.)***.

In the next step we induced eosinophil migration by using DK-PGD_2, which binds and specifically activates CRTH2. The DK-PGD_2-induced migration was inhibited by OC-459 treatment (2), however MK0524 showed no significant effects compared to the vehicle-treated cells. Used in combination, MK0524 eliminated OC-459's inhibiting effect, however it could not reach the migration level of MK0524-(alone)-treated cells ***(Fig. 13. B.)***.

In our last experimental setting, we used PGD_2 to simultaneously activate both CRTH2 and DP at the same time. OC-459 inhibited the migration of eosinophils (2), but unexpectedly, MK0524 showed a similar inhibiting effect. The combination of the two receptor antagonists caused an equal inhibition on migration ***(Fig. 13. C.)***.

VII. Discussion

1. Background of the studies

In this thesis, we asked the question, whether the CRTH2 receptor had a role in experimental colitis and whether our hypothesis, namely that antagonism of CRTH2 provides anti-inflammatory effects. We also asked whether CRTH2 has any relevance in human IBD, especially regarding a potential development of a PGD_2-receptor-based therapy for IBD-patients. To address these questions, we investigated the effect of pharmacological inhibitors of CRTH2 in UC-like (1) and Crohn's disease-like (2) experimental colitis models in mice, and measured the prostaglandin levels and CRTH2 receptor expression in UC (1) and CD (2) patients and compared them to healthy subjects. We could clearly show that CRTH2 antagonism improved experimental colitis (1), (2).

PGD_2 receptors have been already shown to be involved in the pathomechanism of inflammatory and allergic disorders (185). The two PGD_2 receptors, CRTH2 and DP bind the same agonist, namely PGD_2, but they often show opposing (inducing or inhibiting) characteristics. Interestingly, as detailed in the *introduction* section of the thesis, the pro- or anti-inflammatory roles played by the different PGD_2 receptors are found to be strongly dependent on the type of the disease or the experimental model, the context or time of evaluation, and the possibility of an interaction between them. These facts make the overall picture of the biological role of each receptor more complex. Although there is a growing number of studies showing distinct biological evidences for PGD_2 receptors in experimental inflammation models, little is known about their role in human diseases, specifically in IBD.

In our studies, we attended to clarify the role of CTRH2 and DP in UC (1) and CD (2) by using human samples and two well-characterized experimental mouse colitis models [DSS-(1) and TNBS-colitis (2)], which bear good resemblance to their respective human disorders (186). To test the involvement of the two receptors in experimental IBD we used potent and specific pharmacological inhibitors (receptor antagonists) for CRTH2 {Cay10595 [5 mg/kg, s.c.; Cayman Chemicals] in DSS colitis (1) and OC-459 [0.1 mg/kg, s.c.; Cayman Chemicals] in TNBS colitis (2)} and for DP {MK0524 [1 mg/kg, s.c.; Cayman Chemicals] in DSS and TNBS colitis as well (1), (2)}. The applied concentrations of PGD_2 inhibitors were determined by us with dose-response relationships (Cay10595, *data not shown*) or based on the available literature (MK0524 (178) and OC-459 (180)). We found opposing roles for

CRTH2 and DP in UC and CD, i.e. CRTH2 activity seemed to induced inflammatory processes in both of our experimental colitis models while in contrast, DP activity seemed to block inflammation in colitic animals (1), (2).

2. The PGD_2 - $CRTH_2$ signaling axis is likely active in human IBD

First of all, we focused on CRTH2 and investigated the PGD_2 – CRTH2 signaling axis in IBD patients (1), (2). We determined prostanoid concentrations in the serum of CD (2) and UC patients (1) ***(Fig. 3 and 8.)***, and CRTH2 receptor expression in mucosal biopsies of the colon ***(Fig. 4 and 9.)***. Higher amounts of CRTH2 receptor protein (total protein) were detected in both UC ***(Fig. 4. B.)*** and CD ***(Fig. 9. B.)*** groups as compared to their respective CTRLs (1), (2), which is similar to previous findings by others (174), (164). But the question remained open, whether enhanced *de novo* receptor synthesis or simply the accumulation of CRTH2 proteins from migrated leukocytes had resulted in the increased colonic concentrations of the CRTH2 receptor. A previous study already showed a higher number of CRTH2 positive leukocytes (174) in the colon of UC patients as compared to CTRL subjects, whereas another study demonstrated a slight enhancement in CRTH2 mRNA levels in the colon of IBD patients; however these data were not statistically significant (164). In our Sirius Red staining in colon sections of CD patients we found an increased number of eosinophils in the colon of CD patients ***(Fig. 9. B.)*** (2), which are known to be CRTH2 positive (184). These facts let us hypothesize, that the CRTH2-positive leukocytes recruited into the colon, among them a high number of eosinophils, might be one of the main sources of PGD_2 receptors found in the inflamed colon.

If so, then the next question that arose was, if there were any changes in the expression of CRTH2 on the leukocyte cell surfaces in human IBD? Since the amount of eosinophils is enhanced in UC patients in comparison to CTRLs (187), ***(Fig. 9. B.)*** and since eosinophils are CRTH2-positive leukocytes, we isolated peripheral blood eosinophils from UC patients and healthy subjects and determined their level of CRTH2 expression, i.e. the amount of CRTH2 protein localized in the membrane of the cells ***(Fig. 4. C.)*** (1). We found a decreased receptor expression in the membrane of eosinophils isolated from UC patients in comparison to those of CTRLs (1). There might be at least two reasons for this findings: either a lower protein synthesis, or an active receptor internalization. As detailed in the *introduction*, CRTH2 is a G-protein coupled receptor (GPCR). GPCRs, after ligand-binding get desensitized, i.e. they are internalized upon prolonged activation. In eosinophils isolated from healthy subjects, active receptor internalization was detected after addition of CRTH2 agonists (PGD_2, DK-PGD_2, 11-dhTXB_2 [30 nM]), but not after eotaxin, which acts

independently of CRTH2 ***(Fig. 4. C.)*** (1). These results suggest that active CRTH2 signaling is likely going on in eosinophils during human ulcerative colitis.

Taken together, our findings of **I)** elevated CRTH2 receptor expression in the colon and **II)** active CRTH2 internalization in eosinophils (maybe in other CRTH2 positive leukocytes, such as TH2 cells, as well) suggest a role of CRTH2 in the pathomechanism of IBD.

But besides the receptor proteins, an activating ligand (agonist) is essentially needed for receptor signaling. Therefore, we assessed prostanoid concentrations (that include potent agonists of CRTH2 [PGD_2, 11-dh-TXB_2 or Δ^{12}-PGJ_2] and prostanoids, which metabolize to CRTH2 agonists (185) [PGD_2 metabolizes to Δ^{12}-PGJ_2; and TXB_2 metabolizes to 11-dh-TXB_2] in human sera. ***(Fig. 3 and 8.)***. Our measurements showed higher levels for PGD_2 and TXB_2 in UC patients (1), and higher levels of PGD_2, Δ^{12}-PGJ_2, TXB_2 and 11-dh-TXB_2 in CD patients (2), in comparison to their respective CTRLs. Therefore, we concluded, that prostanoids may be directly affected in IBD and assumed that an active PGD_2 – CRTH2 signaling axis was present in UC and CD patients.

One may ask whether there are any differences in prostanoid production between the UC and CD groups. Since blood sampling was not performed at the same time and also followed slightly different protocols, we did not carry out statistical analysis to compare UC and CD patients although an interesting trend seemed to be crystallizing. PGD_2 concentrations measured in the sera of UC and CD patients were found to be quite different ***(Fig. 3 and 8.)***. In CD, levels of PGD_2 were ~25x higher than in the CTRL cohort, with a concentration of about 25-30 ng/ml (2). In contrast, in UC, only a ~2x increase was noticed for PGD_2 as compared to CRTLs rising only to 0.10 – 0.15 ng/ml (1). Similarly, we detected higher concentrations of TXB_2 (which metabolizes to the CRTH2 agonist 11-dh-TXB_2) in CD patients (2) as compared to UC patients (1). Even PGE_2, which is thought to play an anti-inflammatory role in colitis (188) was found to be increased in CD (2). These tendencies let us assume, that prostanoids might be more implicated in the pathomechanism of CD than UC. It is important to note, that in a European prospective cohort study investigating the effect of regular aspirin intake on IBD, a clear positive correlation was found between CD development and aspirin intake but no correlation between usage of aspirin and the development of UC (189). These facts underline that the contribution of prostanoids to the disease pathophysiology of Crohn's disease may be more intense than in ulcerative colitis.

Our data indicate that new compounds against IBD which could specifically target pro-inflammatory prostaglandin receptors, but do not inhibit potential anti-inflammatory receptors of the same prostaglandin ligand (such as PGD_2), would be worth exploring. Thus,

we asked, if and which of the PGD_2 receptors, function as proinflammatory or anti-inflammatory receptors in experimental and human IBD and whether they could act in concert or even oppose each other.

3. CRTH2 and DP play opposing roles in experimental colitis

To answer these questions, we induced UC-like (1) experimental colitis in mice by applying DSS and CD-like (2) experimental colitis by applying TNBS (186) and evaluated the effects of CRTH2 and DP inhibitors ***(Fig. 5 and 10.)***. Since we surmised that prostanoids would be stronger involved in the pathomechanism of CD than in UC, we applied a CRTH2 antagonist, OC-459 (more potent than Cay10595) in our CD-like TNBS-model (2). As a DP inhibitor we used MK0524 (laropiprant) both in DSS- and TNBS-colitis (once or twice a day 1 mg/kg, s.c., depending on the colitis model used) (1), (2). After using a CRTH2 antagonist, we observed an overall improvement in colitic mice. Application of Cay10595 in DSS mice protected colonic tissue structure, inhibited weight loss, and decreased inflammation scores in comparison to DSS+VEH-treated animals ***(Fig. 5. A-B)***. In contrast to this, the DP antagonist MK0524 showed no effect on weight change, but enhanced the signs of colitis as observed by the high clinical scores when compared to the DSS+VEH cohort ***(Fig. 5. C.)***. Thus, in the DSS-induced (UC-like) experimental model, the CRTH2 inhibitor was found to be protective while the DP inhibitor amplified the severity of inflammation (1). This means, that CRTH2 must function as the proinflammatory PGD_2 receptor, while DP has anti-inflammatory effects in colitis. Similarly, the other CRTH2 antagonist, OC-459, protected colonic tissue in TNBS colitis, and decreased inflammation and inflammatory cytokine production (TNF-α, IL-1β and IL-6), in comparison with the TNBS+VEH-treated group ***(Fig. 10. A-B.)***. Also in the TNBS model, DP antagonism by MK0524 application resulted in higher inflammation scores ***(Fig. 10. C.)*** (2). Consequently, CRTH2 (and also DP) played an identical role in two different experimental colitis models.

To further evaluate signaling mechanisms of CRTH2 and DP and to shed light on possible interactions between these two receptors, we tested the effect of a combination therapy. By combination of DP and CRTH2 antagonists, DP antagonist MK0524 was found to eliminate OC-459's protective effect. Of course, this findings can be either the result of cumulative effects mediated by two totally independent receptors or through a direct interplay between CRTH2 and DP.

4. DP regulates CRTH2 signaling in eosinophils

To test a hypothetic interaction between CRTH2 and DP receptors, we performed cell migration experiments by antagonizing CRTH2 and DP, (alone or in combination) in isolated peripheral blood eosinophils. Eosinophils express both DP and CRTH2 receptors (190). We assessed (**I.**) basal activity of PGD_2 receptors (unstimulated cells), and (**II.**) stimulated PGD_2 receptor activity (single CRTH2 agonism (2) or CRTH2 / DP agonism)

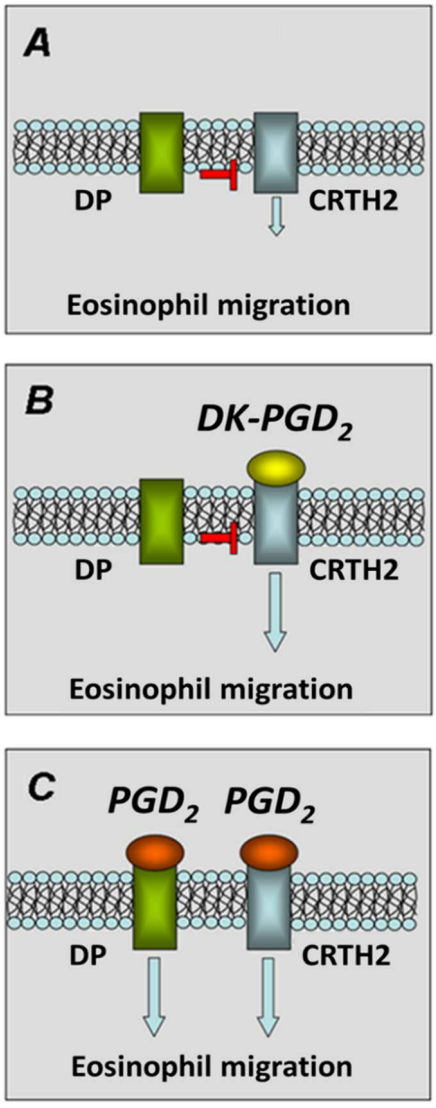

Figure. 14. **Hypothetical model for the interaction between DP and CRTH2.**
A. Without any activating ligands (intrinsic activity),
B. DK-PGD_2 activates CRTH2 alone (CRTH2 agonist),
C. PGD_2 activates CRTH2 and DP at the same time (CRTH2 and DP agonist).
[The figure was created by the author based on his own and co-authors results shown in Fig. 13. (Radnai B, Sturm EM et al. J Crohns Colitis, 2016 (2). The figure contains unpublished results]

In the first experimental setting we evaluated the basal activity of PGD_2 receptors by using CRTH2 (2) and DP antagonists without PGD_2 activation ***(Fig. 13. A.)***. From this first setting we concluded the following points:

1. CRTH2 enhances the migration of eosinophils without ligand binding, but it is permanently blocked by DP ***(Fig. 13. A. and 14. A)***.

2. DP inhibits the migration of eosinophils without ligand binding, because it continuously blocks CRTH2. We hypothesized that DP antagonist MK0524-treatment simply liberated CRTH2 from the constant inhibition by DP ***(Fig. 13. A.; MK)***.

3. Accordingly, under these circumstances, CRTH2 might be a migration activating receptor, while DP is only a regulator of CRTH2. We suggest, that in this situation, CRTH2 is a downstream target of DP ***(Fig. 14. A.)*****.**

4. Both CRTH2 and DP might be constitutively active. CRTH2 would spontaneously activate migration without ligands, but it is controlled by DP. Therefore, in vehicle-treated group of cells ***(Fig. 13. A.)***, no migration was detected, because DP permanently neutralizes CRTH2 activity. But the MK0524 treatment released the DP-induced inhibition and CRTH2 became active (without activating ligand!) ***(Fig. 13. A.; MK)***. Thus CRTH2 and DP must have been constitutively active in this system.

5. During combined inhibition of DP and CRTH2 by the DP antagonist MK0524 and CRTH2 antagonist OC-459, no significant migration was promoted ***(Fig. 13. A.; OC+MK)***. We hypothesized that CRTH2 must have been already blocked by its antagonist OC-459 and DP could not reverse this effect.

Furthermore, if the supposed mechanism is correct, and the receptors are constitutively active without ligands, then the used receptor antagonists should be termed as inverse agonists. As *Kenakin* summarized, the pharmacological definition, “inverse agonism” requires “agonist-independent receptor activity” and it is a “ligand-induced reversal of constitutive activity” (191). Both of the requirements seemed to be fulfilled in our *in vitro* system.

In the next setting, we investigated MK0524 and OC-459 during CRTH2 stimulation with the CRTH2 agonist DK-PGD_2 ***(Fig. 13. B.)***. Predictably, OC-459 inhibited CRTH2 agonist-induced migration of cells (2). MK0524 alone could not further enhance the migratory activity. From these results, we hypothesized, that CRTH2 was overall inhibited

by DP (intrinsic, agonist-independent activity), but this inhibition could be released upon DP antagonism (i.e. inhibition of DP lifts the inhibiting effect of DP on CRTH2, resulting in activation of CRTH2) ***(Fig. 14. B.)***. These findings support the idea, that CRTH2-induced migration could be regulated by DP.

In the case of CRTH2 and DP stimulation by PGD_2 ***(Fig. 13. C.)***, we assume that

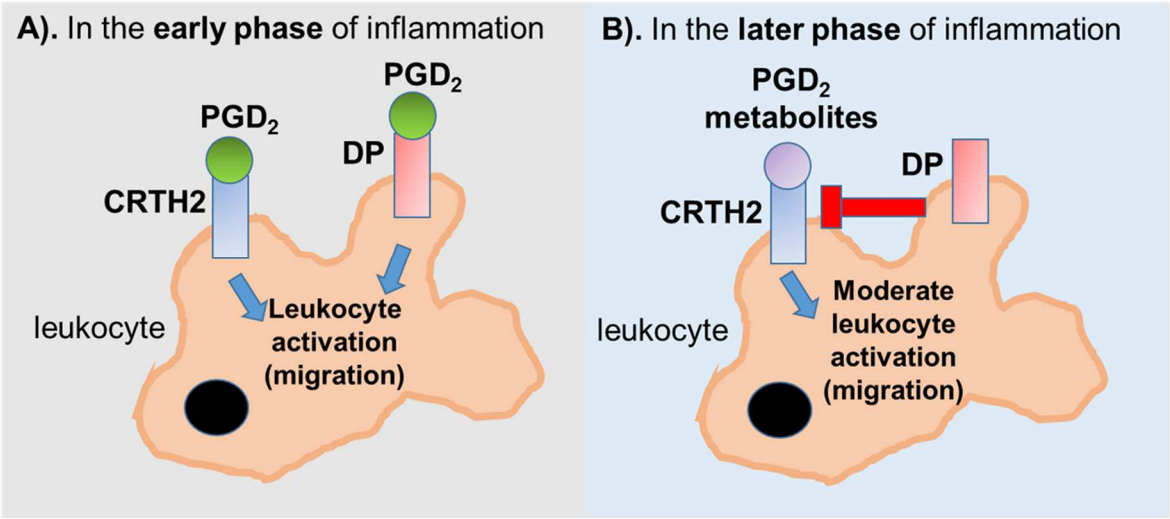

Figure. 15. **Hypothetical time-dependent effects of PGD_2 receptors.**
[The figure was created by the author based on the work of Pettipher R. et al, 2008 (192); and on our own published (1); (2) and unpublished result.]

the DP receptor, in its ligand-free and non-induced state, has a basal inhibitory activity on CRTH2, but after binding of a ligand (PGD_2), it may switch its function and turns on migration ***(Fig. 14. C)***.

Taken together, CRTH2, upon binding a ligand, seems to clearly activate eosinophil migration, whereas DP could both activate and inhibit migration depending on the presence of an activating ligand. In addition, inhibitory effects of DP on migration might be mediated through CRTH2. These facts raise the possibility of the presence of an active interaction between PGD_2 receptors *in vivo* as well. Thus, the clear proinflammatory effects demonstrated for CRTH2 in mice, might be modified by DP.

5. Hypothetical scenarios of PGD_2 receptor involvement in IBD

With keeping in mind that the above described interactions were investigated only in eosinophils in an *in vitro* setting we suggest the following **hypothetical scenarios** that may occur *in vivo* during IBD:

Our two different treatment-settings in the eosinophil migration assay, namely the PGD_2 treatment ***(Fig. 14. C)*** and the DK-PGD_2 treatment ***(Fig. 14. B)*** represent interactions between DP and CRTH2 that very likely also occur *in vivo*.

1. The first scenario (PGD_2 treatment) mimics ***an early (onset) phase of inflammation*** accompanied by a strong PGD_2 production (as we have seen in our human serum IBD samples) and leukocyte activation (1), (2). In this scenario, CRTH2 and DP might be both strongly activated and induce robust leukocyte activation (migration) ***(Fig. 15. A.)***.

2. But on the other hand, as *Pettipher et al.* summarized in their review, many of the PGD_2 metabolites seem to bind and activate rather CRTH2 than DP (192). Therefore, we additionally speculate, that in ***the later stages of inflammation***, when PGD_2 has been largely metabolized to more stable forms, CRTH2 ligands might dominate over DP ligands ***(Fig. 15. B.)***. This situation was meant to be imitated *in vitro* in our eosinophil migration assays by the specific CRTH2 activation (DK-PGD_2) ***(Fig. 13. B.)***, in which the ligand-free DP kept CRTH2 under control and inhibited its migratory activity. We further speculate, that in the early phases of intestinal inflammation, activation of CRTH2 and DP mainly by PGD_2, (imitated by PGD_2-treatment, ***(Fig. 13. C.)***) gives a strong push towards inflammation, but in the later phases, as the PGD_2 metabolites dominantly occur, they rather activate CRTH2 while DP takes a balancing effect over CRTH2 signaling to regulate uncontrolled inflammation ***(Fig. 13. B.)***.

Our next aim was to evaluate which step in the inflammatory process in the colon could have been affected by CRTH2 antagonism. Does the CRTH2 antagonist protect the colonic epithelial barrier or rather, does it regulate the infiltration of immune cells thereby influencing the immune response?

6. CRTH2 is not involved in the PGD_2-induced epithelial damage

Apart from mice, we detected CRTH2 positive cells also in human colonic epithelium ***(Fig. 4. A.)*** (1) and found that Caco-2 (human epithelial colorectal adenocarcinoma) cells also express CRTH2 ***(Fig. 6. A.)***. Therefore, we went on to investigate CRTH2 in Caco-2 epithelial cells. Incubation with PGD_2 induced cell death in Caco-2 cells in a concentration and time dependent manner ***(Fig. 6. B.)***; however, the CRTH2 antagonist Cay10595 had no effect on cell survival ***(Fig. 6. C.)***. Since reactive oxygen species are known to induce prostanoid production (*see introduction*, reactive oxygen species), we treated Caco-2 cells with H_2O_2 to induce oxidative stress and endogen prostaglandin production, however, Cay10595 failed to protect from ROS-induced cell death ***(Fig. 6. C.)***.

Thus, we concluded, that, at least in our hands, PGD_2-induced *in vitro* cell death is independent of CRTH2 activation. An explanation for PGD_2 toxicity in Caco-2 cells is offered by the literature. It could be shown, that 15d-PGJ_2, which is a metabolite of PGD_2 (193), induced cell death in SW480 and LS174T colon cancer cell lines via a peroxisome proliferator-activated receptor-γ (PPAR-γ) mediated way (194). PPAR-γ is a nuclear hormone receptor, which induces the transcription of many genes after ligand binding (195). It has been shown that 15d-PGJ_2 dose dependently inhibited cell proliferation 48 hrs after the treatment, and caused cell death, which could be reversed by PPAR-γ-inhibitor GW9662 (194). Since PGD_2 can metabolize into 15d-PGJ_2 and since Caco-2 cells were shown to possess PPAR-γ (196), this form of cell death is suggestive also for our experiments. In addition, the long time-interval (48 hrs) needed for the massive cell death argues for a transcription factor-(PPAR)-mediated way of action rather than a GPCR-mediated and rapid signaling action.

7. Leukocyte recruitment and CRTH2 antagonism in DSS- and in TNBS-induced experimental colitis

Because we could not observe any involvement of CRTH2 in the PGD_2-induced Caco-2 cell death, we focused on potential immunomodulating effects of CRTH2.

First we measured the effect of CRTH2 antagonists on the recruitment of leukocytes to the colon in our two different models of experimental colitis [DSS-induced ***(Fig. 7.)*** (1) and TNBS-induced ***(Fig. 11.)*** (2)].

Surprisingly, we detected two distinct sets of infiltrated leukocytes that were affected by the CRTH2 antagonism. In the DSS-colitis model, we found lower amounts of lymphocytes and neutrophils after treatment with Cay10595 than in vehicle-treated mice

and a slight tendency toward enhanced monocyte numbers. The extent of eosinophil infiltration in the DSS-colitis model was found to be unchanged between vehicle and Cay10595 treatment ***(Fig. 7.)*** (1). On the other hand, in TNBS-colitis, the only significant alteration in leukocyte infiltration was measured for eosinophils, which were found in lower amounts after OC-459 than vehicle treatment (2). The infiltration of other types of leukocytes was not significantly altered by the treatments ***(Fig. 11.)***.

That means, that by using CRTH2 antagonists in DSS- and TNBS-colitis, we detected different infiltration profiles of the same leukocytes. There might be at least three explanations for these differences:

- **C57BL/6 *vs* CD1:** First, since C57BL/6 mice are highly susceptible to DSS-induced colitis, but resistant to TNBS-induced colitis (197), we used a more appropriate mouse strain (CD1) for our TNBS experiments. It is obvious, that the mouse strains may differ in some parts of the immune response.

- **Cay10595 *vs* OC-459:** The second explanation could be that the CRTH2 antagonists caused different side effects, because two different pharmacological inhibitors (Cay10595 = 5-chloro-1'-[5-chloro-2-fluorophenyl)methyl]-2,2',5'-trioxo-spiro[3H-indole-3,3'pyrrolidine]-1(2H)-acetic acid *vs* OC-459 = 5-fluoro-2-methyl-3-(2-quinolinylmethyl)-1H-indole-1-acetic acid) were used. That means that the different chemical structures might have affected different cellular mechanisms, which remain unknown but might have influenced the antagonistic effect on CRTH2.

- **DSS *vs* TNBS:** On the other hand, if we assume that the inhibitors used were highly specific for CRTH2 and the mice strains did not differ significantly in their immune responses, thus, the only (but quite plausible) explanation for the differences in leukocyte infiltration could be based on the used colitis models (DSS *vs* TNBS). As outlined below the DSS-colitis model is based on an epithelial damage-induced bacterial infection while the TNBS-model is an allergen-induced model, which directly activates immunocytes ***(Fig. 16.)***.

8. Different inflammatory mechanisms of TNBS- and DSS-induced colitis

As *Chassaing B.* and colleagues summarized, DSS is toxic to the epithelial cells and causes strong erosions in the epithelium leading to enhanced epithelial permeability (198). In addition, because of the high amount of negative charges represented by the sulphate groups in the chemical structure of DSS, it inhibits blood coagulation and provokes bleeding. Thus, the DSS-induced inflammation is primarily induced by the epithelial damage and secondary by colonic microbes infiltrating the tissue ***(Fig. 16.)***. In contrast, as *Randhawa PK.* and colleagues summarized, TNBS does not cause direct tissue damage in the colon. It renders small microbial or colonic proteins immunogenic by haptenizing them, i.e. it is the protein/TNBS construct that induces an immune response, but not the protein or TNBS alone (199) ***(Fig. 16.)***.

In conclusion, in the DSS-induced colitis model, the primary epithelial damage

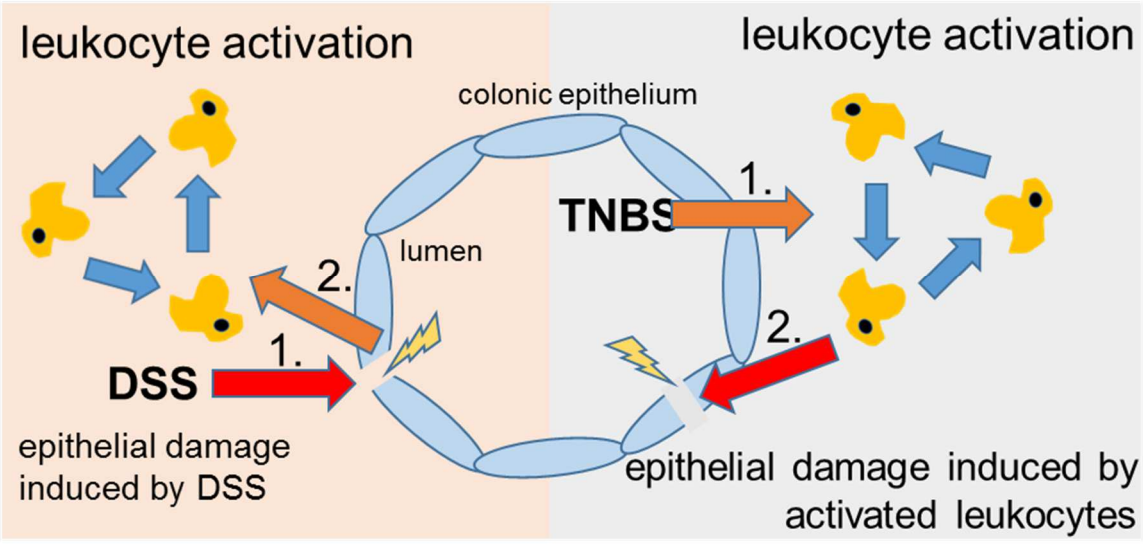

Figure. 16. **Inflammatory mechanisms induced by DSS and TNBS.**
[The figure was created by the author based on the work of Chassaing B. et al. 2014 (198) and Randhawa PK. et al. 2014 (199).]

induces bacterial infiltration and activation of the immune system, in contrast to this, in TNBS-colitis, the primarily activated immune cells cause harmful tissue injury ***(Fig. 16.)***. We hypothesize, that CRTH2 antagonism results in different infiltration profiles of the same leukocytes under different immunological milieus caused by the different models. Just like a single immune cell, which can act pro- or anti-inflammatory depending on a local cytokine milieu.

9. Eosinophils in experimental colitis

According to our experiments, eosinophils are possible targets of CRTH2 antagonists in TNBS-colitis (2), but less in DSS-colitis (1) ***(Fig. 7 and 11.)***. This leads to the question if and in what way are eosinophils involved in the pathomechanism of experimental colitis, and whether they act pro- or anti-inflammatory?

So far, conflicting results have been published in the literature concerning a pro/anti-inflammatory role of eosinophils in IBD. It has been previously demonstrated, that eosinophil peroxidase (EPO) deficient mice were protected in DSS-colitis, thus eosinophils might have an important proinflammatory role in IBD (200). In addition, CD34, which is expressed mainly on eosinophils infiltrating the colon, was found to be crucial for inducing DSS-colitis by mediating eosinophil recruitment, underlining the proinflammatory effects of eosinophils (201). Furthermore, Δdbl-GATA mice, which lack eosinophils, developed less severe signs of inflammation in DSS-colitis, and remarkably, these mice were completely protected against DSS-induced death (202). But in contrast, another type of eosinophil-depleted mice (PHIL-mice) was found to be more susceptible to colitis in the same DSS-model (203). The most obvious difference between the two eosinophil-depleted mice strains is the genetic background. Δdbl-GATA mice were on Balb/c background, whereas PHIL mice were on C57BL/6J background in these studies.

These conflicting data indicate the lack of our knowledge about eosinophils, which could obviously have both pro- and anti-inflammatory roles. Our own results confirmed a pro-inflammatory role of eosinophils in TNBS-colitis and not only in eosinophil-depleted Δdbl-GATA mice, but also in IL-5 transgenic mice, which have life-long eosinophilia ***(Fig. 12.)*** (2). Thus, we suggest, that the main mechanism by which OC-459 improves colitis, is inhibition of activation of proinflammatory eosinophils. However, the other CRTH2 antagonist, Cay10595, attenuated the infiltration of several important proinflammatory leukocytes, but it could not modify the number of eosinophils in the colon during DSS-colitis (1).

It is important to note that, according to our measurements, the participation of eosinophils in TNBS-colitis accounted for about ~9-10%of infiltrated leukocytes in the colon (2), whereas only for about 2-3% in the DSS-model (1). This difference (3-5x) might originate from at least three sources:

- A differential involvement of eosinophils in the pathomechanism of DSS- and TNBS-colitis.
- Leukocytes affect inflammation in the early and/or late-phases of inflammation differentially. Eosinophils might be more involved in the early phase of inflammation and demonstrate higher numbers in the more acute TNBS-inflammation (investigations were performed 3 days after induction) (2) than in DSS-inflammation (investigations were performed 7 days after the first DSS intake) (1).
- Of course it could have also happened, that Cay10595 actually inhibited eosinophil infiltration in DSS-colitis as well. But eosinophils may have already finished their action at the time of investigation. Their purpose might have already been fulfilled (i.e. they may have already degranulated) on the first few days of DSS challenge and eosinophils perhaps were not detectable/involved in the inflammation any more in the later phase.
- Haptenizing colonic and microbial proteins might induce allergy-like processes, which might involve a stronger eosinophil activation in TNBS-colitis in comparison to DSS-colitis.

10. Summary

• In conclusion, we have demonstrated, that pharmacological inhibition of CRTH2 is protective in DSS- or TNBS-induced experimental colitis in mice.

• Specific CRTH2 inhibitors, such as Cay10595 and OC-459 improved intestinal inflammation in mice, in the UC-like (DSS-colitis) and in the CD-like (TNBS-colitis) colitis models, respectively. Thus, CRTH2 is thought to act pro-inflammatory both in our experimental colitis models used.

• The cellular targets of the antagonists might be CRTH2-positive leukocytes, because the antagonists influenced leukocyte recruitment, however they resulted in different leukocyte-profiles in the inflamed colon.

• Colonic epithelial cells were found to be CRTH2-positive, the CRTH2-inhibition had no effects on PGD_2-induced cell death in a CRTH2-expressing colonic epithelial cell-line.

- Inhibitors of DP were not found to be protective in DSS- and TNBS-induced experimental colitis.

- The DP-inhibitor MK0524 was demonstrated to have pro-inflammatory effects in the colitis models used. Thus DP is thought to act as an anti-inflammatory receptor in experimental colitis.

- IBD patients produced higher amounts of prostaglandins (which also act as CRTH2-ligands).

- IBD patients displayed increased numbers of CRTH2 receptors in the colonic tissue (raising the possibility of an existence of an active PGD_2/CRTH2 signaling axis), thus, our findings point at a potential human relevance for CRTH2.

We propose, that pharmacological inhibition of CRTH2 may represent a novel therapeutical approach for future IBD therapy.

VIII. References

1. Sturm EM, Radnai B, Jandl K, Stančić A, Parzmair GP, Högenauer C, et al. Opposing roles of prostaglandin D2 receptors in ulcerative colitis. J Immunol Baltim Md 1950. 2014; 193(2):827–39.

2. Radnai B, Sturm EM, Stančić A, Jandl K, Labocha S, Ferreirós N, et al. Eosinophils Contribute to Intestinal Inflammation via Chemoattractant Receptor-homologous Molecule Expressed on Th2 Cells, CRTH2, in Experimental Crohn's Disease. J Crohns Colitis. 2016; 10(9):1087–95.

3. Zhang Y-Z, Li Y-Y. Inflammatory bowel disease: Pathogenesis. World J Gastroenterol WJG. 2014; 20(1):91–9.

4. M'Koma AE. Inflammatory Bowel Disease: An Expanding Global Health Problem. Clin Med Insights Gastroenterol. 2013; 6:33–47.

5. Molinié F, Gower-Rousseau C, Yzet T, Merle V, Grandbastien B, Marti R, et al. Opposite evolution in incidence of Crohn's disease and ulcerative colitis in Northern France (1988-1999). Gut. 2004; 53(6):843–8.

6. Fumery M, Dauchet L, Vignal C, Gower-Rousseau C. [Epidemiology, risk factors and factors associated with disabling course in inflammatory bowel disease]. Rev Prat. 2014; 64(9):1210–5.

7. Fernandes P, MacSharry J, Darby T, Fanning A, Shanahan F, Houston A, et al. Differential expression of key regulators of Toll-like Receptors in Ulcerative Colitis and Crohn's disease: A role for Tollip and PPARγ? Clin Exp Immunol. 2016; 183(3):358-68

8. Van Der Kraak L, Gros P, Beauchemin N. Colitis-associated colon cancer: Is it in your genes? World J Gastroenterol. 2015; 21(41):11688–99.

9. Kaplan GG. The global burden of IBD: from 2015 to 2025. Nat Rev Gastroenterol Hepatol. 2015; 12(12):720-7

10. Burisch J, Pedersen N, Čuković-Čavka S, Brinar M, Kaimakliotis I, Duricova D, et al. East-West gradient in the incidence of inflammatory bowel disease in Europe: the ECCO-EpiCom inception cohort. Gut. 2014; 63(4):588–97.

11. Hofmarcher MM, Quentin W. Austria: health system review. Health Syst Transit. 2013; 15(7):1–292.

12. Petritsch W, Fuchs S, Berghold A, Bachmaier G, Högenauer C, Hauer AC, et al. Incidence of inflammatory bowel disease in the province of Styria, Austria, from 1997 to 2007: a population-based study. J Crohns Colitis. 2013; 7(1):58–69.

13. Molodecky NA, Kaplan GG. Environmental Risk Factors for Inflammatory Bowel Disease. Gastroenterol Hepatol. 2010; 6(5):339–46.

14. Loddo I, Romano C. Inflammatory bowel disease: genetics, epigenetics, and pathogenesis. Mucosal Immun. 2015; 6:551.

15. Larmonier CB, Shehab KW, Ghishan FK, Kiela PR. T Lymphocyte Dynamics in Inflammatory Bowel Diseases: Role of the Microbiome. BioMed Res Int. 2015;2015:504638.

16. Peters CP, Mjösberg JM, Bernink JH, Spits H. Innate lymphoid cells in inflammatory bowel diseases. Immunol Lett. 2016; 172:124-31.

17. Sartor RB. Microbial Influences in Inflammatory Bowel Diseases. Gastroenterology. 2008; 134(2):577–94.

18. Rossen NG, MacDonald JK, de Vries EM, D'Haens GR, de Vos WM, Zoetendal EG, et al. Fecal microbiota transplantation as novel therapy in gastroenterology: A systematic review. World J Gastroenterol WJG. 2015; 21(17):5359–71.

19. Akiho H, Yokoyama A, Abe S, Nakazono Y, Murakami M, Otsuka Y, et al. Promising biological therapies for ulcerative colitis: A review of the literature. World J Gastrointest Pathophysiol. 2015; 6(4):219–27.

20. DeRoche TC, Xiao S-Y, Liu X. Histological evaluation in ulcerative colitis. Gastroenterol Rep. 2014; 2(3):178–92.

21. Grevenitis P, Thomas A, Lodhia N. Medical Therapy for Inflammatory Bowel Disease. Surg Clin North Am. 2015; 95(6):1159–82.

22. Kühn F, Klar E. Surgical Principles in the Treatment of Ulcerative Colitis. Viszeralmedizin. 2015; 31(4):246–50.

23. Randall CW, Vizuete JA, Martinez N, Alvarez JJ, Garapati KV, Malakouti M, et al. From historical perspectives to modern therapy: a review of current and future biological treatments for Crohn's disease. Ther Adv Gastroenterol. 2015; 8(3):143–59.

24. Campos FGMC de, Kotze PG. Burrill Bernard Crohn (1884-1983): the man behind the disease. ABCD Arq Bras Cir Dig São Paulo. 2013; 26(4):253–5.

25. Ha F, Khalil H. Crohn's disease: a clinical update. Ther Adv Gastroenterol. 2015; 8(6):352–9.

26. Martínez-Montiel MP, Casis-Herce B, Gómez-Gómez GJ, Masedo-González A, Yela-San Bernardino C, Piedracoba C, et al. Pharmacologic therapy for inflammatory bowel disease refractory to steroids. Clin Exp Gastroenterol. 2015; 8:257–69.

27. Büning C, Lochs H. Conventional therapy for Crohn's disease. World J Gastroenterol. 2006; 12(30):4794–806.

28. Klag T, Stange EF, Wehkamp J. Management of Crohn's disease - are guidelines transferred to clinical practice? United Eur Gastroenterol J. 2015; 3(4):371–80.

29. Ford AC, Kane SV, Khan KJ, Achkar J-P, Talley NJ, Marshall JK, et al. Efficacy of 5-aminosalicylates in Crohn's disease: systematic review and meta-analysis. Am J Gastroenterol. 2011; 106(4):617–29.

30. Kappelman MD, Rifas-Shiman SL, Porter C, Ollendorf DA, Sandler RS, Galanko JA, et al. Direct Health Care Costs of Crohn's Disease and Ulcerative Colitis in United States Children and Adults. Gastroenterology. 2008; 135(6):1907–13.

31. Crohn BBMD, Rosenberg HMD. The sigmoidoscopic picture of chronic ulcerative colitis (non-specific). J Med Sci. 1925; 170(2):220–7.

32. Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut. 2001; 48(4):526–35.

33. Low D, Mino-Kenudson M, Mizoguchi E. Recent advancement in understanding colitis-associated tumorigenesis. Inflamm Bowel Dis. 2014; 20(11):2115–23.

34. Salvo Romero E, Alonso Cotoner C, Pardo Camacho C, Casado Bedmar M, Vicario M. The intestinal barrier function and its involvement in digestive disease. Rev Esp Enfermedades Dig Organo Of Soc Esp Patol Dig. 2015; 107(11):686–96.

35. Viggiano D, Ianiro G, Vanella G, Bibbò S, Bruno G, Simeone G, et al. Gut barrier in health and disease: focus on childhood. Eur Rev Med Pharmacol Sci. 2015; 19(6):1077–85.

36. Robinson K, Deng Z, Hou Y, Zhang G. Regulation of the Intestinal Barrier Function by Host Defense Peptides. Vet Infect Dis. 2015; 2:57.

37. Michielan A, D'Incà R. Intestinal Permeability in Inflammatory Bowel Disease: Pathogenesis, Clinical Evaluation, and Therapy of Leaky Gut. Mediators Inflamm. 2015; 2015:628157.

38. Wang S, Liu K, Seneviratne CJ, Li X, Cheung GSP, Jin L, et al. Lipoteichoic acid from an Enterococcus faecalis clinical strain promotes TNF-α expression through the NF-κB and p38 MAPK signaling pathways in differentiated THP-1 macrophages. Biomed Rep. 2015; 3(5):697–702.

39. Płóciennikowska A, Hromada-Judycka A, Borzęcka K, Kwiatkowska K. Co-operation of TLR4 and raft proteins in LPS-induced pro-inflammatory signaling. Cell Mol Life Sci. 2015; 72:557–81.

40. Biedroń R, Peruń A, Józefowski S. CD36 Differently Regulates Macrophage Responses to Smooth and Rough Lipopolysaccharide. PloS One. 2016; 11(4).

41. Riquelme SA, Bueno SM, Kalergis AM. Carbon monoxide down-modulates Toll-like receptor 4/MD2 expression on innate immune cells and reduces endotoxic shock susceptibility. Immunology. 2015; 144(2):321–32.

42. Zou J, Shankar N. Roles of TLR/MyD88/MAPK/NF-κB Signaling Pathways in the Regulation of Phagocytosis and Proinflammatory Cytokine Expression in Response to E. faecalis Infection. PloS One. 2015; 10(8).

43. Veres B, Radnai B, Gallyas F, Varbiro G, Berente Z, Osz E, et al. Regulation of kinase cascades and transcription factors by a poly(ADP-ribose) polymerase-1 inhibitor, 4-hydroxyquinazoline, in lipopolysaccharide-induced inflammation in mice. J Pharmacol Exp Ther. 2004; 310(1):247–55.

44. Han L-P, Li C-J, Sun B, Xie Y, Guan Y, Ma Z-J, et al. Protective Effects of Celastrol on Diabetic Liver Injury via TLR4/MyD88/NF-κB Signaling Pathway in Type 2 Diabetic Rats. J Diabetes Res. 2016; 2016:2641248.

45. Zhang YQ, Zhang JJ, Song HJ, Li DW. Expression and prognostic influence of NF-κB and EGFR in esophageal cancer. Genet Mol Res GMR. 2015; 14(4):16819–26.

46. Li Z, Yang Z, Lapidus RG, Liu X, Cullen KJ, Dan HC. IKK phosphorylation of NF-κB at serine 536 contributes to acquired cisplatin resistance in head and neck squamous cell cancer. Am J Cancer Res. 2015; 5(10):3098–110.

47. Shafik NM, Mohamed DA, Bedder AE, El-Gendy AM. Significance of Tissue Expression and Serum Levels of Angiopoietin-like Protein 4 in Breast Cancer Progression: Link to NF-κB /P65 Activity and Pro-Inflammatory Cytokines. Asian Pac J Cancer Prev APJCP. 2015; 16(18):8579–87.

48. Jurjus A, Eid A, Al Kattar S, Zeenny MN, Gerges-Geagea A, Haydar H, et al. Inflammatory bowel disease, colorectal cancer and type 2 diabetes mellitus: The links. BBA Clin. 2016; 5:16–24.

49. Zhang J-M, An J. Cytokines, Inflammation and Pain. Int Anesthesiol Clin. 2007; 45(2):27–37.

50. Dinarello CA. Historical Review of Cytokines. Eur J Immunol. 2007; 37:S34–45.

51. Francescone R, Hou V, Grivennikov SI. Cytokines, IBD, and colitis-associated cancer. Inflamm Bowel Dis. 2015; 21(2):409–18.

52. Ślebioda TJ, Kmieć Z. Tumour necrosis factor superfamily members in the pathogenesis of inflammatory bowel disease. Mediators Inflamm. 2014; 2014:325129.

53. Rahbar Kafshboran H, Bonyadi M, Miri H, Haghi M, Nikravesh A, Abdolmohammadi R, et al. Association of TNF-α -857 Polymorphism with Inflammatory Bowel Disease in a Group of Iranian Azeri Individuals. Middle East J Dig Dis. 2014; 6(1):28–31.

54. Bonyadi M, Abdolmohammadi R, Jahanafrooz Z, Somy M-H, Khoshbaten M. TNF-alpha gene polymorphisms in Iranian Azari Turkish patients with inflammatory bowel diseases. Saudi J Gastroenterol Off J Saudi Gastroenterol Assoc. 2014; 20(2):108–12.

55. Kinoshita K, Hori M, Fujisawa M, Sato K, Ohama T, Momotani E, et al. Role of TNF-alpha in muscularis inflammation and motility disorder in a TNBS-induced colitis model: clues from TNF-alpha-deficient mice. Neurogastroenterol Motil Off J Eur Gastrointest Motil Soc. 2006; 18(7):578–88.

56. Wang K, Han G, Dou Y, Wang Y, Liu G, Wang R, et al. Opposite role of tumor necrosis factor receptors in dextran sulfate sodium-induced colitis in mice. PloS One. 2012; 7(12).

57. Ludwiczek O, Vannier E, Borggraefe I, Kaser A, Siegmund B, Dinarello CA, et al. Imbalance between interleukin-1 agonists and antagonists: relationship to severity of inflammatory bowel disease. Clin Exp Immunol. 2004; 138(2):323–9.

58. Vounotrypidis P, Kouklakis G, Anagnostopoulos K, Zezos P, Polychronidis A, Maltezos E, et al. Interleukin-1 associations in inflammatory bowel disease and the enteropathic seronegative spondylarthritis. Auto- Immun Highlights. 2013; 4(3):87–94.

59. Ng J, Hirota SA, Gross O, Li Y, Ulke-Lemee A, Potentier MS, et al. Clostridium difficile toxin-induced inflammation and intestinal injury are mediated by the inflammasome. Gastroenterology. 2010; 139(2):542–52.

60. Coccia M, Harrison OJ, Schiering C, Asquith MJ, Becher B, Powrie F, et al. IL-1β mediates chronic intestinal inflammation by promoting the accumulation of IL-17A

secreting innate lymphoid cells and CD4(+) Th17 cells. J Exp Med. 2012; 209(9):1595–609.

61. Ngoh EN, Weisser SB, Lo Y, Kozicky LK, Jen R, Brugger HK, et al. Activity of SHIP, Which Prevents Expression of Interleukin 1β, Is Reduced in Patients With Crohn's Disease. Gastroenterology. 2016; 150(2):465–76.

62. Zhang J, Fu S, Sun S, Li Z, Guo B. Inflammasome activation has an important role in the development of spontaneous colitis. Mucosal Immunol. 2014; 7(5):1139–50.

63. Korolkova OY, Myers JN, Pellom ST, Wang L, M'Koma AE. Characterization of Serum Cytokine Profile in Predominantly Colonic Inflammatory Bowel Disease to Delineate Ulcerative and Crohn's Colitides. Clin Med Insights Gastroenterol. 2015; 8:29–44.

64. Yamamoto M, Yoshizaki K, Kishimoto T, Ito H. IL-6 is required for the development of Th1 cell-mediated murine colitis. J Immunol Baltim Md 1950. 2000; 164(9):4878–82.

65. Kitamura K, Nakamoto Y, Kaneko S, Mukaida N. Pivotal roles of interleukin-6 in transmural inflammation in murine T cell transfer colitis. J Leukoc Biol. 2004; 76(6):1111–7.

66. Sommer J, Engelowski E, Baran P, Garbers C, Floss DM, Scheller J. Interleukin-6, but not the interleukin-6 receptor plays a role in recovery from dextran sodium sulfate-induced colitis. Int J Mol Med. 2014; 34(3):651–60.

67. Kuhn KA, Manieri NA, Liu T-C, Stappenbeck TS. IL-6 stimulates intestinal epithelial proliferation and repair after injury. PloS One. 2014; 9(12).

68. Grivennikov SI, Karin M. Inflammation and oncogenesis: a vicious connection. Curr Opin Genet Dev. 2010; 20(1):65–71.

69. Brighenti E, Calabrese C, Liguori G, Giannone FA, Trerè D, Montanaro L, et al. Interleukin 6 downregulates p53 expression and activity by stimulating ribosome biogenesis: a new pathway connecting inflammation to cancer. Oncogene. 2014; 33(35):4396–406.

70. Zhao G, Yu R, Deng J, Zhao Q, Li Y, Joo M, et al. Pivotal role of reactive oxygen species in differential regulation of lipopolysaccharide-induced prostaglandins production in macrophages. Mol Pharmacol. 2013; 83(1):167–78.

71. Allegra M, D'Acquisto F, Tesoriere L, Attanzio A, Livrea MA. Pro-oxidant activity of indicaxanthin from Opuntia ficus indica modulates arachidonate metabolism and prostaglandin synthesis through lipid peroxide production in LPS-stimulated RAW 264.7 macrophages. Redox Biol. 2014; 2:892–900.

72. Korbecki J, Baranowska-Bosiacka I, Gutowska I, Chlubek D. The effect of reactive oxygen species on the synthesis of prostanoids from arachidonic acid. J Physiol Pharmacol Off J Pol Physiol Soc. 2013; 64(4):409–21.

73. Pravda J. Radical induction theory of ulcerative colitis. World J Gastroenterol. 2005; 11(16):2371–84.

74. Halliwell B. Reactive Species and Antioxidants. Redox Biology Is a Fundamental Theme of Aerobic Life. Plant Physiol. 2006; 141(2):312–22.

75. Nathan C, Cunningham-Bussel A. Beyond oxidative stress: an immunologist's guide to reactive oxygen species. Nat Rev Immunol. 2013; 13(5):349–61.

76. Carneiro PP, Conceição J, Macedo M, Magalhães V, Carvalho EM, Bacellar O. The Role of Nitric Oxide and Reactive Oxygen Species in the Killing of Leishmania braziliensis by Monocytes from Patients with Cutaneous Leishmaniasis. PloS One. 2016;11(2).

77. Shen F, Tang X, Cheng W, Wang Y, Wang C, Shi X, et al. Fosfomycin enhances phagocyte-mediated killing of Staphylococcus aureus by extracellular traps and reactive oxygen species. Sci Rep. 2016; 6:19262.

78. Coady A, Xu M, Phung Q, Cheung TK, Bakalarski C, Alexander MK, et al. The Staphylococcus aureus ABC-Type Manganese Transporter MntABC Is Critical for Reinitiation of Bacterial Replication Following Exposure to Phagocytic Oxidative Burst. PloS One. 2015; 10(9).

79. Mateen S, Moin S, Khan AQ, Zafar A, Fatima N. Increased Reactive Oxygen Species Formation and Oxidative Stress in Rheumatoid Arthritis. PloS One. 2016;11(4).

80. Nesi RT, de Souza PS, Dos Santos GP, Thirupathi A, Menegali BT, Silveira PCL, et al. Physical exercise is effective in preventing cigarette smoke-induced pulmonary oxidative response in mice. Int J Chron Obstruct Pulmon Dis. 2016; 11:603–10.

81. Yu W, Zha W, Ke Z, Min Q, Li C, Sun H, et al. Curcumin Protects Neonatal Rat Cardiomyocytes against High Glucose-Induced Apoptosis via PI3K/Akt Signalling Pathway. J Diabetes Res. 2016; 2016:4158591.

82. Pietrofesa RA, Velalopoulou A, Albelda SM, Christofidou-Solomidou M. Asbestos Induces Oxidative Stress and Activation of Nrf2 Signaling in Murine Macrophages: Chemopreventive Role of the Synthetic Lignan Secoisolariciresinol Diglucoside (LGM2605). Int J Mol Sci. 2016; 17(3).

83. Udwan K, Brideau G, Fila M, Edwards A, Vogt B, Doucet A. Oxidative stress and NF-κB Increase Peritoneal Filtration and Contribute to Ascites Formation in Nephrotic Syndrome. J Biol Chem. 2016; 291(21):11105-13.

84. Turillazzi E, Neri M, Cerretani D, Cantatore S, Frati P, Moltoni L, et al. Lipid peroxidation and apoptotic response in rat brain areas induced by long-term administration of nandrolone: the mutual crosstalk between ROS and NF-kB. J Cell Mol Med. 2016; 20(4):601–12.

85. Fan H-Q, He W, Xu K-F, Wang Z-X, Xu X-Y, Chen H. FTO Inhibits Insulin Secretion and Promotes NF-κB Activation through Positively Regulating ROS Production in Pancreatic β cells. PloS One. 2015; 10(5).

86. Teppner M, Zell M, Husser C, Ernst B, Pähler A. Quantitative profiling of prostaglandins as oxidative stress biomarkers in vitro and in vivo by negative ion online solid phase extraction - Liquid chromatography-tandem mass spectrometry. Anal Biochem. 2016; 498:68–77.

87. Wang Q, Hou Y, Yi D, Wang L, Ding B, Chen X, et al. Protective effects of N-acetylcysteine on acetic acid-induced colitis in a porcine model. BMC Gastroenterol. 2013; 13:133.

88. Cetinkaya A, Bulbuloglu E, Kurutas EB, Ciralik H, Kantarceken B, Buyukbese MA. Beneficial effects of N-acetylcysteine on acetic acid-induced colitis in rats. Tohoku J Exp Med. 2005; 206(2):131–9.

89. Seril DN, Liao J, Ho K-LK, Yang CS, Yang G-Y. Inhibition of chronic ulcerative colitis-associated colorectal adenocarcinoma development in a murine model by N-acetylcysteine. Carcinogenesis. 2002; 23(6):993–1001.

90. Fiorentini C, Falzano L, Rivabene R, Fabbri A, Malorni W. N-acetylcysteine protects epithelial cells against the oxidative imbalance due to Clostridium difficile toxins. FEBS Lett. 1999; 453(1–2):124–8.

91. Guijarro LG, Mate J, Gisbert JP, Perez-Calle JL, Marin-Jimenez I, Arriaza E, et al. N-acetyl-L-cysteine combined with mesalamine in the treatment of ulcerative colitis: randomized, placebo-controlled pilot study. World J Gastroenterol. 2008; 14(18):2851–7.

92. Gryglewski RJ. Prostacyclin among prostanoids. Pharmacol Rep PR. 2008; 60(1):3–11.

93. Dennis EA, Norris PC. Eicosanoid storm in infection and inflammation. Nat Rev Immunol. 2015; 15(8):511–23.

94. Martin SA, Brash AR, Murphy RC. The Discovery and Early Structural Studies of Arachidonic Acid. J Lipid Res. 2016; 57(7)

95. Lenihan-Geels G, Bishop KS, Ferguson LR. Cancer Risk and Eicosanoid Production: Interaction between the Protective Effect of Long Chain Omega-3 Polyunsaturated Fatty Acid Intake and Genotype. J Clin Med. 2016; 5(2).

96. Smyth EM, Grosser T, Wang M, Yu Y, FitzGerald GA. Prostanoids in health and disease. J Lipid Res. 2009; 50:S423-428.

97. Smith T, Hutchison P, Schrör K, Clària J, Lanas A, Patrignani P, et al. Aspirin in the 21st century—common mechanisms of disease and their modulation by aspirin: a report from the 2015 scientific conference of the international aspirin foundation, 28 August, London, UK. ecancermedicalscience [Internet]. 2015; 9. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4631575/

98. Caughey GE, Cleland LG, Penglis PS, Gamble JR, James MJ. Roles of cyclooxygenase (COX)-1 and COX-2 in prostanoid production by human endothelial cells: selective up-regulation of prostacyclin synthesis by COX-2. J Immunol Baltim Md 1950. 2001; 167(5):2831–8.

99. Hamberg M, Svensson J, Samuelsson B. Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. Proc Natl Acad Sci U S A. 1975; 72(8):2994–8.

100. Yang W, Yan A, Zhang T, Shao J, Liu T, Yang X, et al. Thromboxane A2 Receptor Stimulation Enhances Microglial Interleukin-1β and NO Biosynthesis Mediated by the Activation of ERK Pathway. Front Aging Neurosci. 2016; 8:8.

101. Csató V, Pető A, Koller Á, Édes I, Tóth A, Papp Z. Hydrogen peroxide elicits constriction of skeletal muscle arterioles by activating the arachidonic acid pathway. PloS One. 2014; 9(8).

102. Smith JB, Ingerman C, Silver MJ. Persistence of thromboxane A2-like material and platelet release-inducing activity in plasma. J Clin Invest. 1976; 58(5):1119–22.

103. Lei X, Li Q, Rodriguez S, Tan SY, Seldin MM, McLenithan JC, et al. Thromboxane synthase deficiency improves insulin action and attenuates adipose tissue fibrosis. Am J Physiol Endocrinol Metab. 2015; 308(9):E792-804.

104. Wang M-J, Huang Y, Huang R-Y, Chen X-M, Zhou Y-Y, Yu W-L, et al. Determination of role of thromboxane A2 in rheumatoid arthritis. Discov Med. 2015; 19(102):23–32.

105. Sobolesky PM, Halushka PV, Garrett-Mayer E, Smith MT, Moussa O. Regulation of the tumor suppressor FOXO3 by the thromboxane-A2 receptors in urothelial cancer. PloS One. 2014; 9(9).

106. Rosenblum WI, El-Sabban F. Topical prostacyclin (PGI2) inhibits platelet aggregation in pial venules of the mouse. Stroke J Cereb Circ. 1979; 10(4):399–401.

107. Bayer BL, Blass KE, Förster W. Anti-aggregatory effect of prostacyclin (PGI2) in vivo. Br J Pharmacol. 1979; 66(1):10–2.

108. Lang IM, Gaine SP. Recent advances in targeting the prostacyclin pathway in pulmonary arterial hypertension. Eur Respir Rev Off J Eur Respir Soc. 2015; 24(138):630–41.

109. Zhang H, Li X, Huang J, Li H, Su Z, Wang J. Comparative Efficacy and Safety of Prostacyclin Analogs for Pulmonary Arterial Hypertension: A Network Meta-Analysis. Medicine (Baltimore). 2016; 95(4).

110. Koyama A, Fujita T, Gejyo F, Origasa H, Isono M, Kurumatani H, et al. Orally active prostacyclin analogue beraprost sodium in patients with chronic kidney disease: a randomized, double-blind, placebo-controlled, phase II dose finding trial. BMC Nephrol. 2015; 16:165.

111. Wang P, Guan P-P, Yu X, Zhang L-C, Su Y-N, Wang Z-Y. Prostaglandin I2 Attenuates Prostaglandin E2-Stimulated Expression of Interferon γ in a β-Amyloid Protein- and NF-κB-Dependent Mechanism. Sci Rep. 2016; 6:20879.

112. Klein T, Benders J, Roth F, Baudler M, Siegle I, Kömhoff M. Expression of Prostacyclin-Synthase in Human Breast Cancer: Negative Prognostic Factor and Protection against Cell Death In Vitro. Mediators Inflamm. 2015; 2015:864136.

113. Keith RL, Miller YE, Hudish TM, Girod CE, Sotto-Santiago S, Franklin WA, et al. Pulmonary prostacyclin synthase overexpression chemoprevents tobacco smoke lung carcinogenesis in mice. Cancer Res. 2004; 64(16):5897–904.

114. Euler US v. Über die Spezifische Blutdrucksenkende Substanz des Menschlichen Prostata- und Samenblasensekretes. Klin Wochenschr. 1935; 14(33):1182–3.

115. Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid Receptors: Structures, Properties, and Functions. Physiol Rev. 1999; 79(4):1193–226.

116. Smith WL. Prostanoid biosynthesis and mechanisms of action. Am J Physiol. 1992; 263(2 Pt 2):F181-191.

117. Le Loupp A-G, Bach-Ngohou K, Bourreille A, Boudin H, Rolli-Derkinderen M, Denis MG, et al. Activation of the prostaglandin D2 metabolic pathway in Crohn's disease: involvement of the enteric nervous system. BMC Gastroenterol. 2015; 15:112.

118. Kojima F, Frolov A, Matnani R, Woodward JG, Crofford LJ. Reduced T cell-dependent humoral immune response in microsomal prostaglandin E synthase-1 null mice is mediated by nonhematopoietic cells. J Immunol Baltim Md 1950. 2013; 191(10):4979–88.

119. Lee ES, Rasmussen CA, Filla MS, Slauson SR, Kolb AW, Peters DM, et al. Prospects for lentiviral vector mediated prostaglandin F synthase gene delivery in monkey eyes in vivo. Curr Eye Res. 2014; 39(9):859–70.

120. Jones R, Giembycz M, Woodward D. Prostanoid receptor antagonists: development strategies and therapeutic applications. Br J Pharmacol. 2009; 158(1):104–45.

121. Urade Y, Fujimoto N, Hayaishi O. Purification and characterization of rat brain prostaglandin D synthetase. J Biol Chem. 1985; 260(23):12410–5.

122. Urade Y, Eguchi N, Hayaishi O. Lipocalin-Type Prostaglandin D Synthase as an Enzymic Lipocalin [Internet]. Landes Bioscience; 2013. Available from: http://www.ncbi.nlm.nih.gov/books/NBK6621/

123. Joo M, Sadikot RT. PGD synthase and PGD2 in immune resposne. Mediators Inflamm. 2012; 2012:503128.

124. Eguchi Y, Eguchi N, Oda H, Seiki K, Kijima Y, Matsu-ura Y, et al. Expression of lipocalin-type prostaglandin D synthase (beta-trace) in human heart and its accumulation in the coronary circulation of angina patients. Proc Natl Acad Sci U S A. 1997; 94(26):14689–94.

125. Nagata N, Fujimori K, Okazaki I, Oda H, Eguchi N, Uehara Y, et al. De novo synthesis, uptake and proteolytic processing of lipocalin-type prostaglandin D synthase, beta-trace, in the kidneys. FEBS J. 2009; 276(23):7146–58.

126. Hokari R, Kurihara C, Nagata N, Aritake K, Okada Y, Watanabe C, et al. Increased expression of lipocalin-type-prostaglandin D synthase in ulcerative colitis and exacerbating role in murine colitis. Am J Physiol Gastrointest Liver Physiol. 2011; 300(3):G401-408.

127. Uehara Y, Makino H, Seiki K, Urade Y, L-PGDS Clinical Research Group of Kidney. Urinary excretions of lipocalin-type prostaglandin D synthase predict renal injury in type-2 diabetes: a cross-sectional and prospective multicentre study. Nephrol Dial Transplant Off Publ Eur Dial Transpl Assoc - Eur Ren Assoc. 2009; 24(2):475–82.

128. Ogawa M, Hirawa N, Tsuchida T, Eguchi N, Kawabata Y, Numabe A, et al. Urinary excretions of lipocalin-type prostaglandin D2 synthase predict the development of proteinuria and renal injury in OLETF rats. Nephrol Dial Transplant Off Publ Eur Dial Transpl Assoc - Eur Ren Assoc. 2006; 21(4):924–34.

129. Kanekiyo T, Ban T, Aritake K, Huang Z-L, Qu W-M, Okazaki I, et al. Lipocalin-type prostaglandin D synthase/beta-trace is a major amyloid beta-chaperone in human cerebrospinal fluid. Proc Natl Acad Sci U S A. 2007; 104(15):6412–7.

130. Inui T, Ohkubo T, Emi M, Irikura D, Hayaishi O, Urade Y. Characterization of the unfolding process of lipocalin-type prostaglandin D synthase. J Biol Chem. 2003; 278(5):2845–52.

131. Tanaka T, Urade Y, Kimura H, Eguchi N, Nishikawa A, Hayaishi O. Lipocalin-type Prostaglandin D Synthase (β-Trace) Is a Newly Recognized Type of Retinoid Transporter. J Biol Chem. 1997; 272(25):15789–95.

132. Elias E, Benrick A, Behre CJ, Ekman R, Zetterberg H, Stenlöf K, et al. Central nervous system lipocalin-type prostaglandin D2-synthase is correlated with orexigenic neuropeptides, visceral adiposity and markers of the hypothalamic-pituitary-adrenal axis in obese humans. J Neuroendocrinol. 2011; 23(6):501–7.

133. Ragolia L, Palaia T, Hall CE, Maesaka JK, Eguchi N, Urade Y. Accelerated glucose intolerance, nephropathy, and atherosclerosis in prostaglandin D2 synthase knock-out mice. J Biol Chem. 2005; 280(33):29946–55.

134. Evans JF, Islam S, Urade Y, Eguchi N, Ragolia L. The lipocalin-type prostaglandin D2 synthase knockout mouse model of insulin resistance and obesity demonstrates early hypothalamic-pituitary-adrenal axis hyperactivity. J Endocrinol. 2013; 216(2):169–80.

135. Saleem S, Shah ZA, Urade Y, Doré S. Lipocalin-prostaglandin D synthase is a critical beneficial factor in transient and permanent focal cerebral ischemia. Neuroscience. 2009; 160(1):248–54.

136. Coquenlorge S, Van Landeghem L, Jaulin J, Cenac N, Vergnolle N, Duchalais E, et al. The arachidonic acid metabolite 11β-ProstaglandinF2α controls intestinal epithelial healing: deficiency in patients with Crohn's disease. Sci Rep. 2016; 6:25203.

137. Jowsey IR, Thomson AM, Flanagan JU, Murdock PR, Moore GB, Meyer DJ, et al. Mammalian class Sigma glutathione S-transferases: catalytic properties and tissue-specific expression of human and rat GSH-dependent prostaglandin D2 synthases. Biochem J. 2001; 359(Pt 3):507–16.

138. Murata T, Aritake K, Tsubosaka Y, Maruyama T, Nakagawa T, Hori M, et al. Anti-inflammatory role of PGD2 in acute lung inflammation and therapeutic application of its signal enhancement. Proc Natl Acad Sci U S A. 2013; 110(13):5205–10.

139. Iwanaga K, Nakamura T, Maeda S, Aritake K, Hori M, Urade Y, et al. Mast cell-derived prostaglandin D2 inhibits colitis and colitis-associated colon cancer in mice. Cancer Res. 2014; 74(11):3011–9.

140. Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. Arterioscler Thromb Vasc Biol. 2011; 31(5):986–1000.

141. Sedej M, Schröder R, Bell K, Platzer W, Vukoja A, Kostenis E, et al. D-type prostanoid receptor enhances the signaling of chemoattractant receptor-homologous molecule expressed on T(H)2 cells. J Allergy Clin Immunol. 2012; 129(2):492–500, 500.e1-9.

142. Boie Y, Sawyer N, Slipetz DM, Metters KM, Abramovitz M. Molecular cloning and characterization of the human prostanoid DP receptor. J Biol Chem. 1995; 270(32):18910–6.

143. Kim ND, Luster AD. Regulation of Immune Cells by Eicosanoid Receptors. Sci World J. 2007; 7:1307–28.

144. Vong L, Ferraz JGP, Panaccione R, Beck PL, Wallace JL. A pro-resolution mediator, prostaglandin D2, is specifically up-regulated in individuals in long-term remission from ulcerative colitis. Proc Natl Acad Sci U S A. 2010; 107(26):12023–7.

145. Wright DH, Ford-Hutchinson AW, Chadee K, Metters KM. The human prostanoid DP receptor stimulates mucin secretion in LS174T cells. Br J Pharmacol. 2000; 131(8):1537–45.

146. Moreland RB, Nehra A, Kim NN, Min K-S, Albadawi H, Watkins MT, et al. Expression of functional prostaglandin D (DP) receptors in human corpus cavernosum smooth muscle. Int J Impot Res. 2002; 14(6):446–52.

147. Kanai K, Okano M, Fujiwara T, Kariya S, Haruna T, Omichi R, et al. Effect of prostaglandin D2 on VEGF release by nasal polyp fibroblasts. Allergol Int Off J Jpn Soc Allergol. 2016; 65(4):414-419

148. Fujita T, Narumiya S. Roles of hepatic stellate cells in liver inflammation: a new perspective. Inflamm Regen. 2016; 36:1.

149. Gervais FG, Cruz RP, Chateauneuf A, Gale S, Sawyer N, Nantel F, et al. Selective modulation of chemokinesis, degranulation, and apoptosis in eosinophils through the PGD2 receptors CRTH2 and DP. J Allergy Clin Immunol. 2001; 108(6):982–8.

150. Monneret G, Gravel S, Diamond M, Rokach J, Powell WS. Prostaglandin D2 is a potent chemoattractant for human eosinophils that acts via a novel DP receptor. Blood. 2001; 98(6):1942–8.

151. Nagata K, Tanaka K, Ogawa K, Kemmotsu K, Imai T, Yoshie O, et al. Selective expression of a novel surface molecule by human Th2 cells in vivo. J Immunol Baltim Md 1950. 1999; 162(3):1278–86.

152. Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, et al. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. J Exp Med. 2001; 193(2):255–61.

153. Kataoka N, Satoh T, Hirai A, Saeki K, Yokozeki H. Indomethacin inhibits eosinophil migration to prostaglandin D2 : therapeutic potential of CRTH2 desensitization for eosinophilic pustular folliculitis. Immunology. 2013; 140(1):78–86.

154. Davoine F, Sim A, Tang C, Fisher S, Ethier C, Puttagunta L, et al. Eosinophils in human oral squamous carcinoma; role of prostaglandin D2. J Inflamm Lond Engl. 2013; 10(1):4.

155. El-Shazly AE, Begon DY, Kustermans G, Arafa M, Dortu E, Henket M, et al. Novel association between vasoactive intestinal peptide and CRTH2 receptor in recruiting eosinophils: a possible biochemical mechanism for allergic eosinophilic inflammation of the airways. J Biol Chem. 2013; 288(2):1374–84.

156. Moon TC, Campos-Alberto E, Yoshimura T, Bredo G, Rieger AM, Puttagunta L, et al. Expression of DP2 (CRTh2), a prostaglandin D_2 receptor, in human mast cells. PloS One. 2014; 9(9).

157. Matsuoka T, Hirata M, Tanaka H, Takahashi Y, Murata T, Kabashima K, et al. Prostaglandin D2 as a Mediator of Allergic Asthma. Science. 2000; 287(5460):2013–7.

158. Spik I, Brénuchon C, Angéli V, Staumont D, Fleury S, Capron M, et al. Activation of the prostaglandin D2 receptor DP2/CRTH2 increases allergic inflammation in mouse. J Immunol Baltim Md 1950. 2005; 174(6):3703–8.

159. Jandl K, Stacher E, Bálint Z, Sturm EM, Maric J, Peinhaupt M, et al. Activated prostaglandin D2 receptors on macrophages enhance neutrophil recruitment into the lung. J Allergy Clin Immunol. 2016; 137(3):833–43.

160. Kanai K, Okano M, Fujiwara T, Kariya S, Haruna T, Omichi R, et al. Effect of prostaglandin D2 on VEGF release by nasal polyp fibroblasts. Allergol Int Off J Jpn Soc Allergol. 2016; 65(4):414-419.

161. Maicas N, Ibáñez L, Alcaraz MJ, Úbeda A, Ferrándiz ML. Prostaglandin D2 regulates joint inflammation and destruction in murine collagen-induced arthritis. Arthritis Rheum. 2012; 64(1):130–40.

162. Sarashina H, Tsubosaka Y, Omori K, Aritake K, Nakagawa T, Hori M, et al. Opposing immunomodulatory roles of prostaglandin D2 during the progression of skin inflammation. J Immunol Baltim Md 1950. 2014; 192(1):459–65.

163. Ma J, Yang Q, Wei Y, Yang Y, Ji C, Hu X, et al. Effect of the PGD2-DP signaling pathway on primary cultured rat hippocampal neuron injury caused by aluminum overload. Sci Rep. 2016; 6:24646.

164. Vong L, Ferraz JGP, Panaccione R, Beck PL, Wallace JL. A pro-resolution mediator, prostaglandin D(2), is specifically up-regulated in individuals in long-term remission from ulcerative colitis. Proc Natl Acad Sci U S A. 2010; 107(26):12023–7.

165. Zamuner SR, Bak AW, Devchand PR, Wallace JL. Predisposition to colorectal cancer in rats with resolved colitis: role of cyclooxygenase-2-derived prostaglandin d2. Am J Pathol. 2005; 167(5):1293–300.

166. Ajuebor MN, Singh A, Wallace JL. Cyclooxygenase-2-derived prostaglandin D(2) is an early anti-inflammatory signal in experimental colitis. Am J Physiol Gastrointest Liver Physiol. 2000; 279(1):G238-244.

167. Tsubosaka Y, Nakamura T, Hirai H, Hori M, Nakamura M, Ozaki H, et al. A deficiency in the prostaglandin D2 receptor CRTH2 exacerbates adjuvant-induced joint inflammation. J Immunol Baltim Md 1950. 2014; 193(12):5835–40.

168. Wojno EDT, Monticelli LA, Tran SV, Alenghat T, Osborne LC, Thome JJ, et al. The prostaglandin D_2 receptor CRTH2 regulates accumulation of group 2 innate lymphoid cells in the inflamed lung. Mucosal Immunol. 2015; 8(6):1313–23.

169. Bartemes KR, Kephart GM, Fox SJ, Kita H. Enhanced innate type 2 immune response in peripheral blood from patients with asthma. J Allergy Clin Immunol. 2014; 134(3):671–678.e4.

170. Straumann A, Hoesli S, Bussmann C, Stuck M, Perkins M, Collins LP, et al. Anti-eosinophil activity and clinical efficacy of the CRTH2 antagonist OC000459 in eosinophilic esophagitis. Allergy. 2013; 68(3):375–85.

171. Ishii M, Asano K, Namkoong H, Tasaka S, Mizoguchi K, Asami T, et al. CRTH2 is a critical regulator of neutrophil migration and resistance to polymicrobial sepsis. J Immunol Baltim Md 1950. 2012; 188(11):5655–64.

172. Satoh T, Moroi R, Aritake K, Urade Y, Kanai Y, Sumi K, et al. Prostaglandin D2 plays an essential role in chronic allergic inflammation of the skin via CRTH2 receptor. J Immunol Baltim Md 1950. 2006; 177(4):2621–9.

173. Shiraishi Y, Asano K, Nakajima T, Oguma T, Suzuki Y, Shiomi T, et al. Prostaglandin D2-induced eosinophilic airway inflammation is mediated by CRTH2 receptor. J Pharmacol Exp Ther. 2005; 312(3):954–60.

174. Matsuzaki K, Hokari R, Kato S, Tsuzuki Y, Tanaka H, Kurihara C, et al. Differential expression of CCR5 and CRTH2 on infiltrated cells in colonic mucosa of patients with ulcerative colitis. J Gastroenterol Hepatol. 2003; 18(9):1081–8.

175. Ma H-L, Goodwin D, Fish S, Napierata L, Morgan P, Page K, et al. CRTH2 is Critical to the Development of Colitis Induced by Dextran Sodium Sulfate (DSS). J Clin Cell Immunol [Internet]. 2012; 3:131.

176. Dignass A, Eliakim R, Magro F, Maaser C, Chowers Y, Geboes K, et al. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 1: definitions and diagnosis. J Crohns Colitis. 2012; 6(10):965–90.

177. Assche GV, Dignass A, Panes J, Beaugerie L, Karagiannis J, Allez M, et al. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Definitions and diagnosis. J Crohns Colitis. 2010; 4(1):7–27.

178. Cheng K, Wu T-J, Wu KK, Sturino C, Metters K, Gottesdiener K, et al. Antagonism of the prostaglandin D2 receptor 1 suppresses nicotinic acid-induced vasodilation in mice and humans. Proc Natl Acad Sci U S A. 2006; 103(17):6682–7.

179. Kimball ES, Wallace NH, Schneider CR, D'Andrea MR, Hornby PJ. Vanilloid receptor 1 antagonists attenuate disease severity in dextran sulphate sodium-induced colitis in mice. Neurogastroenterol Motil Off J Eur Gastrointest Motil Soc. 2004; 16(6):811–8.

180. Straumann A, Hoesli S, Bussmann C, Stuck M, Perkins M, Collins LP, et al. Anti-eosinophil activity and clinical efficacy of the CRTH2 antagonist OC000459 in eosinophilic esophagitis. Allergy. 2013; 68(3):375–85.

181. Schicho R, Bashashati M, Bawa M, McHugh D, Saur D, Hu H-M, et al. The atypical cannabinoid O-1602 protects against experimental colitis and inhibits neutrophil recruitment. Inflamm Bowel Dis. 2011; 17(8):1651–64.

182. Böhm E, Sturm GJ, Weiglhofer I, Sandig H, Shichijo M, McNamee A, et al. 11-Dehydro-thromboxane B2, a stable thromboxane metabolite, is a full agonist of chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2) in human eosinophils and basophils. J Biol Chem. 2004; 279(9):7663–70.

183. Liu F, Gonzalo JA, Manning S, O'Connell LE, Fedyk ER, Burke KE, et al. Pharmacological characterization of guinea pig chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). Prostaglandins Other Lipid Mediat. 2005; 76(1–4):133–47.

184. Fujishima H, Fukagawa K, Okada N, Takano Y, Tsubota K, Hirai H, et al. Prostaglandin D2 induces chemotaxis in eosinophils via its receptor CRTH2 and eosinophils may cause severe ocular inflammation in patients with allergic conjunctivitis. Cornea. 2005; 24:S66–70.

185. Schuligoi R, Sturm E, Luschnig P, Konya V, Philipose S, Sedej M, et al. CRTH2 and D-type prostanoid receptor antagonists as novel therapeutic agents for inflammatory diseases. Pharmacology. 2010; 85(6):372–82.

186. Alex P, Zachos NC, Nguyen T, Gonzales L, Chen T-E, Conklin LS, et al. Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. Inflamm Bowel Dis. 2009; 15(3):341–52.

187. Al-Haddad S, Riddell RH. The role of eosinophils in inflammatory bowel disease. Gut. 2005; 54(12):1674–5.

188. Montrose DC, Nakanishi M, Murphy RC, Zarini S, McAleer JP, Vella AT, et al. The role of PGE2 in intestinal inflammation and tumorigenesis. Prostaglandins Other Lipid Mediat. 2015; 116–117:26–36.

189. Chan SSM, Luben R, Bergmann MM, Boeing H, Olsen A, Tjonneland A, et al. Aspirin in the aetiology of Crohn's disease and ulcerative colitis: a European prospective cohort study. Aliment Pharmacol Ther. 2011; 34(6):649–55.

190. Schratl P, Royer JF, Kostenis E, Ulven T, Sturm EM, Waldhoer M, et al. The role of the prostaglandin D2 receptor, DP, in eosinophil trafficking. J Immunol Baltim Md 1950. 2007; 179(7):4792–9.

191. Kenakin T. Principles: receptor theory in pharmacology. Trends Pharmacol Sci. 2004; 25(4):186–92.

192. Pettipher R. The roles of the prostaglandin D(2) receptors DP(1) and CRTH2 in promoting allergic responses. Br J Pharmacol. 2008; 153:S191-199.

193. Scher JU, Pillinger MH. 15d-PGJ2: the anti-inflammatory prostaglandin? Clin Immunol Orlando Fla. 2005; 114(2):100–9.

194. Shen D, Deng C, Zhang M. Peroxisome proliferator-activated receptor gamma agonists inhibit the proliferation and invasion of human colon cancer cells. Postgrad Med J. 2007; 83(980):414–9.

195. Dou X, Xiao J, Jin Z, Zheng P. Peroxisome proliferator-activated receptor-γ is downregulated in ulcerative colitis and is involved in experimental colitis-associated neoplasia. Oncol Lett. 2015; 10(3):1259–66.

196. Wächtershäuser A, Loitsch SM, Stein J. PPAR-gamma is selectively upregulated in Caco-2 cells by butyrate. Biochem Biophys Res Commun. 2000; 272(2):380–5.

197. te Velde AA, Verstege MI, Hommes DW. Critical appraisal of the current practice in murine TNBS-induced colitis. Inflamm Bowel Dis. 2006; 12(10):995–9.

198. Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran sulfate sodium (DSS)-induced colitis in mice. Curr Protoc Immunol. 2014; 104:15.25.

199. Randhawa PK, Singh K, Singh N, Jaggi AS. A review on chemical-induced inflammatory bowel disease models in rodents. Korean J Physiol Pharmacol Off J Korean Physiol Soc Korean Soc Pharmacol. 2014; 18(4):279–88.

200. Forbes E, Murase T, Yang M, Matthaei KI, Lee JJ, Lee NA, et al. Immunopathogenesis of experimental ulcerative colitis is mediated by eosinophil peroxidase. J Immunol Baltim Md 1950. 2004; 172(9):5664–75.

201. Maltby S, Wohlfarth C, Gold M, Zbytnuik L, Hughes MR, McNagny KM. CD34 is required for infiltration of eosinophils into the colon and pathology associated with DSS-induced ulcerative colitis. Am J Pathol. 2010; 177(3):1244–54.

202. Vieira AT, Fagundes CT, Alessandri AL, Castor MGM, Guabiraba R, Borges VO, et al. Treatment with a novel chemokine-binding protein or eosinophil lineage-ablation protects mice from experimental colitis. Am J Pathol. 2009; 175(6):2382–91.

203. Masterson JC, McNamee EN, Fillon SA, Hosford L, Harris R, Fernando SD, et al. Eosinophil-mediated signalling attenuates inflammatory responses in experimental colitis. Gut. 2015; 64(8):1236–47.

IX. Appendix

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June 29, 2017

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