

Dissertation

**Cannabinoid receptor 2 activation as a novel priming
factor for human and mouse eosinophils**

submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

Abstract

Background and Methods:

Allergic inflammation is characterized by an accumulation of activated eosinophils in the affected tissue. Recently, the endocannabinoid system, via activation of CB₂ by the endogenous ligand 2-arachidonoylglycerol was shown to induce migration of human eosinophils. However, further reports identified several other mechanisms like cytokine priming and metabolism of 2-AG into eicosanoids that might contribute to this effect. Thus, in this study I focused on the contribution of selective CB₂ activation by the agonist JWH-133 on human and mouse eosinophil function *in vitro* and *in vivo*.

Eosinophil responsiveness was studied in assays of adhesion, migration, respiratory burst, degranulation and Ca²⁺ release. Furthermore, potentially altered CB₂ expression under allergic condition was analyzed. The contribution of the endocannabinoid system to allergic airway inflammation was assessed in established mouse models.

Results and Conclusion:

CB₂ expression was significantly enhanced in eosinophils of allergic donors with respiratory symptoms, compared to healthy control. Selective activation of CB₂ only induced a moderate migratory response in human eosinophils but greatly enhanced the effects of chemoattractants on shape change, migration, expression of surface integrins, adhesion and reactive oxygen species production. Using the selective cannabinoid receptor 2 antagonist SR144528 I could confirm that these effects are indeed mediated by CB₂ activation rather than eicosanoid metabolism. Systemic application of JWH-133 in a setting of directed eosinophil migration and ovalbumin-induced allergic asthma in mice clearly elevated eosinophil influx into the airways and airway hyperresponsiveness in a CB₂ dependent manner. Interestingly the latter effect was completely absent in eosinophil-deficient Δ dblGATA mice.

To summarize, the data obtained clearly demonstrates that activation of CB₂ on eosinophils represents a novel priming mechanisms and may therefore contribute to the pathogenesis of eosinophil driven diseases. Furthermore, it provides new details on the molecular machinery associated with CB₂ induced priming. Concluding, antagonism of CB₂ might represent an interesting molecular target for the treatment of allergic inflammation.

Zusammenfassung

Hintergrund und Methoden:

Entzündung im Rahmen einer Allergie ist durch die Einwanderung und Anreicherung aktivierter eosinophiler Granulozyten charakterisiert. Kürzlich konnte gezeigt werden, dass das Endocannabinoidsystem, über Aktivierung von CB₂ durch den endogenen Liganden 2-AG, eine Rolle bei der Rekrutierung von humanen eosinophilen Granulozyten spielt. Später veröffentlichte Studien zeigten allerdings, dass mehrere Prozesse, unter anderem Voraktivierung durch Zytokine sowie Metabolisierung von 2-AG zu Arachidonsäure und letztendlich zu Eicosanoiden daran beteiligt sind. In dieser Studie wurde der Fokus auf die potentielle Beteiligung einer selektiven CB₂ Aktivierung und ihre Folgen auf die Funktion eosinophiler Granulozyten gesetzt.

Eosinophile Granulozyten wurden in verschiedenen Experimenten auf Adhäsion, Chemotaxis, Produktion von reaktiven Sauerstoffradikalen (ROS), Degranulierung sowie Ca²⁺ Freisetzung untersucht. Des Weiteren wurde eine mögliche Änderung der CB₂ Expression durch eine Allergie oder allergisches Asthma untersucht. Zuletzt wurde die Beteiligung des Endocannabinoidsystems an der allergischen Entzündung der Atemwege in etablierten Mausmodellen erforscht.

Ergebnisse und Zusammenfassung:

Es konnte gezeigt werden, dass die Expression von CB₂ auf Eosinophilen von Allergikern mit respiratorischen Symptomen, gegenüber Gesunden signifikant erhöht ist. Selektive Aktivierung von CB₂ Rezeptoren führte zu einer mäßigen chemotaktischen Antwort, konnte aber die Effekte von Chemokinen wie Eotaxin-2 auf die Aktivierung, Chemotaxis, Integrinexpression, Adhäsion sowie Produktion von ROS signifikant erhöhen. Durch den Einsatz des selektiven CB₂ Antagonisten SR144528 konnten diese beobachteten Effekte auf die Aktivierung des Rezeptors zurückgeführt werden. Metabolisierung und Eicosanoidproduktion konnte ausgeschlossen werden, da JWH-133 kein Substrat für MAGL ist. Des Weiteren konnte im Asthma-Modell gezeigt werden, dass systemische Verabreichung von JWH-133 zu einer erhöhten Einwanderung von eosinophilen Granulozyten in die Luftwege führt. Zusammenfassend zeigen die Resultate dieser Studie, dass Aktivierung von CB₂ einen bis jetzt unbekannten „Priming-Mechanismus“ darstellt und deshalb möglicherweise an der Pathogenese allergischer sowie eosinophiler

Erkrankungen beteiligt ist. Weiters konnte ein Überblick über die durch die CB₂ Aktivierung induzierten molekularen Mechanismen gegeben werden. Abschließend wurde gezeigt, dass Antagonismus von CB₂ ein mögliches neues Ziel in der Behandlung allergischer Entzündung darstellen könnte.

Abbreviations

<i>Abbreviation</i>	<i>Definition</i>
2-AG	2-arachidonoylglycerol
AA	arachidonic acid
AEA	anandamide
AHR	airway hyperresponsiveness
BAL	bronchoalveolar lavage
C5a	complement factor 5a
cAMP	cyclic adenosine monophosphate
CB	cannabinoid receptor
CI	chemotactic index
COX-1, 2	cyclooxygenase-1, 2
CRTH2	chemoattractant receptor homologous model expressed on T _h 2 cells
cysLTs	cysteinyl leukotrienes
eCB	endocannabinoids
ECM	extracellular matrix
ECP	eosinophil cationic protein
ED	eosinophil-derived neurotoxin
EGF	epidermal growth factor
EPO	eosinophil peroxidase
ERK	extracellular signal regulated kinase
E-selectin	endothelial-selectin

FAAH	fatty acid amid hydrolase
FSC	forward scatter
G-CSF	granulocyte-colony stimulating factor
GPCR	G-protein coupled receptor
hEoPs	eosinophil lineage-committed progenitor population
ICAM-1	intercellular cell adhesion molecule-1
IgE	immunoglobulin E
IL	Interleukin
IP3	inositol triphosphate
LOX	lipoxygenase
MAC-1	macrophage-1 antigen, CD11b/CD18
MAGL	monoacylglycerol lipase
MAPK	mitogen-activated protein kinase
MAPK	mitogen-activated protein kinases
MBP	major basic protein
MCP	monocyte chemotactic protein
OVA	ovalbumin
PAF	platelet-activating factor
PAMPS	pathogen-associated molecular patterns
PECAM-1	platelet-endothelial cell adhesion molecule-1
PGD ₂	prostaglandin D ₂
PGE ₂	prostaglandin E ₂
PI3K	phosphoinositide 3-kinase
PLC	phospholipase C

PSGL-1	P-selectin glycoprotein ligand 1
PTX	pertussis toxin
ROS	reactive oxygen species
SSC	side scatter
Th ₂	T-helper cell type 2
THC	Δ^9 -tetrahydrocannabinol
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4

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Introduction

I. Eosinophils

The scientific history of the eosinophil goes back to the early 19th century where they were first described by Wharton Jones. The term “Eosinophil” was coined 30 years later by Paul Ehrlich who first recognized the intense staining of their granules with the dye eosin (Gleich and Adolphson, 1986). This simple but specific staining technique for eosinophils was deployed in blood analysis and gave the first clues of eosinophil involvement in asthma and parasitic diseases. Subsequent studies extended the knowledge about eosinophils and they are now considered multifunctional leukocytes involved in the modulation of the adaptive immune system, tissue homeostasis, and innate immunity to certain microbes. Furthermore they are efficient producers of cytokines and participate actively in Th₂-immune responses (Kita, 2011).

Life Cycle

Blood eosinophil numbers are tightly regulated and comprise 1-3% of the total leukocytes under healthy conditions (Spry, 1988). Their life cycle can be divided into 3 phases depending on their site of residency which are the bone marrow, blood, intestine and other tissues. The first phase in the life cycle of an eosinophil is its production or eosinophilopoiesis, which takes mostly part in the bone marrow, although there are some exceptions during inflammatory diseases (Rådinger and Lötvall, 2009), and is regulated via the transcription factors GATA-1, PU.1, C/EBP and FOG-1 (McNagny and Graf, 2002). Gene expression studies have shown that this event is highly dependent on the balance of those factors, where subtle disruptions change the cells either to a myeloid or erythroid destiny (Akashi et al., 2000). Previously it was thought that human eosinophils develop from the granulocyte/macrophage progenitor cell but recent studies identified a stem cell-derived CD34⁺, interleukin-5 receptor subunit alpha (IL-5RA)⁺ eosinophil lineage-committed progenitor population (hEoPs) (Mori et al., 2009). Additionally to IL-5RA

hEoPs express IL-3RA and granulocyte-macrophage colony stimulating factor receptor subunit alpha (GM-CSF-R-alpha) that compose the receptors for the cytokines granulocyte macrophages colony stimulating factor (GM-CSF), IL-3 and IL-5 which are subsequently regulating eosinophil differentiation (Ackerman and Du, 2013). GM-CSF and IL-3 are multipotent cytokines acting on many myeloid lineages and are required for the initial expansion of the EoP pool, whereas IL-5 is required in promoting eosinophil lineage-committed EoP cell proliferation and terminal differentiation (Clutterbuck and Sanderson, 1990).

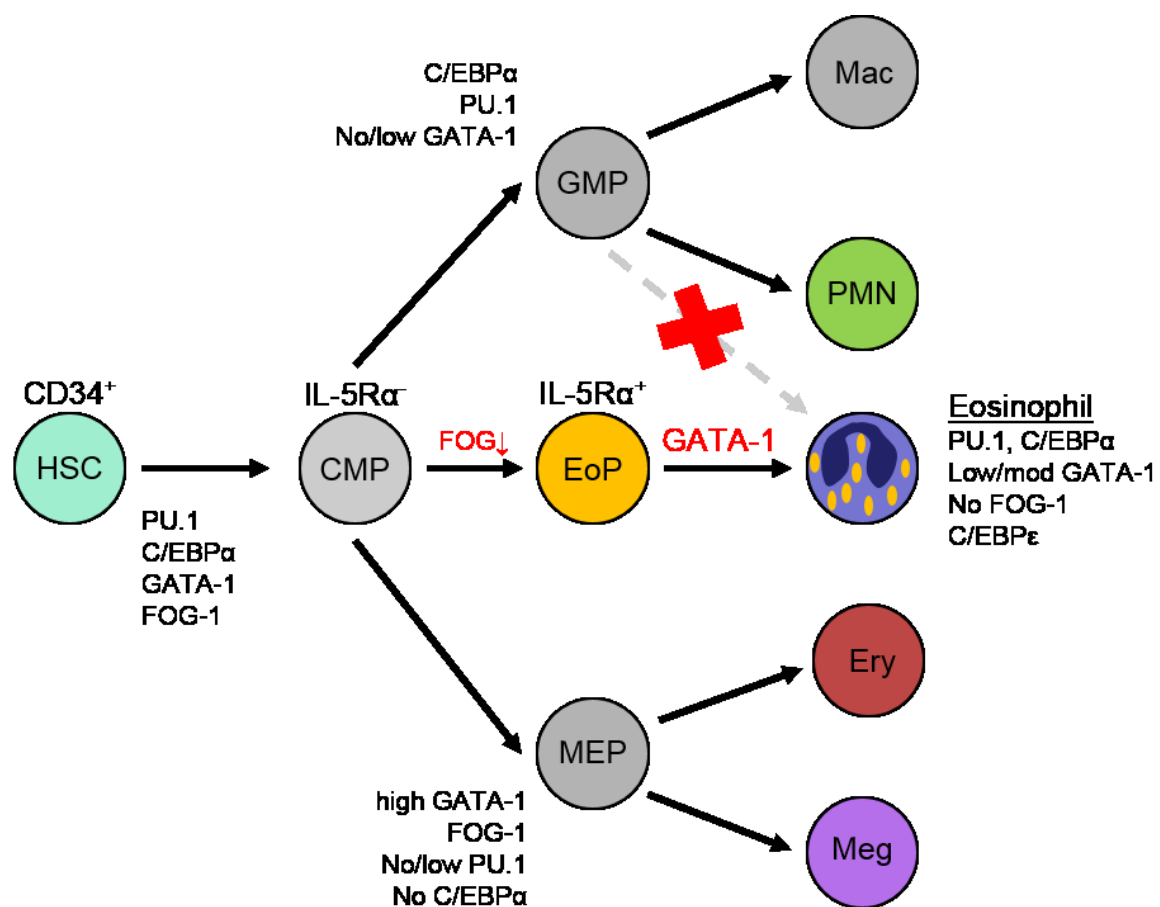


Figure I-1. Action of transcription factors required for eosinophil lineage commitment and terminal differentiation. Modified from (McNagny and Graf, 2002).

Finally, mature eosinophils approach the second phase of their life cycle and enter the circulation, where they reside for a mean blood transit time of about 26 hours (Steinbach et al., 1979) before migrating to their target tissue.

Eosinophil Trafficking

Under healthy conditions eosinophils reside mostly the gastrointestinal tract except the esophagus, mammary glands, uterus or the thymus and other lymphoid organs (Mishra et al., 1999) but they can be recruited to any other organ under parasitic or allergic inflammatory disease states. This recruitment is achieved by the tight interaction of cytokines, eosinophil activating chemokines and adhesion molecules and their respective ligands (Blanchard and Rothenberg, 2009). Moreover, it involves a complex multistage cascade which can be divided into following processes (Zimmermann and Zhu, 2013)

- (1) Priming
- (2) Rolling along endothelial cells
- (3) Firm adhesion to the endothelium
- (4) Transendothelial diapedesis
- (5) Chemotaxis to the inflammatory site

Priming

The response of leukocytes is primed when a stimulus, e.g. cytokines, does not cause the response by itself but amplifies the response to other subsequent stimuli (Koenderman, 2013). Regarding eosinophils several soluble priming factors have been described including chemotactic lipids (platelet activating factor, PAF), chemokines (eotaxin), cytokines like GM-CSF, IL3 and IL-5 as wells as complement factors and pathogen-associated molecular patterns (PAMPS). On the one hand they facilitate responses like tissue homing, adhesion, migration and chemotaxis, but on

the other hand they are powerful primers of phagocytosis, reactive oxygen species (ROS) production and degranulation (Coffer and Koenderman, 1997; Koenderman et al., 1996). Previously it was also shown that the process of adhesion, especially binding of $\beta 2$ integrins can induce priming (Kaneko et al., 1995). But not only soluble factors and adhesion can evoke primed responses in eosinophils but also environmental factors, although still poorly defined, have been shown to cause priming during isolation and *in vitro* handling (Koenderman, 2013). In disease states it has been reported that eosinophils in the peripheral blood of allergic asthmatics are primed in the context of adhesion/chemotaxis (Håkansson et al., 1997; Warringa et al., 1992), ROS production (Sannohe et al., 2003), degranulation (Carlson et al., 1992) and cytokine release (Koenderman, 2013)(Koenderman, 2013). Therefore, it can be concluded that priming is a critical regulatory step not only in the distribution of eosinophils but also in the activation of cytotoxic responses under inflammatory conditions (Koenderman, 2013).

Rolling along endothelial cells

Rolling of eosinophils along endothelial cells is mainly a selectin-mediated process but also involves the integrin $\alpha 4\beta 1$ in its low affinity state (Ebnet et al., 1996). Selectins are glycoproteins that consist of multiple domains: two or more homologous to repeats found in complement component 3/4, one homologous to EGF (epidermal growth factor) and one homologous to lectins. Studies done with freshly isolated eosinophils revealed that the expressing of L-selectin (CD-62L) plays an important role in the rolling of eosinophils through carbohydrate binding (Matsumoto and Bochner, 2013a). The attachment to carbohydrates also increases the accessibility of proteoglycan-bound chemokines, creating gradients on the endothelium and in the tissue interstitium (Shikata et al., 1999). Another important mechanism of eosinophil rolling is the P-Selectin CD162 [P-selectin glycoprotein ligand 1 (PSGL-1)] interaction. On activated endothelial cells, P-Selectin is rapidly upregulated on the cell surface via various cytokines including TNF- α and also the TH₂ cytokine IL-13. Interestingly, the IL-13-induced upregulation of P-selectin enhances eosinophil but

not neutrophil attachment to the vessel wall, although they express similar amounts of PSGL-1 on their cell surface. Thus, this capability of eosinophil-specific recruitment is thought to be a key event in allergic diseases (Woltmann et al., 2000).

Firm adhesion to the endothelium

Eosinophil rolling on the endothelium is followed by firm adhesion through integrins. They consist of two non-covalently bound transmembrane glycoprotein subunits called α and β , which are both contributing to the binding function (Matsumoto and Bochner, 2013a). Upon binding to extracellular matrix (ECM) proteins or other cells, integrins can mediate the assembly of actin filaments and thereby regulate morphological changes by their talin and α -actinin binding sites located on the intracellular tail of the β subunit. Up to now, eosinophils have been reported to express seven integrin molecules: $\alpha 4\beta 1$, $\alpha 6\beta 1$, $\alpha L\beta 2$, $\alpha M\beta 2$, $\alpha X\beta 2$, $\alpha D\beta 2$ and $\alpha 4\beta 7$. Binding to vascular cell adhesion molecule-1 (VCAM-1) and fibronectin (FN), an ECM protein, is mediated by the integrin $\alpha 4\beta 1$ (Matsumoto and Bochner, 2013a). The binding to laminin (LN), an ECM protein that plays a part in eosinophilopoiesis, is $\alpha 6\beta 1$ dependent (Georas et al., 1993). $\alpha L\beta 2$, $\alpha M\beta 2$ and $\alpha X\beta 2$ show high binding affinity to intercellular adhesion molecule 1 (ICAM-1) and are involved in the transendothelial migration of eosinophils (Ebisawa et al., 1992; Matsumoto and Bochner, 2013a). $\alpha D\beta 2$ binds to VCAM-1 and ICAM-3 (Grayson et al., 1998), whereas various eosinophil functions depend on the binding of $\alpha M\beta 2$ to ECM proteins like fibrinogen and complement factors (Horie and Kita, 1994; Kaneko et al., 1995; Kato et al., 2000; Matsumoto and Bochner, 2013a). Lastly, $\alpha 4\beta 7$ is participating in the binding to mucosal addressin cell adhesion molecule-1 (MAdCAM-1), VCAM-1 and FN. Interestingly, integrins can change their affinity to their target within seconds, which can be achieved by conformational changes of the α and β subunit (Sung et al., 1997). These changes from low to high affinity can be regulated by cytokines or chemokines but even by the process of adhesion itself (Barthel et al., 2006). Changing the affinity of certain integrins also alters their binding properties and thereby they have to be transient and strictly controlled. For example the transmigration of eosinophils requires the firm adhesion to the endothelium through $\alpha 4\beta 1$ – VCAM-1 interactions at first but later the affinity of $\beta 2$ integrins has to

be increased in order to bind to ICAM-1, which subsequently also requires the detachment of the VCAM-1 binding (Kuijpers et al., 1993; Sung et al., 2000; Tachimoto et al., 2000).

Transendothelial diapedesis and chemotaxis to the inflammatory site

During allergic inflammation cytokines and chemokines like IL-4, IL-13, TNF- α and eotaxins induce the expression of the previously noted adhesion molecules on both eosinophils and endothelial cells. Specialized cell organelles called Weibel-Palade-bodies in endothelial cells store preformed P-selectin as well as eotaxin-3, which is released upon stimulation with inflammatory mediators (Cook-Mills, 2013; Inomata et al., 2009). Furthermore activated endothelial cells produce GM-CSF which enhances eosinophilopoiesis and increases $\alpha 4\beta 1$ affinity on eosinophils (Nagata et al., 2000; Yamamoto and Nagata, 1999). Further cytokines released from activated endothelial cells include CCL5 (RANTES), eotaxin, eotaxin-2, monocyte chemotactic protein 1 (MCP-1) and MCP-4, which bind to heparin and heparin sulfate covering the endothelial cell surface and are thereby creating a fixed gradient regulating leukocyte recruitment (Cook-Mills, 2013). As eosinophils sense those cytokines and start binding to selectins and ultimately VCAM-1, it is the binding to VCAM-1 which activates intracellular signaling cascades in endothelial cells leading to actin polymerization and intercellular gap formation (Carman and Springer, 2004; Matheny et al., 2000; van Wetering et al., 2003). The majority of leukocytes migrate through these intercellular gaps but under severe inflammation also transcellular migration was reported (Cernuda-Morollón et al., 2010).

Eosinophils in Asthma

Asthma is generally characterized by a variety of symptoms which are all linked and interdependent like airflow obstruction, bronchial/airway hyperresponsiveness (AHR) and airway inflammation. Already at the end of the 19th century the eosinophil, has

been identified as a prominent cell in the pathogenesis of asthma (Golasch, 1889). These first observations were based on autopsies performed on patients who died from status asthmaticus and revealed eosinophil infiltrations in the airways (Thomas and Busse, 2013). First clinical evidence of eosinophil involvement in asthma resulted from peripheral blood counts performed on asthma patients showing eosinophilia, which could be correlated with enhanced airflow obstruction, bronchial hyperresponsiveness and a more severe disease status (Kay, 2015; Taylor and Luksha, 1987; Thomas and Busse, 2013). Those early blood count staining techniques still exploited the ability of eosin to specifically stain the granules of eosinophils. But those granules are not merely a marker but their content, such as major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein and eosinophil-derived neurotoxin (EDN), has been found to produce pathological features connected with asthma (Gleich and Adolphson, 1986; Thomas and Busse, 2013). MBP which makes up about 55% of the total granule content was shown to be toxic to parasites but can also elicit cytotoxic effects on the respiratory epithelium (Frigas et al., 1981, 1980). Subsequently not only MBP but also ECP and EPO were found to cause damage to the respiratory tract cells and elevated levels could be found in bronchoalveolar lavage fluid (BALF) of asthmatic patients after allergen challenge (De Monchy et al., 1985; Motojima et al., 1989; Thomas and Busse, 2013). Damage to epithelial cells is linked to reduced release of bronchodilatory substances like prostaglandin E₂ (PGE₂) and is thereby resulting in enhanced airflow obstruction. Another mediator shown to damage epithelial cells is ROS which can be produced by all phagocytes including eosinophils and its production has previously been shown to be enhanced in asthma (Chanez et al., 1990; Henricks and Nijkamp, 2001). Aside from their role as powerful effector cells, eosinophils can release a range of inflammatory cytokines and lipid mediators. In asthma, generation of LTC₄ by eosinophils can increase vascular permeability and bronchoconstriction (Dahlén et al., 1980; Isono et al., 1985). Another cytokine which is produced by eosinophils and can be found in increased concentrations in asthma is transforming growth factor β (TGF- β). This pro-fibrotic cytokine can stimulate fibroblasts involved in the thickening of the reticular lamina of the airways, a process called structural remodeling (Minshall et al., 1997). The secretion of IL-3 and GM-CSF can provide an autocrine loop, prolonging the survival of eosinophils (Kita et al., 1991). IL-4 released from eosinophils in asthmatics is connected to the upregulation of VCAM on

endothelial cells, isotype switching to immunoglobulin E (IgE), whereas IL-5 release is connected to enhanced eosinophil terminal differentiation and survival. (Broide et al., 1992; Moqbel et al., 1995).

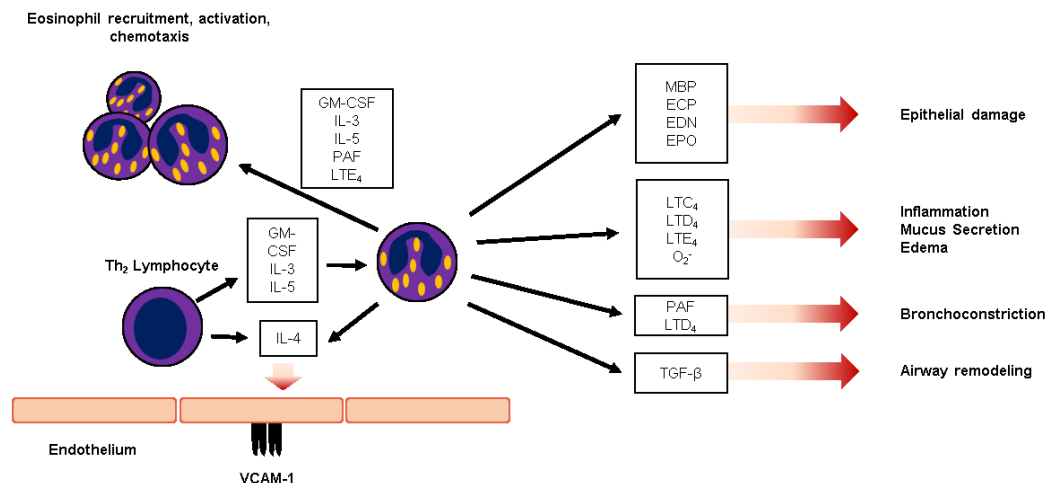


Figure I-4: Eosinophils in Asthma. Modified from (Thomas and Busse, 2013).

Targeting eosinophils in asthma

The role of eosinophils as key players in human asthma is not only established by their mere presence but also eosinophil counts in sputum and in the airways is closely correlated to controlled, uncontrolled or severe asthma. Moreover, treatment strategies that are based on sputum eosinophil counts have proven to be more effective in controlling asthma and reducing exacerbations. The most frequently used drugs for the first-line treatment of eosinophilic disorders are corticosteroids, a class of nonspecific anti-inflammatory drugs targeting multiple cellular and humoral sites in the inflammatory cascade (Straumann, 2013). But the chronic inflammation happening in asthma requires long term control and especially prolonged corticosteroid use is limited by side effects. Invention of new corticosteroids with minimal systemic exposure, like beclomethasone fluticasone, and mometasone and their application via aerosols, managed to nearly completely avoid those side effects. But controlling exacerbations in severe asthma often requires orally applied

corticosteroids, resulting in potentially harmful side effects and thus limit their therapeutic use (Lieberman et al., 1972; Nair, 2013). Another approach in the treatment addresses the fact that eosinophilic asthma shows mainly T_H2 characteristics and targets a receptor present on the associated cells, namely chemoattractant receptor homologous model expressed on T_H2 cells (CRTH2) (Gleich, 2000; Pettipher et al., 2007; Straumann, 2013). CRTH2 which is a receptor for PGD_2 , is a G-protein coupled receptor and is involved in the recruitment and activation of eosinophils, basophils and T_H2 lymphocytes (Pettipher et al., 2007). First trials with the selective CRTH2 inhibitor OC000459 showed promising results in patients with moderate persistent asthma and was able to improve airflow limitations and eosinophilic infiltration in asthma, providing first clinical evidence as possible novel oral treatment option (Barnes et al., 2012; Pettipher et al., 2014). The third approach is targeting eosinophils directly, e.g. through interference with their development, differentiation or apoptosis (Straumann, 2013). In this regard, the most promising target is IL-5 which is crucial for eosinophil-specific differentiation, maturation and survival (Ackerman and Du, 2013; Clutterbuck and Sanderson, 1990). Up to now, there are 2 humanized monoclonal antibodies available that target and inactivate IL-5, these are mepolizumab and reslizumab. Benralizumab, a third monoclonal antibody, is targeting the α subunit of the IL-5 receptor, thereby inhibiting the action of IL-5 on eosinophils and is also causing antibody-mediated cytotoxicity resulting in apoptosis induction (Straumann, 2013; Varricchi et al., 2016). Recent trials with the anti-IL-5 antibody mepolizumab revealed an oral glucocorticoid sparing effect and significantly reduced exacerbations rates in patients with persistent eosinophilic asthma (Bel et al., 2014; Ortega et al., 2014). Recently Nucala®, the first commercially available drug, based on mepolizumab, received FDA approval for the treatment of severe eosinophilic asthma.

II. The endocannabinoid system

Overview

Cannabis sativa, from which the name of the endocannabinoid system is derived was already recognized as a pain and cramps reliever 3000 years BC in China (Mechoulam, 1986). Till the mid of the 20th century the field of cannabinoid research was rather small but changed when Gaoni and Mechoulam identified the structure of the main ingredient of the marijuana plant Δ^9 -tetrahydrocannabinol (THC) (Mechoulam and Gaoni, 1965). Till then it was believed that marijuana which contains more than 60 cannabinoids (Begg et al., 2005), mediates its effects through perturbation of membrane lipids due to its high lipophilicity (Lawrence and Gill, 1975). While studying THC, Hollister and Jones discovered its structural stereo selectivity which gave hints to drug-receptor interactions (Hollister, 1974; Jones et al., 1974). The generation of synthetic cannabinoids, which furthermore allowed the production of radioligands, facilitated mapping of brain cannabinoid receptors and peaked in the identification and cloning of an orphan G-protein coupled receptor (GPCR) as the first cannabinoid receptor, named CB₁ (Matsuda et al., 1990). Three years later, the second cannabinoid receptor (CB₂) was cloned from rat spleen (Munro et al., 1993). Interestingly the expression patterns of both receptors differ, with CB₁ being mainly expressed in the central nervous system, and CB₂ mostly in peripheral organs, on hematopoietic and immune cells (Pacher et al., 2006). Apart from CB₁ and CB₂, a few other receptors also show endocannabinoid binding properties like GPR55, GPR119 and the transient receptor potential cation channel subfamily V member 1 (TRPV1), although they are not yet officially classified as cannabinoid receptors (Pacher and Mechoulam, 2011). Arachidonoyl ethanolamide (anandamide [AEA]), the first described endogenous ligand, was discovered in porcine brain in 1992 (Devane et al., 1992) shortly followed by the discovery of the second endogenous ligand 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). According to their binding properties to the two main CB receptors, 2-AG is specified as a moderate affinity full agonist at both receptors whereas AEA is a high affinity partial agonist only at CB₁ but can furthermore activate TRPV1 (Di Marzo and De Petrocellis, 2012). While the expression pattern of CB receptors is ubiquitous in most

mammals, CB receptor KO mice show little to almost no functional consequences, suggesting minimal activity under normal conditions (Pacher et al., 2006; Pacher and Kunos, 2013; Pacher and Mechoulam, 2011). But enhanced or decreased cannabinoid activity, resulting from altered CB receptor expression, synthesis or metabolism has been described for various pathological states like cardiovascular conditions, metabolic dysfunctions, gastrointestinal inflammation, neurodegenerative disorders and pain. (Di Marzo, 2008; Miller and Devi, 2011; Pacher et al., 2006; Pacher and Kunos, 2013).

Cannabinoid receptor 1

Of the two main cannabinoid receptors, the CB₁ receptor is mostly expressed in the CNS and is by far the most abundant receptor in the brains of mammals (Begg et al., 2005). Further expression sites are cells of the immune system, heart, vascular system and testis (Begg et al., 2005; Bonz et al., 2003; Gérard et al., 1991; Liu et al., 2000; Pertwee, 1997). CB₁ is a G-protein coupled receptor, mostly associated with G_{i/o} proteins and its activation leads to inhibition of adenylyl cyclase (AC), activation of mitogen-activated protein kinases (MAPK), inhibition of voltage-dependent Ca²⁺ channels and activation of inwardly rectifying potassium (K⁺) channels (Demuth and Molleman, 2006; Hudson et al., 2010). However, CB₁ can not only signal via G_{i/o} but also in a G_s and G_{q/11} dependent manner and thereby AC activating or Ca²⁺ increasing pathways have been described (Glass and Felder, 1997; Lauckner et al., 2005; Maneuf and Brotchie, 1997; McIntosh et al., 2007). In addition to its diverse signaling capabilities, CB₁ receptor ligands show a high pharmacological and structural diversity and are divided into four distinct classes (Hudson et al., 2010): 1. Classical cannabinoids, which include the plant-derived substances and its synthetic derivatives; 2. Nonclassical cannabinoids, which comprise those substances related to classical cannabinoids with the exception that they lack the dihydropyran ring; 3. Eicosanoid derived cannabinoids, including the endocannabinoids AEA and 2-AG; 4. Aminoalkylindoles and similar compounds (Howlett et al., 2002; Pertwee, 2006). Interestingly, these different cannabinoid classes can signal through different G-proteins, e.g. only Aminoalkylindoles are releasing intracellular Ca²⁺ through G_{q/11} upon CB₁ stimulation (Hudson et al., 2010; Lauckner et al., 2005; McIntosh et al.,

2007). This agonist directed signaling may be of use in inventing therapeutics targeting just one specific signaling pathway of CB₁ (Hudson et al., 2010).

Cannabinoid receptor 2

The CB₂ receptor was identified three years after CB₁ in a cDNA library from promyelocytic HL60 cells (Munro et al., 1993). Its expression was previously reported to be limited to hematopoietic and immune cells but recent studies revealed low levels of CB₂ in specific brain regions, myocardium, gut, bone, pancreas, endothelium and the reproductive system (Pacher and Kunos, 2013; Pacher and Mechoulam, 2011). Like CB₁, with whom CB₂ is sharing about 44% of its amino acid sequence (Munro et al., 1993), the cannabinoid receptor 2 is also a GPCR and its activation is linked to G_{i/o} or G_{q/11} dependent AC inhibition, regulation of Ca²⁺ and potassium channels but also MAPK/ERK activation (Brailoiu et al., 2014; Derocq et al., 1995; Mackie and Stella, 2006; Walter et al., 2003). Of note, similar to CB₁, CB₂ also shows ligand-directed trafficking. The study of Shoemaker et al. examined various agonists at CB₂ by looking at their effects on AC inhibition, Ca²⁺ release and MAPK activation. Their results showed that although all tested ligands elicited the same effects they displayed a rank order of potency, e.g. at the same concentrations 2-AG was most potent in activating ERK/MAPK but showed lesser effect on AC inhibition and Ca²⁺ released compared to CP55-940 and noladin ether (Shoemaker et al., 2005). *In vivo*, the main ligand for CB₂ is the eicosanoid derived endocannabinoid 2-AG (Pacher et al., 2006).

Endocannabinoids: Synthesis and degradation

The most bioactive endocannabinoids described are 2-AG and AEA, although the endocannabinoid (eCB) family also includes virodhamine, noladin ether, N-arachidonoyldopamine (NADA), homo-linolenylethanolamide (HEA), docosatetraenylethanolamide (DEA), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) (Battista et al., 2012).

The precursor molecule for AEA is N-acylphosphatidyl-ethanolamine (NAPE), which is produced by Ca^{2+} dependent/independent N-acyl transferases by acylation of phosphatidylethanolamines (Jin et al., 2007; Natarajan et al., 1982; Turcotte et al., 2015). From here on 4 distinct pathways for AEA generation have been described: 1. Hydrolysis of NAPE by type D phospholipases (Di Marzo et al., 1994; Hansen et al., 1995; Leung et al., 2006; Okamoto et al., 2004); 2. Hydrolysis of NAPE by a PLC which generates a phosphor-AEA intermediate that is subsequently dephosphorylated by phosphatases (Liu et al., 2006); 3. Production of lyso-NAPE by PLA_2 followed by hydrolysis by a lyso-PLD (Sun et al., 2004); 4. Same as in 3, but hydrolysis into glycerophospho-N-arachidonoyl-ethanolamine by the serine esterase ABHD4 with following activation of glycerophosphodiesterase 1 (Simon and Cravatt, 2006; Turcotte et al., 2015).

AEA shows higher selectivity for CB_1 receptors, whereas 2-AG is capable of activating both CB receptors. Therefore it is not surprising that their levels are regulated differently (Di Marzo and De Petrocellis, 2012; Di Marzo and Petrosino, 2007). 2-AG can be produced via several pathways, where most of them have the generation of 1, 2-diacylglycerol (DAG) in common. E.g. DAG can be synthesized by the hydrolysis of glycerol-phosphatidylinositol by PLC activity (Kondo et al., 1998; Prescott and Majerus, 1983) or by the combination of PLD and phosphatidic acid phosphatase acting on glyceryl-phosphatidylcholine (Bisogno et al., 1999). Finally 2-AG is generated out of arachidonic acid-containing DAG species by the DAG-lipase- α or $-\beta$ (Bisogno et al., 2003; Tanimura et al., 2010) but can also be produced by the de-phosphorylation of arachidonoyl-lysophosphatidic acid (Nakane et al., 2002).

Both 2-AG and AEA are synthesized on demand, which is triggered by enhanced intracellular Ca^{2+} concentrations, and are immediately released into the extracellular space where they are rapidly cleared by cellular uptake, hydrolysis, or a combination of both (Di Marzo and Petrosino, 2007; Turcotte et al., 2015). After their uptake, both 2-AG and AEA can be hydrolyzed by serine esterases into glycerol/ethanolamine and AA, or they are converted to several metabolites by several enzymes involved in eicosanoid metabolism, like lipoxygenases (LOX) cyclooxygenases (COX) and epoxygenases (Turcotte et al., 2015).

The main enzymes for the degradation of AEA to ethanolamine and AA are the membrane associated molecules fatty acid amid hydrolase (FAAH)-1 and -2. They

differ slightly in their tissue distribution with FAAH-1 being mainly located in the brain, kidney, liver, lung, prostate, testis and small intestine, whereas FAAH-2 can be found in the heart, kidney, liver, lung, prostate and ovary (Turcotte et al., 2015).

2-AG is metabolized into AA and glycerol mainly via monoacylglycerol lipase (MAGL). MAGL activity is responsible for about 85% of 2-AG hydrolysis, whereas α/β -hydrolase domain (ABHD)-6, ABHD-12 and also FAAH-1 are metabolizing the remaining 15% (Turcotte et al., 2015).

Interestingly the AA moiety of 2-AG and AEA makes them prone to metabolism by enzymes involved in the eicosanoid biosynthesis. The products obtained from that pathways have mostly little or no affinity towards CB receptors but can activate a variety of eicosanoid related receptors hence giving them great importance in the modulation of inflammatory responses (Turcotte et al., 2015).

The first description of AEA being a substrate for COX-2 but not COX-1 was noted in 1997 by Yu et.al (Yu et al., 1997). Additional studies revealed the generation of several AEA prostaglandin (PG) ethanolamides (EA) by COX-2: PGG₂-EA, PGH₂-EA, PGD₂-EA, PGE₂-EA, PGF_{2 α} -EA and PGI₂-EA (Koda et al., 2004; Kozak et al., 2002a; Yang et al., 2005). It was furthermore shown that PG-EA can activate PPAR- γ and EP-receptors (Matias et al., 2004; Rockwell and Kaminski, 2004) but are poor activators of CB receptors (Berglund et al., 1999).

Similar to AEA, oxygenation of 2-AG by COX-2 but not COX-1 is generating the respective prostaglandin-glycerols (PG-G) and PGE₂-G was shown to be a potent cell activator by acting on specific PG-G receptors (Kozak et al., 2000; Nirodi et al., 2004; Turcotte et al., 2015).

Furthermore AEA and 2-AG can be used as substrates by leukocyte type 12-LOX and 15-LOX (Hampson et al., 1995; Kozak et al., 2002b; Moody et al., 2001; Ueda et al., 1995). AEA metabolites seem to exert their actions via TRPV receptors, whereas little is known about the bioactivity of 2-AG metabolites (Turcotte et al., 2015).

Taken together the regulatory/modulatory capacity of 2-AG and AEA regarding immune function is most likely an interplay involving cannabinoid, eicosanoid and endocannabinoid metabolite receptors (Turcotte et al., 2015).

the immune response favors pathogen proliferation and cancer cell growth (Klein et al., 2000; McKallip et al., 2005; Turcotte et al., 2015).

Pro-inflammatory effects

Apart from their anti-inflammatory effects, there are also several studies describing pro-inflammatory effects of cannabinoids especially in inflammation connected with hypersensitivity (Maestroni, 2004), nephropathy (Mukhopadhyay et al., 2010a), cardiomyopathy (Mukhopadhyay et al., 2010b) and dermatitis (Oka et al., 2006, 2005). The mechanism of these pro-inflammatory effects are associated with increased inflammatory cell recruitment and enhanced ROS and cytokine production (Turcotte et al., 2015). Interestingly almost all pro-inflammatory effects of endocannabinoids are attributed to 2-AG but not AEA. Accordingly 2-AG has been described to participate in the recruitment of leukocytes under inflammation. So far, only endocannabinoids but not cannabinoids have been shown to activate and recruit leukocytes in experimental models, indicating that 2-AG may modulate immune functions through its numerous metabolites independent of CB receptor activation (Turcotte et al., 2015).

Summarized, effects of cannabinoids on experimental models of inflammation are a complex interplay and may involve a variety of CB dependent and independent actions. Moreover effects may differ dependent on the micro-milieu, location and cell types involved (Turcotte et al., 2015).

Cannabinoids in allergy

Regarding allergy and its main cell type involved, i.e. the eosinophil, only a few studies addressed the effects of cannabinoids with conflicting outcomes. Oka et al. could show that 2-AG is a chemotactic agent for EoL-1 cells and human peripheral blood eosinophils although 2-AG was significantly weaker compared to other chemoattractants (Kishimoto et al., 2004; Oka et al., 2004). Another group confirmed this minimal effect of 2-AG on eosinophil chemotaxis and, moreover, suggested that this process may involve CB receptor-independent factors like cytokine priming, metabolism into eicosanoids and generation of 15-LOX metabolites (Larose et al., 2014a). The effects of cannabinoids on eosinophils *in vivo* are also conflicting as

literature reports both pro-inflammatory effects on ovalbumin (OVA)-induced lung eosinophilia (Lunn et al., 2006a) and oxazolone-induced contact dermatitis (Oka et al., 2006), and beneficial effects of the CB₁/CB₂ receptor agonist CP55,940 on OVA-induced allergic asthma in guinea pigs (Giannini et al., 2008). Furthermore, THC was shown to reduce cytokine and IgE levels as well as mucus production in OVA-challenged mice (Jan et al., 2003), although it was later shown that the effects of THC were CB receptor independent (Braun et al., 2011).

III Materials

All reagents were from Sigma (Vienna, Austria), unless specified. Assay buffer was made from Dulbecco's modified PBS (with 0.9mmol/L Ca^{2+} and 0.5mmol/L Mg^{2+} ; Invitrogen, Vienna, Austria), 0.1% BSA, 10 mmol/L HEPES, and 10mmol/L glucose, pH 7.4. Human eotaxin-2/CCL24 was from R&D Systems (Minneapolis, USA). IL-8 was from Peprotech (London, United Kingdom). PGD₂, SR144528, JWH-133, 2-APB, U-73122, U-73343 and 2-AG were purchased from Tocris (Bristol, UK). Anti-CD16-PE-Cy5, anti-HLA-DR-FITC, anti-CD123-PE and anti-CD11b-PE (ICRF44) were from Becton Dickinson (Vienna, Austria). Polyclonal rabbit anti-human CB₂ Ab was from Abcam (Cambridge, United Kingdom) and rabbit IgG isotype control was supplied from Santa Cruz Biotechnology (Heidelberg, Germany). The PI3K inhibitor LY294002 was supplied from Biomol (Hamburg, Germany). The MEK1/2 inhibitor U-0126, the p38 MAPK inhibitor SB 202190 and the ROCK inhibitor Y-27632 were purchased from Tocris (Bristol, UK). The MEK1/2 inhibitor PD 184161 was from Cayman Chemicals (Ann Arbor, MI). CellFix and FACS-Flow were from Becton Dickinson (Vienna, Austria). PVP-free polycarbonate filters were from NeuroProbe (Gaithersburg, USA) and 96-well chemotaxis plates were from Corning Life Sciences (Radnor, USA). Fluo-3-AM was supplied from Life Sciences (Vienna, Austria).

Fixative solution was prepared by adding 9ml of distilled water and 30ml of FACS-Flow to 1ml of CellFix. Assay buffer was made from PBS (with or without $\text{Ca}^{2+}/\text{Mg}^{2+}$) amended with 0.1% BSA, 10mM glucose, 10mM HEPES, adjusted to a pH of 7.4 with NaOH.

Blood donors:

Blood was collected in- and off-season from healthy and allergic volunteers according to a protocol approved by the Ethics Committee of the Medical University of Graz. Medical history and allergic symptoms were assessed using a standardized questionnaire asking for physician-diagnosed allergies and the presence, nature and frequency of allergic symptoms. In this study, only healthy non-allergic subjects and

subjects allergic to aeroallergens with respiratory symptoms were included (Frei et al., 2016).

Mouse strains:

Female C57Bl6/N mice were obtained from Charles River (Sulzfeld, Germany). Breeding pairs of the interleukin-5 transgenic (IL-5Tg) and eosinophil-deficient $\Delta db/GATA$ strains (BALB/c background) were a kind gift of Dr. Helene Rosenberg and maintained on-site. Mice were housed in individually ventilated cages (5 per cage) under controlled conditions of temperature (set point 21 °C), air humidity (set point 50%) and a 12 h light/dark cycle (lights on at 6:00 a.m.). Standard chow (altromin 1324 FORTI, Altromin, Lage, Germany) and water were provided ad libitum. The experimental procedure used in this study was approved by the Austrian Federal Ministry of Science, Research and Economy (protocol numbers: BMWF-66.010/0094-II/3b/2013; BMWFW-66.010/0020-WF/V/3b/2015) conform to Directive 2010/63/EU, and was performed in accordance with national and international guidelines. Mice were randomly assigned before treatment.

IV Methods

Preparation of human eosinophils:

Blood was sampled from healthy non-atopic volunteers according to a protocol approved by the Ethics Committee of the Medical University of Graz and processed as described previously (Schratl et al., 2006). For some experiments blood from volunteers, suffering from allergy and/or allergic asthma, determined by questionnaires, was used. In short, citrated whole blood was centrifuged for 20min at 400xg following the removal of platelet-rich plasma. Polymorphonuclear leukocytes (PMNL, containing eosinophils and neutrophils) and peripheral blood mononuclear cells (PBMC) were purified by means of dextran sedimentation of erythrocytes and remaining platelets followed by centrifugation on a Histopaque 1.077 layer. Eosinophils were further purified by negative magnetic selection (MACS Separation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the protocol provided by the manufacturer. Purity of eosinophils was assessed using a hemocytometer and viability was determined by a flow cytometric Annexin-V, PI apoptosis assay. Both parameters were greater than 95%.

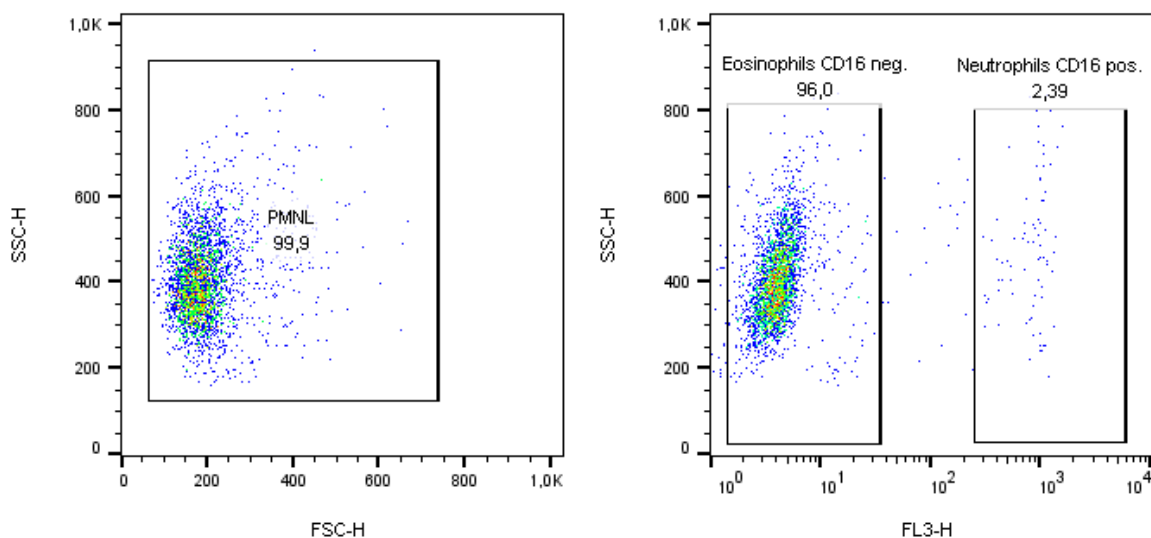


Figure IV-1: Purity of isolated human eosinophils: Freshly isolated and purified human eosinophils were stained with an anti CD16-PE-Cy5 antibody to detect neutrophils. As depicted in the cytometry plot above, purity was greater than 95%.

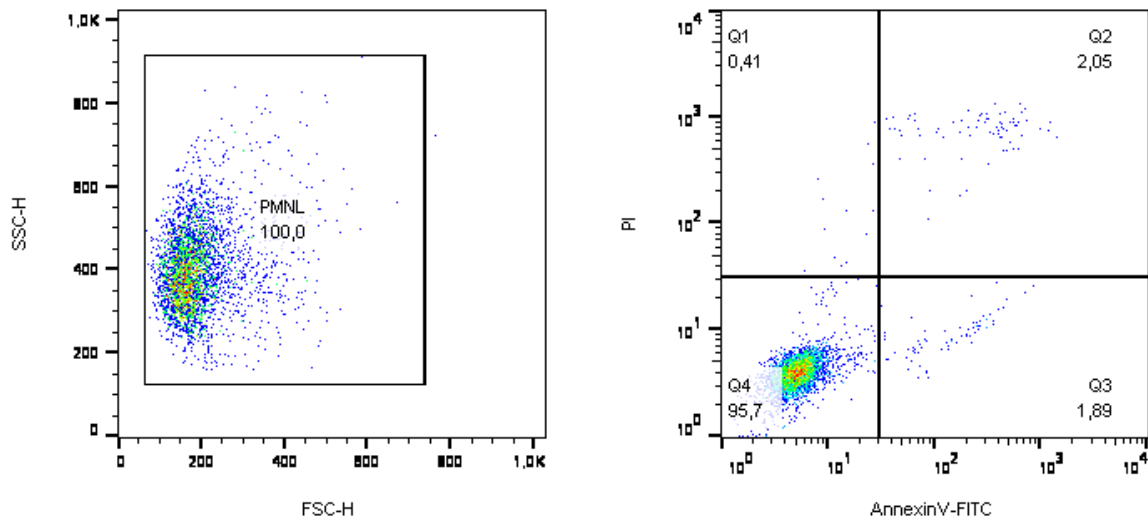


Figure IV-2: Viability of isolated human eosinophils: Freshly isolated and purified human eosinophils were stained with AnnexinV-FITC and propidium iodide to detect apoptosis. As shown above, viability is greater than 95%.

Preparation of mouse eosinophils:

Eight-week-old female BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Bone marrow-derived eosinophils (bmEos) were differentiated *ex vivo* from unselected bone marrow progenitors (Dyer et al., 2008). Progenitors were cultivated for 4 days in the presence of recombinant mouse Fms-related tyrosine kinase 3 ligand (rmFLT3-L) and recombinant mouse stem cell factor (rmSCF) followed by treatment with rmlL-5 from day 4 onwards. By day 10-12 our culture yielded high-purity (90-100%) bmEos as estimated by visual inspection of Diff-Quik stained cytospins. Experimental procedures were approved by the Austrian Federal Ministry of Science and Research (protocol number: BMWF-66.010/0143-II/3b/2012) and performed in accordance with international guidelines.

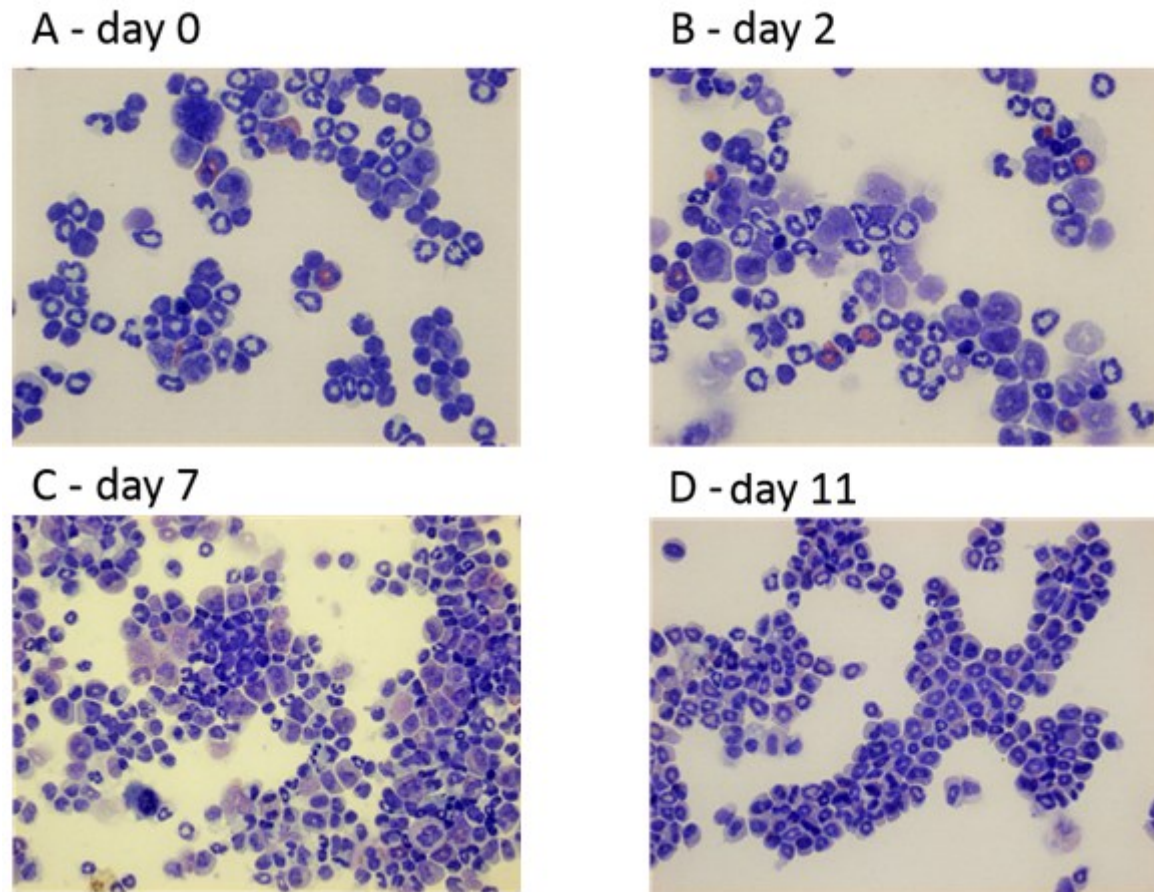


Figure IV-3: Diffquick stained cytopins of cultured bmEos. (Images thankfully obtained from Thomas Silly)

Flow cytometric staining of CB₂ receptors on eosinophils:

Human peripheral blood eosinophils were purified by negative magnetic isolation and resuspended at 1×10^6 cells/ml in assay buffer (without $\text{Ca}^{2+}/\text{Mg}^{2+}$) supplemented with 5% fetal calf serum (FCS). Three x 100 μl cell suspension were either stained with a rabbit polyclonal antibody to CB₂ (10 $\mu\text{g}/\text{ml}$), normal rabbit IgG isotype control or left unstained for 45min in the dark on ice. After washing twice with assay buffer, cells were incubated with goat anti-rabbit IgG conjugated with an Alexafluor (AF) 647 fluorochrome or buffer for 45min on ice in the dark. After washing the cells again twice with assay buffer, samples were fixed with 200 μl fixative solution and analyzed by flow cytometry.

Shape change assay:

The shape change assay was essentially carried out as described by Sabroe et al., 1998. Polymorphonuclear leukocytes (PMNL), peripheral blood mononuclear cells (PBMCs) or isolated peripheral blood eosinophils were resuspended in assay buffer (with $\text{Ca}^{2+}/\text{Mg}^{2+}$) at a concentration of 2×10^6 cells/ml. Cells were divided into two or more fractions and pretreated as indicated. Thereafter, aliquots were mixed with chemoattractants or CB receptor agonists and incubated for 4min at 37°C . Samples were immediately transferred to ice and $150\mu\text{l}$ fixative solution was added to preserve cell structure until analysis by flow cytometry. Eosinophils were either distinguished from neutrophils by their higher auto-fluorescence in the FL2 (585/42nm) channel or an anti-CD16 PE/Cy-5 conjugated antibody was used to stain neutrophils and identify them as highly positive cells in the FL3 (670LP) channel. Basophils were identified as $\text{CD123}^+/\text{HLA-DR}^-$ cells in PBMC preparations. Shape change was recorded by the increase in forward scatter.

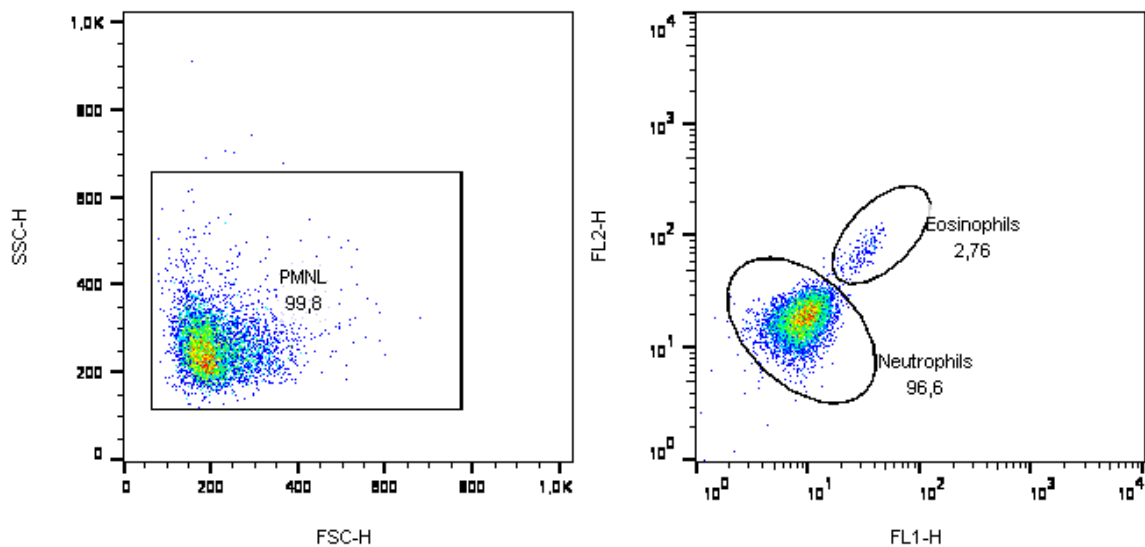


Figure IV-3: Differentiation of eosinophils and neutrophils according to their auto-fluorescence.

Migration:

Chemotaxis: Human peripheral blood eosinophils, purified by negative magnetic isolation or bmEos were resuspended at a concentration of 2×10^6 cells/ml. Cells were pre-treated as indicated, 50 μ l were placed into each of the top wells of a 5 μ m pore-sized 48-well micro-Boyden chamber (human) or a 96-well chemotaxis plate (bmEos) and were allowed to migrate towards the indicated chemoattractant for 1 hour (human) or 3 hours (bmEos) at 37 °C (Dyer et al., 2008; Heinemann et al., 2003).

Chemokinesis: Same procedure was followed as above but after pretreatment purified human eosinophils were allowed to migrate towards assay buffer instead of chemoattractants. After the respective time points, cells that have migrated to the bottom wells of the micro-Boyden chamber were transferred to small FACS tubes and 150 μ l fixative solution was added. Analysis was done by flow cytometry.

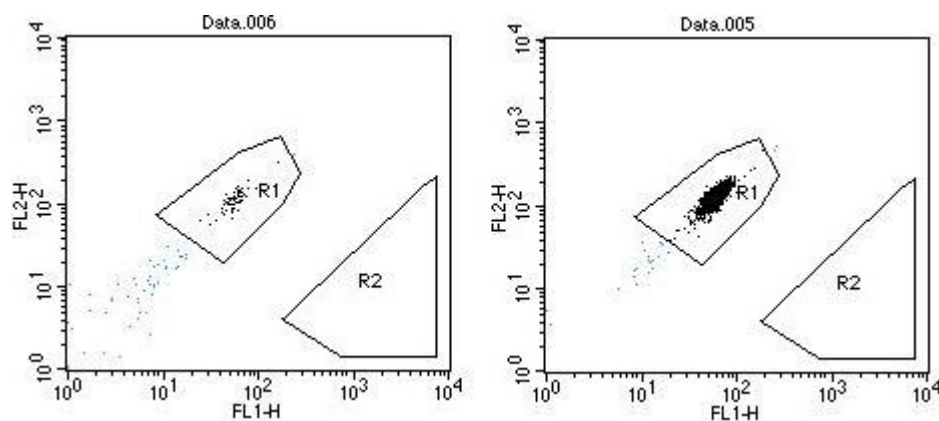


Figure IV-4: Original plots from a chemotaxis experiment. *On the left side you can see cells that had migrated towards vehicle and on the right side you can notice the extensive increase of cells in the bottom wells when migrating towards eotaxin-2.*

Calcium Flux:

Human peripheral blood eosinophils, bmEos or PMNL were resuspended in 1ml assay buffer (without Ca^{2+} / Mg^{2+}) at a concentration of 10×10^6 cells/ml. The Ca^{2+} sensitive dye Fluo-3 in its acetoxymethylester form was added to give a final concentration of 2 μ M in the presence of 0.02% pluronic F-127 for 60min at room temperature in the dark (Heinemann et al., 2003; Monneret et al., 2001). After a washing step with assay buffer, the eosinophils were resuspended in 500 μ l assay

buffer (without $\text{Ca}^{2+}/\text{Mg}^{2+}$) and 5 μl of an anti-CD16 PE/Cy-5 AB was added. This mixture was incubated for 15min at room temperature in the dark. Following a final washing step, cells were resuspended in assay buffer (with $\text{Ca}^{2+}/\text{Mg}^{2+}$) at a concentration of 1×10^6 cells/ml and were divided to a distinct amount of FACS tubes according to the experiment. In some experiments cells were pretreated with various antagonists/inhibitors or EGTA before measurement. Baseline fluorescence was acquired for 1 min and then the respective chemoattractants / CB agonists were added and intracellular Ca^{2+} release was measured as fluorescence increase in the FL-1 (530/30) channel on a FACSCalibur flow cytometer.

Adhesion assay under flow:

Vena8™ biochips (Cellix Ltd., Dublin, Ireland) were coated with 10 $\mu\text{g}/\text{ml}$ ICAM-1 and coated channels were superfused with 2×10^6 purified eosinophils at 0.5 dyne/cm^2 for 5min at 37 °C in an OKOLAB H201-T1 heated cage. Adhesion was monitored on a Zeiss Axiovert 40 CFL microscope equipped with a Zeiss A-Plan 103/0.25 Ph1 lens, using a Hamamatsu ORCA-03G digital camera and CellixVenaFlux software. Cell adhesion was quantified by computerized image analysis using DucoCell analysis software (Cellix Ltd, Dublin, Ireland).

CD11b-upregulation:

Whole blood samples or PMNL were pre-treated as indicated and incubated with agonists for 30min at 37 °C (Konya et al., 2010). Samples were stained with anti-CD16-PE-Cy5 and anti-CD11b-PE AB. CD11b upregulation was analyzed by flow cytometry and expressed as percent of the vehicle response. Eosinophils were identified as CD16⁺ cells in the granulocyte forward scatter /side scatter region.

Respiratory burst:

Human peripheral blood eosinophils were purified by negative magnetic isolation and resuspended at a concentration of 1×10^6 cells/ml in assay buffer (with $\text{Ca}^{2+}/\text{Mg}^{2+}$). Afterwards cells were pretreated with a CB_2 agonists and/or antagonist and then stimulated with serial dilutions of eotaxin-2 in the presence of $50 \mu\text{M}$ dichlorofluorescein diacetate (DCFDA) for 20min at 37°C . The reaction was stopped by the addition of $100 \mu\text{l}$ fixative solution. Eosinophil respiratory burst was measured as an increase in fluorescence of the FL1- (530/30nm) channel due to generation of the fluorophore 2', 7' -dichlorofluorescein (DCF) in the presence of reactive oxygen species (ROS) (Konya et al., 2014). Responses were expressed as percent of the vehicle response.

CD63 expression:

Human peripheral blood eosinophils were purified by negative magnetic selection and resuspended at a concentration of 1×10^6 cells/ml in assay buffer (with $\text{Ca}^{2+}/\text{Mg}^{2+}$) in the presence of an anti-CD63-FITC AB. Cells were then pre-treated for 10min at room temperature as indicated and cytochalasin B ($5 \mu\text{g}/\text{ml}$) was added for 5min at 37°C . Degranulation was induced with serial dilutions of C5a for 20 min at 37°C . The reaction was stopped by transferring the cells to ice and further addition of $150 \mu\text{l}$ fixative solution. CD63 expression was detected by flow cytometry and expressed as percent of the respective control responses (Heinemann et al., 2003).

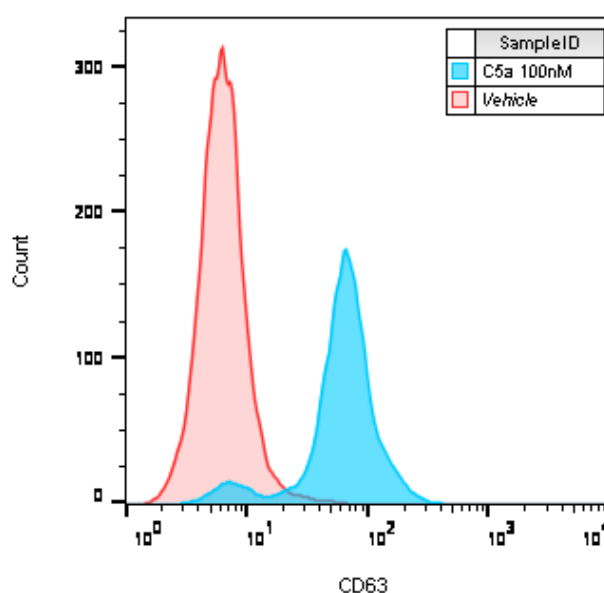


Figure IV-5: Example histogram of CD63 upregulation on eosinophils by C5a.

EPO release:

Human peripheral blood eosinophils were purified by negative magnetic isolation and resuspended at a concentration of 1×10^6 cells/ml in assay buffer (with $\text{Ca}^{2+}/\text{Mg}^{2+}$). Cells were pretreated with CB₂ receptor agonist and/or antagonist and cytochalasin B (10 $\mu\text{g}/\text{ml}$) was added. 50 μl aliquots were transferred into the wells of a 96-well microplate (Schuligoi et al., 2009). After stimulation with serial dilutions of C5a for 20min at 37 °C, H_2O_2 (1mM) was added to start the peroxidase reaction. Tetramethylbenzidine (2.8mM) was added to detect the reaction and color development was terminated by addition of 4M acetic acid. EPO release was quantified on a bench reader at a wavelength of 630nm and data was expressed as percent of the respective control responses.

***In vivo* chemotaxis:**

Eight week-old Interleukin-5 transgenic (IL-5Tg) mice on the Balb/c background (kind gift of Dr. Helene Rosenberg) were treated with JWH-133 (5mg/kg/d) or vehicle intra

peritoneal (i.p.) for three days. Finally, 4µg eotaxin-2 were applied via intranasal instillation and bronchoalveolar lavage fluid (BALF) was collected after 5h. Samples were stained with an anti-mouse Siglec-F AB conjugated to AF647 and an anti-mouse CD11c AB conjugated to FITC. Eosinophils were identified as Siglec-F⁺/CD11c⁻ cells and migration of mouse eosinophils into the lung was assessed by flow cytometric counting.

Ovalbumin (OVA)-induced pulmonary inflammation model:

Eight week old female C57Bl6/N mice were immunized by i.p. injections of 10µg of OVA adsorbed to Al(OH)₃ on days 0 and 7. Mice were challenged with an aerosol of 0.5% (w/v) of OVA in saline for 30min on days 14 and 16. In between, mice received a daily i.p. injection of a combination of JWH-133 and/or SR144528 (10 mg/kg) or vehicle from day 9 onwards till day 16. On day 17 either airway hyperresponsiveness (AHR) to methacholine was recorded with the FlexiVent system (Scireq, Montreal, CA) or BALF was taken using 3ml PBS/EDTA (1mM), and analyzed by flow cytometry. Eosinophils in BALF were identified as highly granular (high side scatter) CD11c⁻/Siglec F⁺ cells, T-Cells as CD3e⁺, B-Cells as I-A/I-E⁺/B220⁺, macrophages as CD11c⁺/Siglec F⁺, monocytes as I-A/I-E⁺/CD11c⁻ and neutrophils as Siglec F⁻ cells in the granulocyte region. For histological analysis, lung tissue was fixed in 4% formaldehyde and embedded in paraffin. Ten-µm sections were stained with H&E and pictures were taken at a 100x magnification on an Olympus IX70 microscope with an Olympus DP50 camera.

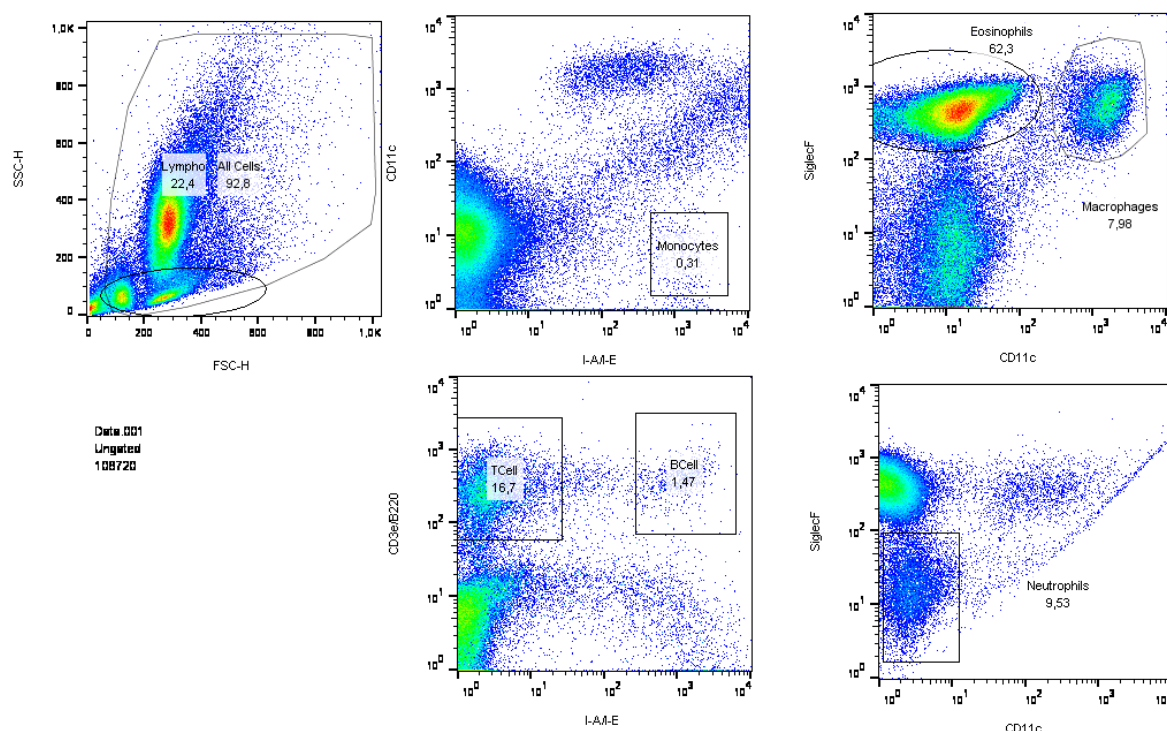


Figure IV-6: Gating strategy used in the determination of BALF cells.

LC-MS analysis of BALF metabolites:

A stable isotope dilution liquid chromatography-mass spectrometry (LC-MS) method was used, previously reported elsewhere (Balgoma et al., 2013), with slight modification to quantify cysteinyl leukotrienes (CysLTs) LTC₄, LTD₄ and LTE₄. The lipid mediator nomenclature is provided in **Table 1**. (*Mass spectrometry was kindly done by Alexander Fauland in the Craig Wheelock laboratory, Stockholm, SE*)

SPE extraction

Extraction of analytes was carried out in 3cc/60 mg HLB Oasis SPE cartridges (Waters). Cartridges were conditioned with 2ml of methanol followed by 2ml of water. A cocktail of deuterated internal standards (10µL; **Table 2**) was added to 800 to 900 µL BALF sample, followed by 10 µl BHT/EDTA solution (0.2mg/ml, dissolved in methanol/water 50:50, v/v) and 900µl extraction buffer (citric acid: 0.2M Na₂HPO₄, pH5.6) and loaded onto the SPE cartridge. Samples were washed with 2ml of water/methanol (80:20, v/v) and the cartridges were dried in the manifold under

vacuum-induced air stream at -30 kPa for 30min. Analytes were eluted from the columns with 2.5ml of methanol in cryotubes containing 10µl of glycerol 30% in methanol. After evaporation of the eluates under vacuum, samples were resuspended in 60µl of methanol. Additionally 10µl of water was added to the samples. Solution was filtered by centrifugation in Amicon Ultrafree-MC, PVDF 0.1µm (Millipore) and transferred to autosampler vials with inserts before injection.

UHLC-MS/MS measurement

Liquid chromatography coupled to mass spectrometry (LC-MS/MS) separation and quantification was carried out on a UPLC Acquity-Xevo TQS mass spectrometer system (Waters, Milford, MA). The autosampler and column were kept at 5°C and 60°C, respectively. Injection volume was set to 7.5µl. Separation was achieved with an Acquity UPLC BEH C18 (2.1 × 150 mm, 1.7µm, Waters).

For negative electrospray ionization (ESI) a gradient of solvents A (water with 0.1% of acetic acid) and B (acetonitrile/isopropanol 90:10, v/v) at a flow of 0.5ml min⁻¹ was used. The gradient initiated with 80% of A, which was decreased linearly to 65% at 2.5 min, to 60% at 4.5min, to 58% at 6min, to 50% at 8min, to 35% at 14min, to 27.5% at 15.50min and to 0% at 16.60min. The column was then washed with solvent B for 0.9min and equilibrated to initial conditions. For positive ESI, a gradient of solvents A (water with 0.2% of formic acid) and B (acetonitrile/isopropanol 90:10, v/v + 0.2% formic acid) at a flow of 0.45 ml min⁻¹ was used. The gradient initiated with 60% of A, which was decreased linearly to 50% at 4.25min and to 5% at 4.5min. The column was then washed with 95 % of solvent B for 3min and equilibrated to initial conditions.

Data was collected on a Waters Xevo TQS-MS mass spectrometer using positive and negative electrospray ionization (ESI) and scheduled multiple reaction monitoring (MRM) mode. Dwell time was automatically adjusted in order to acquire 20 points per chromatographic peak; capillary voltage was 2.20kV in positive and negative ESI. Desolvation temperature and gas flow were set according to the instrument recommendations for the chromatographic flow. Detailed MRM transition and chromatographic retention time for each compound is provided in **Table 3**.

Table 1. Nomenclature for lipid mediators

Abbreviation	Common name	Systematic name
LTC ₄	leukotriene C ₄	5S-hydroxy-6R-(S-glutathionyl)-7E,9E,11Z,14Z-eicosatetraenoic acid
LTD ₄	leukotriene D ₄	5S-hydroxy-6R-(S-cysteinyglycynyl)-7E,9E,11Z,14Z-eicosatetraenoic acid
LTE ₄	leukotriene E ₄	5S-hydroxy-6R-(S-cysteiny)-7E,9E,11Z,14Z-eicosatetraenoic acid

Table 2. Calibration levels of the lipid mediators (LTC₄, LTD₄ and LTE₄) analyzed and concentration of internal standard ([²H₅]-LTD₄, [²H₅]-LTC₄, and [²H₅]-LTE₄) after adding to the sample or to the calibration levels.

[illegible]

Table 3. Analytical characterization of the lipid mediator standards. Ionization mode, chromatographic retention time (RT), parent ion, product ion, the internal standard (IS) assigned to every lipid mediator and the on-column injected linear range are reported.

Lipid mediator	ionization mode	RT	Parent ion	Product ion	IS	Linear range (fmol)
		(min)	(m/z)	(m/z)		
LTC ₄	[M+H] ⁺	3.61	626.2	308.2	[² H ₅]-LTC ₄	0.2-2226
LTD ₄	[M+H] ⁺	3.46	497.1	189.2	[² H ₅]-LTD ₄	0.3-2804
LTE ₄	[M-H] ⁻	7.6	438.4	333.3	[² H ₅]-LTE ₄	0.3-3169
[² H ₅]-LTC ₄	[M+H] ⁺	3.58	631.1	308.2		
[² H ₅]-LTD ₄	[M+H] ⁺	3.43	502.2	194.2		
[² H ₅]-LTE ₄	[M-H] ⁻	7.55	443.1	338.1		

Statistical analysis:

Data are shown as mean ± SEM for n observations. Comparisons of groups were performed using one-way ANOVA or two-way ANOVA for repeated measurements followed by Bonferroni's post-hoc test to determine the levels of significance for each group. Probability values of p < 0.05 were considered as statistically significant.

The Results of this thesis have been published in agreement with the Curriculum of the PhD Program at the Medical University of Graz:

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Cannabinoid receptor 2 augments eosinophil responsiveness and aggravates allergen-induced pulmonary inflammation in mice.

Allergy 2016; DOI: 10.1111/all.12858.

V Results Part I (Cannabinoids *in vitro*)*

V.I. CB₂ expression is increased on eosinophils from allergic donors

The expression of the cannabinoid receptor 2 has so far been reported on several leukocytes including monocytes, macrophages, B cells, NK cells, basophils and eosinophils (Galiègue et al., 1995; Graham et al., 2010; Oka et al., 2004). Furthermore CB₂ was found on T cells, although at much lower levels compared to other leukocyte populations. Interestingly, its expression on human peripheral blood neutrophils is reported controversially. A study done by Galiègue et al. reported CB₂ mRNA expression in neutrophils (Galiègue et al., 1995), but a 10 year later performed analysis done by PCR and western blotting revealed only negligible CB₂ expression on human neutrophils (Oka et al., 2004) . One explanation for this contradictory findings may be over-amplification of mRNA, as the main invading cell type in neutrophil isolation techniques, the eosinophil, expresses plenty of CB₂ mRNA. Moreover the removal of contaminating eosinophils led to a considerable diminishment of CB₂ mRNA amplification (Turcotte et al., 2015)

The first study reporting the protein expression of CB₂ but not CB₁ on human peripheral blood eosinophils and initial observation that the endocannabinoid 2-AG is a chemotactic factor suggested participation of the endocannabinoid system in the regulation of eosinophil associated disorders through selective activation of CB₂ (Oka et al., 2004). Subsequent studies reported the upregulation of the CNR2 gene by bronchoalveolar lavage (BAL) eosinophils after whole lung allergen challenge (Esnault et al., 2013).

Here I could confirm the initial observation that CB₂ protein can be found on the surface of human eosinophils by flow cytometric staining. Interestingly, when I compared eosinophils isolated from the blood of allergic volunteers with seasonal respiratory symptoms to those from healthy controls a ~3.4 fold higher CB₂ protein level could be observed. Of note, this effect vanished when blood was taken from allergic donors off-season of the triggering allergen (**Fig. V-1**). The initial observation that CB₂ is upregulated locally in the lung after allergen challenge is extended by our results, suggesting that allergen exposure of sensitized subjects is directly linked with an over-active endocannabinoid system, leading to priming of peripheral blood eosinophils.

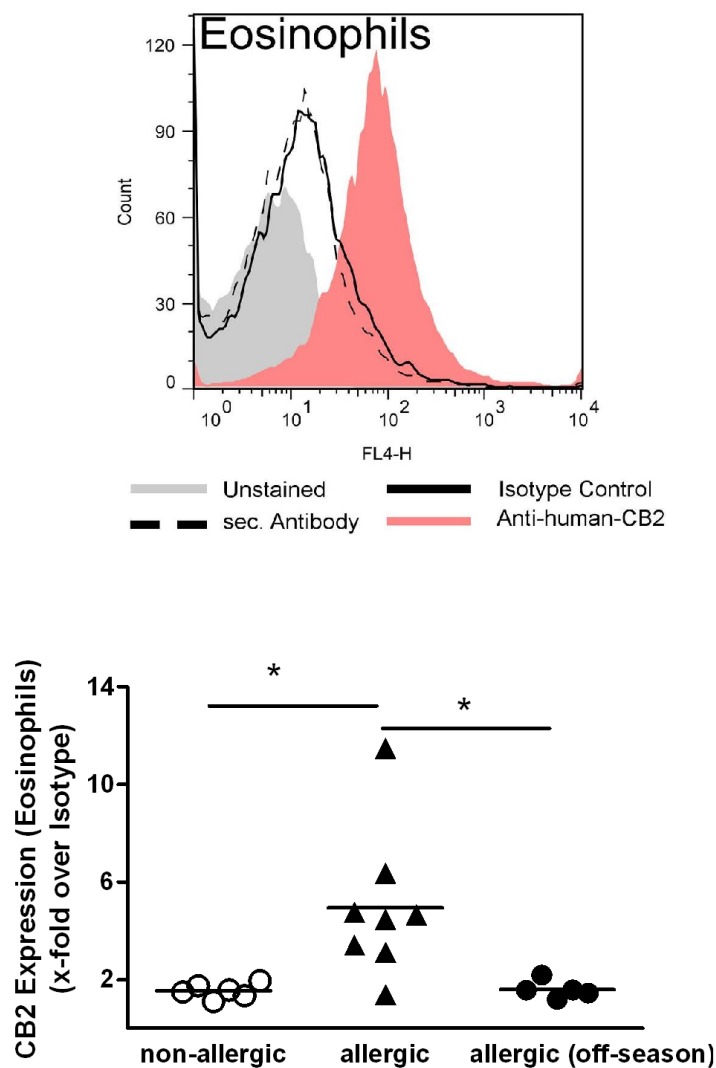


Figure V-2. CB₂ receptor expression is increased on eosinophils from allergic donors:

Human peripheral blood eosinophils from donors (non-allergic, allergic with acute symptoms or allergic off-season) were purified by negative magnetic isolation and resuspended at 1×10^6 cells/ml in assay buffer (without $\text{Ca}^{2+}/\text{Mg}^{2+}$) supplemented with 5% fetal calf serum (FCS). $3 \times 100 \mu\text{l}$ cell suspension were either stained with a rabbit polyclonal antibody to CB₂ ($10 \mu\text{g}/\text{ml}$), normal rabbit IgG isotype control or left unstained for 45min in the dark on ice. After washing two times with assay buffer, cells were incubated with goat anti-rabbit IgG conjugated with an Alexafluor (AF) 647 fluorochrome ($4 \mu\text{g}/\text{ml}$) or buffer for 45min on ice in the dark. After washing the cells

again twice with assay buffer, samples were fixed with 200µl fixative solution and analyzed by flow cytometry. Data were expressed as increase over the respective isotype control.

V.II. The selective CB₂ receptor agonist JWH-133 enhances human eosinophil function

As the presence of CB₂ receptors on eosinophils was confirmed in the first part of this work our next aim was to determine its functional role *in vitro*. Previous work done in mice showed that application of the selective inverse cannabinoid receptor 2 agonist Sch.336 diminished chemokine- and antigen-induced leukocyte trafficking *in vivo*. Interestingly, this effect would suggest that activation of CB₂ is not only directly inducing eosinophil chemotaxis, but also modulates leukocyte recruitment (Lunn et al., 2006b).

Thus, to explore the direct effects of CB₂ activation on eosinophils, I chose the selective agonist JWH-133 and tested its effect in an eosinophil shape change assay. Leukocytes respond to contact with chemotactic factors with a quick rearrangement of their cytoskeleton in order to allow their attachment to microvascular endothelial cells. Flow cytometry is providing an excellent tool to monitor such morphological changes, can easily be detected as alterations in the forward scatter (FSC) properties of a cell (Sabroe et al., 1999). Representative images of data acquisition by flow cytometry is show in **Fig. V-2**.

Our first aim was to establish an effective working concentration for JWH-133 according to the shape change data. Thus, human eosinophils were isolated by negative magnetic selection and treated with increasing concentrations of JWH-133 or vehicle following stimulation with the eosinophil specific chemokine eotaxin-2 (**Fig.V-3-B**). In parallel, the same experiment was conducted with the endocannabinoid 2-AG in order to elucidate similarities or possible differences in the mode of action (**Fig.V-3-A**). As shown in Fig. V, 2-AG dose-dependently increases the response of eotaxin-2 with a maximum at a concentration of 500nM. On the other hand eosinophils are only showing a minimal response when treated with 2-AG alone (**Fig.V-3-A**). In contrast to 2-AG, JWH-133 is showing almost no effect when used

alone, but is comparably efficient when used together with eotaxin-2, showing its maximum response at 100nM (**Fig.V-3-B**).

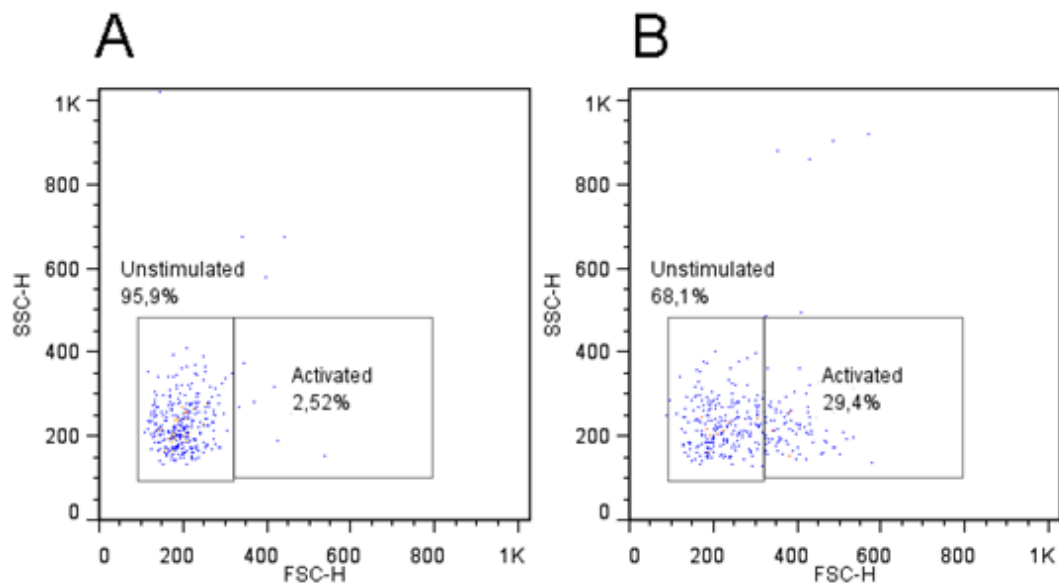


Figure V-2. Representative plots of shape change acquisition

A: FSC/SSC plot of unstimulated isolated human eosinophils. **B:** FSC/SSC plot of isolated human eosinophils stimulated with 0.6nM eotaxin-2/CCL24. Due to the rearrangement of the cytoskeleton upon activation, the cell size increases, which is detectable as an increase of the FSC

Having determined the most effective concentration of JWH-133, I pre-treated eosinophils with 100nM JWH-133 and shape change was induced by serial dilutions of eotaxin-2. As illustrated in **Fig.V-3-C**, eosinophil sensitivity to eotaxin-2 was significantly increased up to three-fold.

Due to the fact that cannabinoids have frequently been reported to show effects beside from CB receptor activation, I exposed the cell to the selective CB2 antagonist SR144528 at a concentration of 1 μ M for 10min prior to JWH-133 treatment. **Fig.V-3-D** reveals that pre-treatment with the antagonist completely reversed the effects of JWH-133 verifying that the direct activation of the CB₂ receptor is responsible for the observed shape change responses.

Off note, JWH-133 is not only able to enhance the effects of eotaxin-2, but also those of eotaxin-1 and prostaglandin D₂ (PGD₂) (**Fig. V3-E/F**).

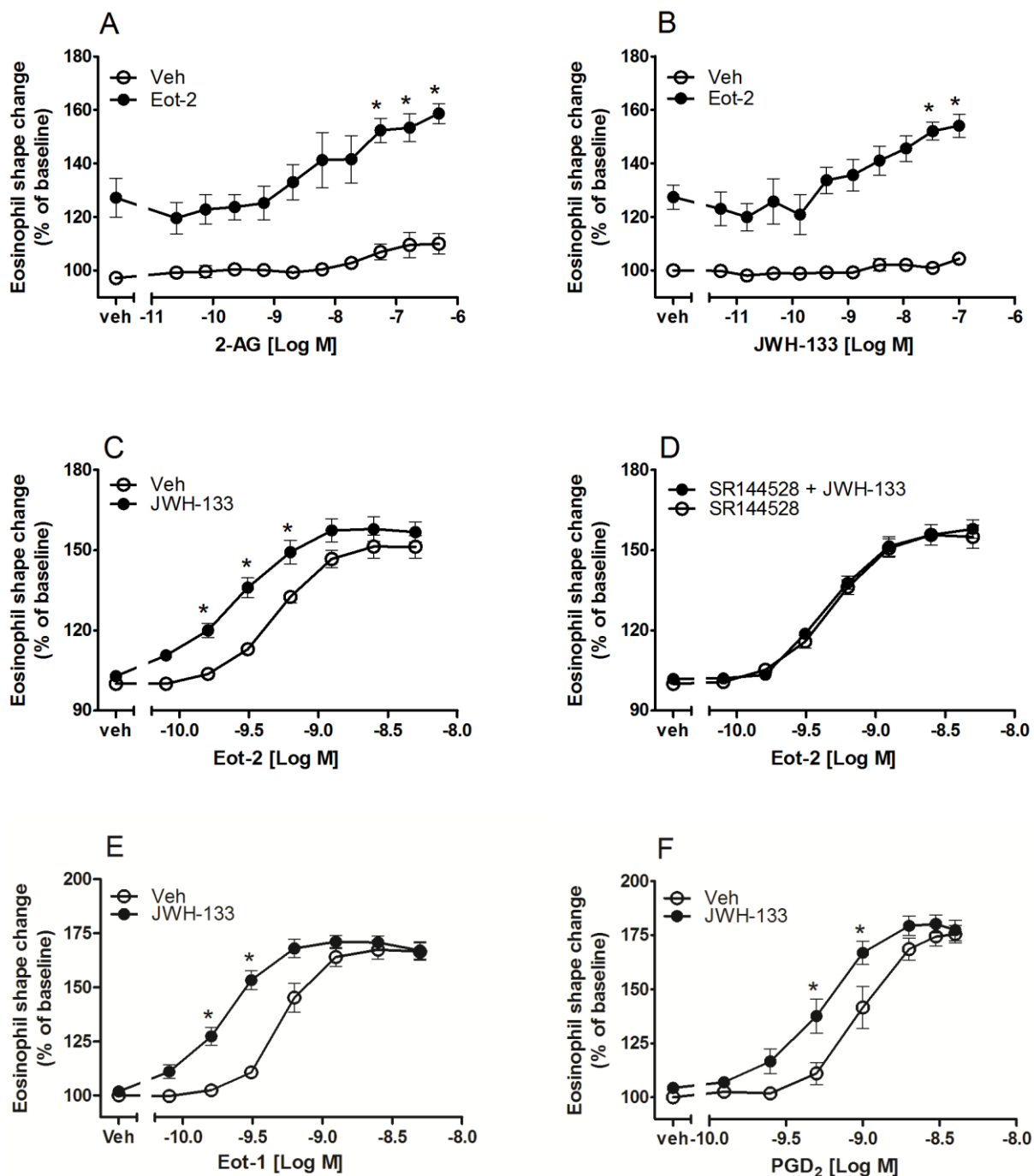


Figure V-3 JWH-133 is enhancing human eosinophil responses induced by eotaxin-2

*Isolated peripheral blood eosinophils were resuspended in assay buffer (with $\text{Ca}^{2+}/\text{Mg}^{2+}$) at a concentration of 2×10^6 cells/ml. **A:** Eosinophils were either pretreated with serial dilutions of 2-AG or vehicle for 5min at room temperature. Thereafter, aliquots were mixed with eotaxin-2 (Final concentration: 0.6nM) or vehicle for 4min at 37°C. Immediately thereafter, samples were transferred to ice and 150µl fixative solution was added to preserve cell structure until analysis by flow cytometry. **B:** Same as in **A** but JWH-133 was used instead of 2-AG. **C:** Isolated human eosinophils were pretreated with JWH-133 (100nM) for 5min at room temperature. Thereafter aliquots were mixed with serial dilutions of eotaxin-2 for 4min at 37°C. Samples were immediately transferred to ice and 150µl fixative solution was added to preserve cell structure until analysis by flow cytometry. **D:** Same as in **C** but a 10min incubation with the selective CB_2 receptor antagonist SR144528 was added before JWH-133 stimulation. **E** and **F:** Same as in **C** but eotaxin-1 or PGD_2 were used instead of eotaxin-2. Data are shown as mean $\pm$ SEM. * $p < 0.05$ versus vehicle $n = 4-7$.*

V.III. JWH-133 shows no effect on human neutrophils and basophils

The previously described effects of the endocannabinoid 2-AG on human neutrophils are controversial. In 2006 Kurihara et al. reported that 2-AG and JWH015 are inducing shape change in HL-60 neutrophil like cells but are decreasing N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) induced actin polarization and migration (Kurihara et al., 2006) in human peripheral blood neutrophils. Opposing effects were described five years later by Chouinard et al. who discovered that 2-AG activates human neutrophils due to its hydrolysis to arachidonic acid and glycerol and subsequent metabolism to leukotriene B_4 (Chouinard et al., 2011).

Regarding basophils, apart from one paper showing reduced CD63 expression upon 2-AG treatment, little is known about the impact of cannabinoids (Vannacci et al., 2002).

To address the possibility of cannabinoid hydrolysis I used the synthetic CB_2 agonist JWH-133, which is not a substrate for monoacylglycerol-lipase (MAGL), the main degrading enzyme of 2-AG, and does not contain an arachidonic acid domain in its molecular structure. **Fig.V-4-A** shows that JWH-133 pre-treatment of human

neutrophils does not influence their response to interleukin-8 (IL-8) used as stimulant. Basophils show slightly enhanced, although insignificant, responses to eotaxin-2 when treated with JWH-133 (**Fig.V-4-B**)

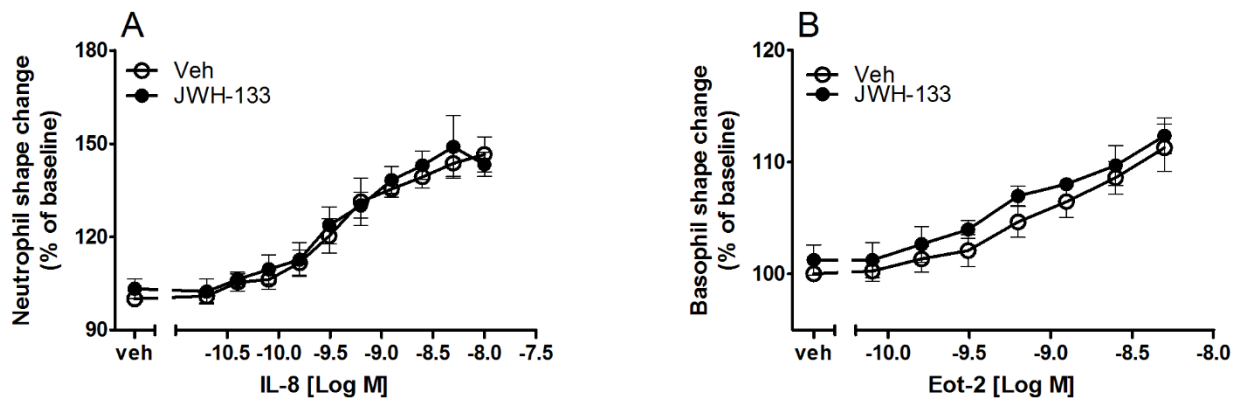


Figure V-4 JWH-133 shows no effect on neutrophil and basophil shape change

Polymorphonuclear leukocytes (PMNL) or blood mononuclear cells (PBMC) were resuspended in assay buffer (with $\text{Ca}^{2+}/\text{Mg}^{2+}$) at a concentration of 2×10^6 cells/ml. **A:** Neutrophils were either pretreated with JWH-133 (100nM) or vehicle for 5min at room temperature. Thereafter, aliquots were mixed with serial dilutions of IL-8 for 4min at 37°C . Immediately after, samples were transferred to ice and 150 μl fixative solution was added to preserve cell structure until analysis by flow cytometry. **B:** Same as in **A** but PBMC preparations were used instead of PMNL. Neutrophils were distinguished from eosinophils by their lower auto-fluorescence in the FL2 (585/42nm) channel. Basophils were identified as $\text{CD123}^+/\text{HLA-DR}^-$ cells in PBMC preparations. Data are shown as mean $\pm$ SEM. * $p < 0.05$ versus vehicle $n = 4-7$.

Taken together enhanced responses to chemoattractants following CB_2 receptor activation seem to be eosinophil specific, as neither neutrophils nor basophils show enhanced shape change activity.

V.IV. JWH-133 primes eosinophils for enhanced CD11b expression

The data obtained from the previous shape change experiments suggested JWH-133 as a novel priming factor for human eosinophils. Priming is the process of enhancing a specific cellular response by a molecule or process that is not inducing the response itself but is amplifying a subsequent stimulus generated by chemokines/chemoattractants. The best described priming factor for eosinophils is IL-5. In previous studies done with eosinophils isolated from allergic patients it was shown that BAL eosinophils display higher chemotactic responses to fMLP and PAF, are more likely to degranulate and show enhanced expression of CD11b on their cell surface. Interestingly when eosinophils from healthy donors were treated with IL-5 *in vitro*, similarly enhanced responses were obtained, suggesting IL-5 to be a major contributing factor in the pathogenesis of allergic diseases by priming eosinophils (Sampson, 2001).

After the priming process, the next step in the inflammatory cascade is the interaction with endothelial cells, a selectin/integrin mediated process which ultimately leads to the tight adhesion to the vessel wall. Eosinophils have been reported to express 7 different integrin molecules (Matsumoto and Bochner, 2013a), of which the expression of $\alpha M\beta_2$ (CD11b/18; MAC-1) was shown to be rapidly increased after exposure to cytokines, chemotactic or inflammatory factors (Walker et al., 1993). Interestingly, increased expression of $\alpha M\beta_2$ has been associated with allergic diseases like atopic dermatitis or bronchial asthma. Moreover, a “hyperadhesive” eosinophil phenotype that relies greatly on enhanced $\alpha M\beta_2$ levels has been reported after allergen challenge in asthmatic patients (Barthel et al., 2006).

Thus, I investigated the contribution of JWH-133 to integrin upregulation by labeling the αM subunit (CD11b) of MAC-1 with an antibody conjugated to phycoerythrin (PE). To this end, whole blood samples were treated with 1 μ M of JWH-133 before stimulation with eotaxin-2 for 30min. **Fig. V-5** shows that pre-treatment with JWH-133 resulted in enhanced CD11b upregulation by eotaxin-2. Especially at lower concentrations of eotaxin-2, JWH-133 led to a 50% increase of CD11b on the surface of human eosinophils which may then further contribute to increased adhesion to endothelial cells by interacting with its physiological ligand ICAM-1.

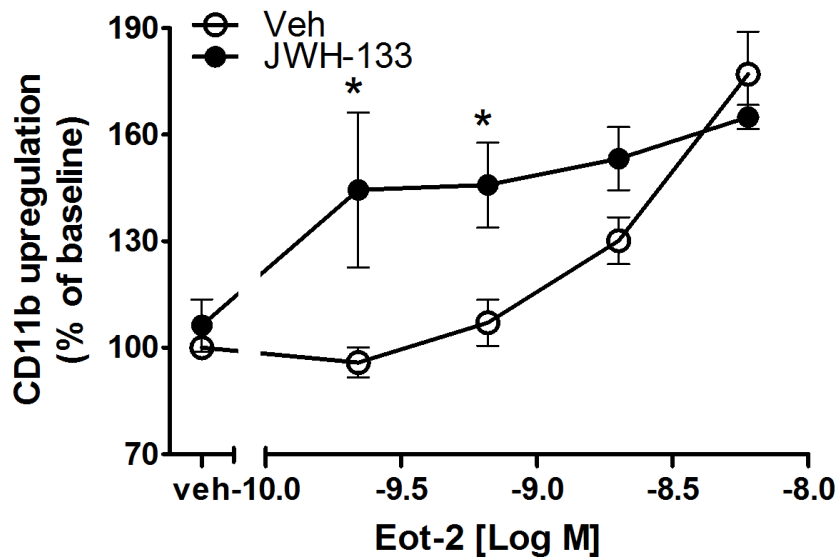


Figure V-5 JWH-133 increases CD11b expression on human eosinophils induced by eotaxin-2

Whole blood samples were pre-treated with JWH-133 (1 μ M) or vehicle for 10min at room temperature. CD11b upregulation was induced by incubating the cells in the presence of serial eotaxin-2 concentrations for 30min at 37 °C. Samples were stained with anti-CD16-PE-Cy5 and anti-CD11b-PE Ab. CD11b upregulation was analyzed by flow cytometry and expressed as percent of the vehicle response. Eosinophils were identified as CD16⁺ cells in the granulocyte forward scatter /side scatter region. Data are shown as mean $\pm$ SEM. * $p < 0.05$ versus vehicle $n=6$

V.V. JWH-133 increases eosinophil adhesion to ICAM-1 under flow conditions

Binding and firm adhesion of human eosinophils to endothelial cells via α M β ₂-ICAM-1 interaction is of great importance in transendothelial migration (Ebisawa et al., 1992). As eosinophil priming with JWH-133 followed by eotaxin-2 stimulation showed significantly enhanced CD11b levels on human eosinophils, the next step would be to prove that enhanced expression actually means enhanced adhesion. An elegant method to study leukocytes and their interactions with endothelial cells or

recombinant ligands or ECM proteins is the Cellix™ system. Biochips, containing eight channels, can be coated with endothelial cells or proteins of choice. After loading a reservoir on the chip with the desired amount of leukocytes, a micro-pump is connected to the opposing end of the channels and individually controllable fluid shear stress is applied.

For the experiment shown in **Fig. V-6**, channels were coated with 10µg/ml recombinant human ICAM-1 and fluid shear stress was set to 0.5 dyne/cm², resembling typical shear stress on the venous side of the circulation. After pre-treating the eosinophils with either vehicle or the CB₂ antagonist SR144528 (1µM) for 5min at room temperature, cells were stimulated with either eotaxin-2 (0.6nM) or JWH-133 (250nM) or a combination of both. Following eosinophil superfusion, adhesion was monitored using a Hamamatsu ORCA-03G digital camera.

As illustrated in **Fig. V-6A** treating eosinophils with a combination of JWH-133 and eotaxin-2 potently enhanced adhesion (up to ~2.3 fold) to ICAM-1. CB₂ specificity was again confirmed with the selective antagonist SR144528, which completely reverse the effect of JWH-133.

Fig. V-6B and **C** shows representative images taken during the superfusion. Tightly adherent eosinophils can be differentiated from rolling cells by their increased size and structure when compared to smaller/round shaped rolling eosinophils. For statistical analysis only tightly adherent cells were counted.

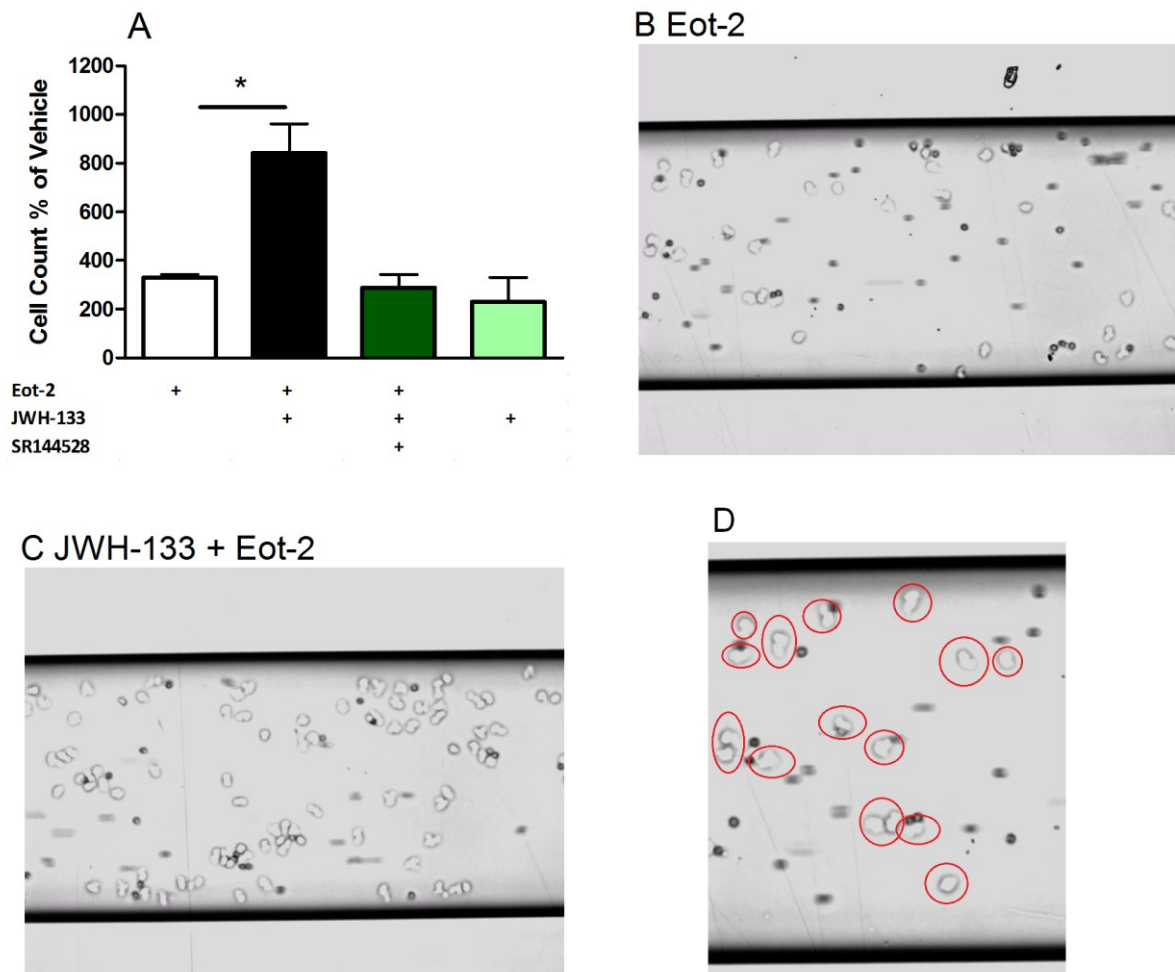


Figure V-6 Eotaxin-2 induced adhesion to ICAM-1 coated channels is increased by JWH-133

Vena8™ biochips were coated with 10 $\mu\text{g/ml}$ ICAM-1 and coated channels were superfused with 2×10^6 purified eosinophils at 0.5 dyne/cm^2 for 5min at 37 °C in an OKOLAB H201-T1 heated cage. Before eosinophils were superfused they were treated with a combination of eotaxin-2 (0.6nM), JWH-133 (250nM) and SR144528 (1 μM). Adhesion was monitored on a Zeiss Axiovert 40 CFL microscope equipped with a Zeiss A-Plan 103/0.25 Ph1 lens, using a Hamamatsu ORCA-03G digital camera and CellixVenaFlux software. Cell adhesion was quantified by computerized image analysis using DucoCell analysis software (**D**: Tightly adherent cells (red circles) were counted). Data are shown as mean $\pm$ SEM. * $p < 0.05$ versus vehicle $n=5$

V.VI. Migratory responses are enhance by JWH-133

All experiments shown here so far provided evidence for a regulatory role of the cannabinoid receptor CB2 in the inflammatory process. The final step in this process is the migration or chemotaxis of inflammatory cells to the affected tissue. At the inflammatory site various inflammation-associated cytokines are released resulting in a cytokine gradient. Subsequently, adhesion molecules like selectins or integrins are upregulated on endothelial cells and the homing of leukocytes into the target tissue is initiated (Matsumoto and Bochner, 2013a).

The first report of significant CB2-induced human eosinophil migration was published in 2004 by Oka et al. Interestingly in 2014 it was stated that 2-AG alone induces only minimal migration and requires a co-stimulus by either IL-3, IL-5 or GM-CSF. Furthermore, the authors discovered that the MAGL inhibitor JZL-184 partially inhibited eosinophil chemotaxis induced by 2-AG, suggesting that hydrolysis of 2-AG into arachidonic acid and glycerol is involved. Arachidonic acid is a well-recognized substrate for the COX and LOX pathways responsible for the generation of a variety of chemotactic active substances (Turcotte et al., 2015). Of note, they found that the 15-LOX inhibitor PD146176 could completely inhibit the 2-AG mediated chemotaxis.

For the chemotaxis experiment shown in **Fig. V-6** I used the synthetic cannabinoid receptor 2 agonist JWH-133 which cannot be metabolized by MAGL excluding the possibility of metabolite influence on the generated results.

We discovered that JWH-133 itself is inducing a moderate migratory response in human eosinophils. The maximal effect (chemotactic index ~ 3.9) was observed at a concentration of 100nM. At higher concentrations a chemokinetic effect (chemotactic index~1.8) that peaked at 3µM was detected (**Fig. V-6A**).

In order to elucidate a possible priming effect, human eosinophils were pretreated with a low nanomolar concentration of JWH-133 (5nM) and chemotaxis was induced by 1nM eotaxin-2. In this setting JWH-133 was able to increase the responses towards eotaxin-2 by up to 80% (**Fig. V-6B**)

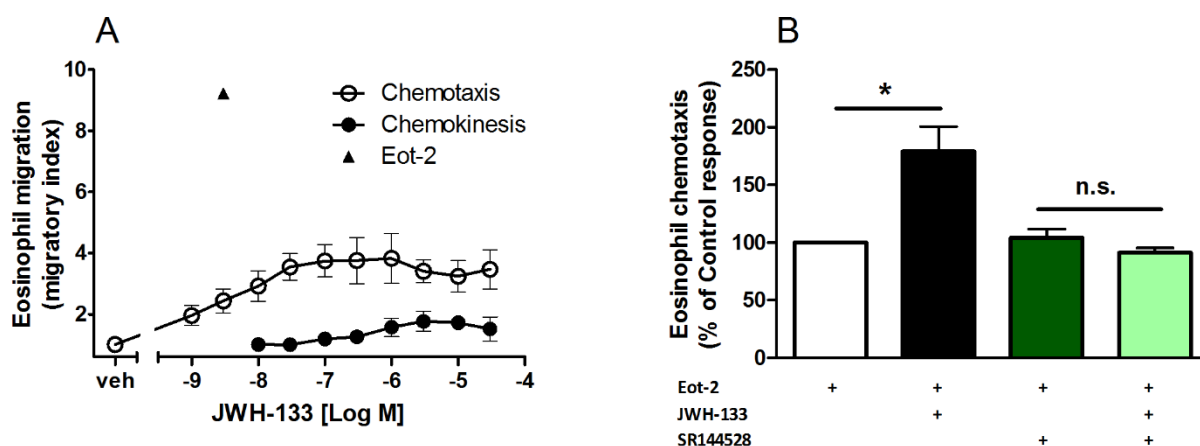


Figure V-7 JWH-133 and its effects on human eosinophil migratory behavior

Chemotaxis: **A** and **B:** Human peripheral blood eosinophils, purified by negative magnetic selection were resuspended at a concentration of 2×10^6 cells/ml. **A:** 50 μ l aliquots of cell suspension were placed into the top wells of a 5 μ m pore-sized 48-well micro-Boyden chamber were allowed to migrate towards serial dilutions of JWH-133 for 1 hour at 37 °C. Chemokinesis: Same as above but after the pretreatment of purified human eosinophils was done with serial dilutions of JWH-133 and they were allowed to migrate towards assay buffer. **B:** Same as in **A** but pre-treatment was done with 5nM JWH-133, SR144528 (1 μ M) or a combination and cells were allowed to migrate towards 1nM eotaxin-2. After the respective time points, cells that have migrated to the bottom wells of the micro-Boyden chamber were transferred to small FACS tubes and 150 μ l fixative solution was added. Analysis was done by flow cytometry. Data are shown as mean $\pm$ SEM. * $p < 0.05$ versus vehicle $n = 6$

V.VII. JWH-133 enhances ROS production but not EPO release or degranulation

Tissue injury is an important mechanism in the pathogenesis of allergic asthma, causing airway hyper-reactivity (AHR) and airway remodeling. Even bronchoconstriction can result from damaging epithelial cells, as the release of bronchodilatory substances like PGE₂ was shown to be diminished (Chanez et al., 1990). During respiratory burst, human eosinophils can generate reactive oxygen

species (ROS) including superoxide and hydrogen peroxide. Increased ROS production has been associated with allergic asthma and was furthermore shown to cause tissue damage (Thomas and Busse, 2013).

In **Fig. V-8** I could show that the endocannabinoid system plays a role in eosinophil ROS production, as pre-treatment with JWH-133 led to increased generation of ROS up to 50% after eotaxin-2 stimulation (**Fig. V-8A**)

Further, eosinophil derived mediators discovered to damage airway tissue, are the three major eosinophil associated proteins located in their secondary granules: major basic protein (MBP), eosinophil derived neurotoxin (EDN) and eosinophil peroxidase (EPO). All three were found at increased levels in the BAL fluid of allergic patients and were further shown to be damaging respiratory tract cells (Thomas and Busse, 2013). To assess eosinophil degranulation, I used an antibody against CD63 which is located within the granule membrane of eosinophils but not on their cell surface. During eosinophil degranulation, the granule membrane fuses with the cell membrane and CD63 is exposed at the cell surface and allows fluorophore labeled antibodies to bind. Another method to assess eosinophil degranulation is the detection of released EPO activity, using tetramethylbenzidine (TMB) which is oxidized by EPO and develops a permanent dark blue reaction product.

Interestingly, although JWH-133 is significantly affecting eosinophil recruitment and ROS production, I could not detect enhancement of eosinophil degranulation induced by serial dilutions of complement factor 5a (C5a), neither by means of CD63 upregulation on the cell surface nor by EPO release (**Fig. V-8B and C**).

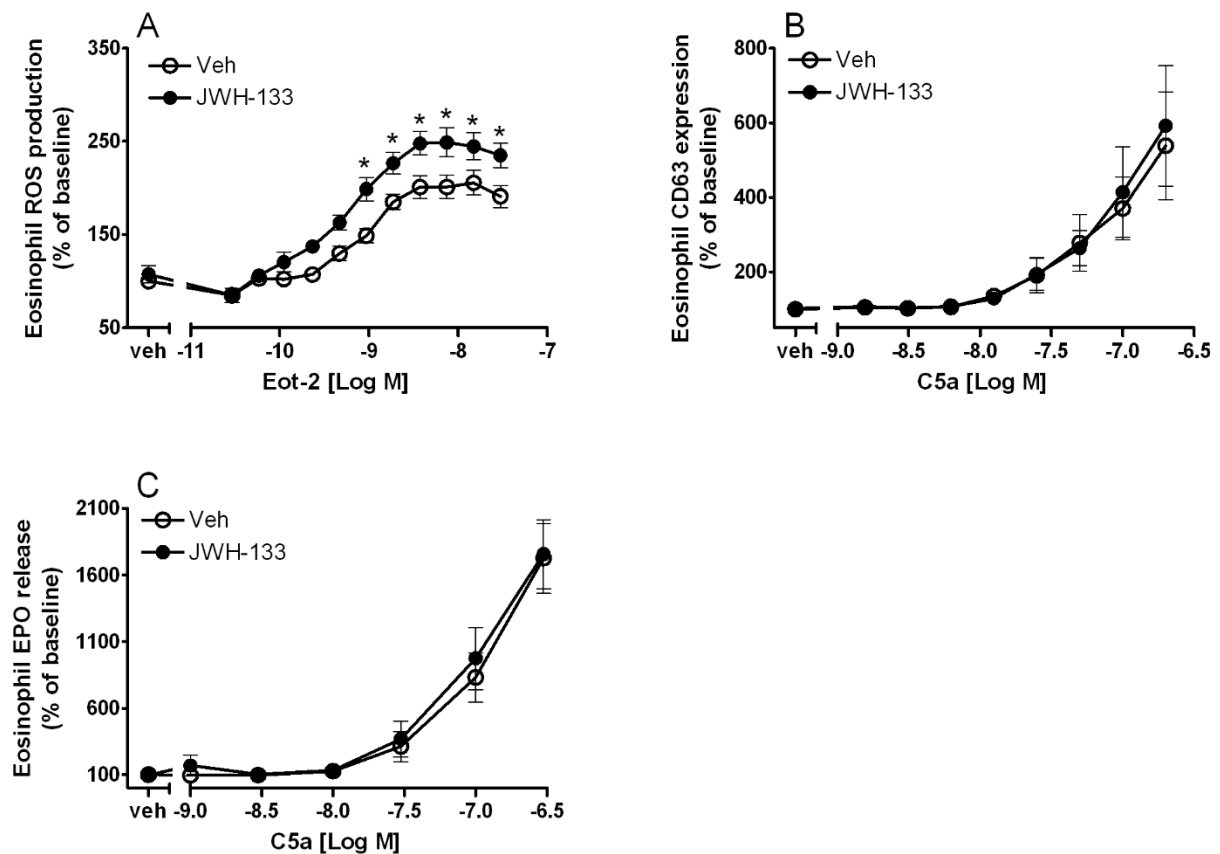


Figure V-8 Eosinophil ROS production is enhanced by JWH-133

A: Human peripheral blood eosinophils were purified by negative magnetic selection and resuspended at a density of 1×10^6 cells/ml in assay buffer (with $\text{Ca}^{2+}/\text{Mg}^{2+}$). Cells were pretreated with 250nM JWH-133 and then stimulated with serial dilutions of eotaxin-2 in the presence of 50 μM dichlorofluorescein diacetate (DCFDA) for 20min at 37°C. Eosinophil respiratory burst was measured as an increase in fluorescence of the FL1- (530/30nm) channel due to generation of the fluorophore DCF in the presence of reactive oxygen species (ROS). Responses were expressed as percent of the vehicle response. **B:** Purified human eosinophils were resuspended at 1×10^6 cells/ml in assay buffer (with $\text{Ca}^{2+}/\text{Mg}^{2+}$) in the presence of an anti-CD63-FITC antibody. Cells were then pre-treated for 10min at room temperature with 250nM JWH-133 and cytochalasin B (5 $\mu\text{g}/\text{ml}$) was added for 5min at 37 °C. Degranulation was induced with serial dilutions of C5a for 20min at 37 °C. CD63 expression was detected by flow cytometry and expressed as percent of the respective control responses. **C:** Eosinophils were purified and resuspended at 1×10^6 cells/ml in assay buffer (with $\text{Ca}^{2+}/\text{Mg}^{2+}$). Cells were pretreated with JWH-133 (250nM) and

*cytochalasin B (10µg/ml) was added. 50µl aliquots were transferred into the wells of a 96-well microplate. After stimulation with serial dilutions of C5a for 20min at 37 °C, H₂O₂ (1mM) was added to start the peroxidase reaction. Tetramethylbenzidine (2.8mM) was added to detect the reaction and color development was terminated by addition of 4M acetic acid. EPO release was quantified on a bench reader at a wavelength of 630nm and data was expressed as percent of the respective control responses. Data are shown as mean ± SEM. * p<0.05 versus vehicle n=5*

V.VIII. The modulatory effects of CB₂ activation are PTX insensitive

CB₂ receptors are known to activate heterotrimeric G_{α_{i/o}} type G proteins, leading to the inhibition of the adenylyl cyclase (AC) and a reduction in intracellular cyclic AMP. Furthermore, the initial study describing the chemotactic effect of 2-AG on eosinophils also reported this response to be mediated via a G_{α_{i/o}} dependent pathway as pertussis toxin (PTX) completely inhibited eosinophil chemotaxis towards 2-AG. Alternatively, CB₂ signalling can be mediated through MAP kinases (p38 and p42/44) and PI3K, or influence gene transcription by reducing the DNA-binding affinity of CREB (Atwood et al., 2012). To investigate if the JWH-133-mediated effects on eosinophil function were dependent on G_{α_i} protein activation, I performed shape change experiments with the G_{α_i} protein inhibitor pertussis toxin (PTX). PTX catalyzes ADP-ribosylation of the G_{α_i} protein as a result of which the G_i subunit remains locked in its GDP bound, inactive state, thus making it unable to inhibit its target, adenylyl cyclase (AC).

In **Fig. V-9** shape change was either induced by PGD₂ (PTX insensitive, as CTRH₂ signaling involves G_q (Stubbs et al., 2002)) or eotaxin-2 (PTX sensitive as CCR3 is also coupled to G_i). Notably, I discovered that pretreatment with PTX is not affecting the modulatory effects of JWH-133 on PGD₂ induced shape change (**Fig. V-9A-B**). In a control experiment, PTX completely abrogated the eotaxin-2 induced responses, but not the priming effect of JWH-133 (**Fig. V-9C-D**). Interestingly, a similar heterogeneity in signaling pathways has already been described for CB₁, which is able to activate G_i, G_s or G_q resulting in activation/inhibition of AC or Ca²⁺ release (Glass and Felder, 1997; Lauckner et al., 2005; McIntosh et al., 2007).

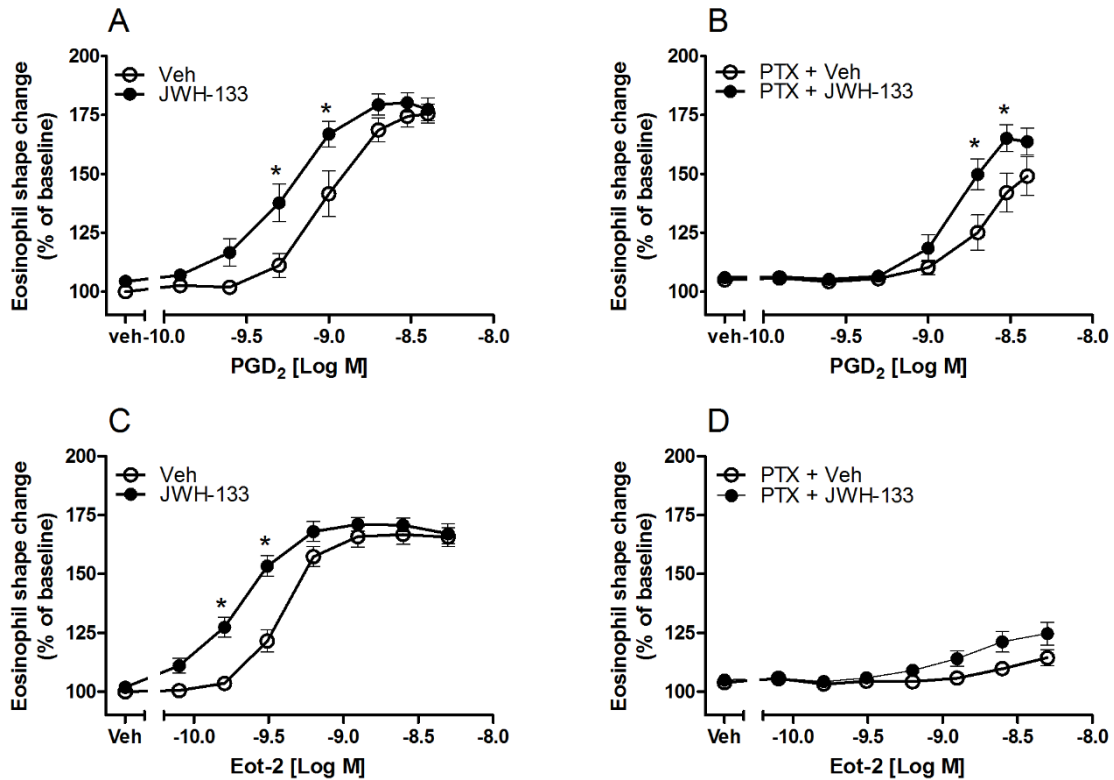


Figure V-9 JWH-133 is enhancing eosinophil functions in a PTX insensitive manner

Isolated peripheral blood eosinophils were resuspended in assay buffer (with $\text{Ca}^{2+}/\text{Mg}^{2+}$) to a concentration of 2×10^6 cells/ml. **A:** Eosinophils were either pretreated with JWH-133 (100nM) or vehicle for 5min at room temperature. Thereafter, aliquots were mixed with serial dilutions of PGD_2 for 4min at 37°C . Samples were immediately transferred to ice and 150 μl fixative solution was added to preserve cell structure until flow cytometric analysis. **B:** Same as in **A**, but prior to JWH-133 treatment eosinophils were exposed to PTX (5 $\mu\text{g}/\text{ml}$) for 20min at 37°C . **C:** Isolated human eosinophils were pretreated with JWH-133 (100nM) for 5 min at room temperature. Thereafter aliquots were mixed with serial dilutions of eotaxin-2 for 4min at 37°C . **D:** Same as in **B** but eotaxin-2 was used instead of PGD_2 . Data are shown as mean $\pm$ SEM. * $p < 0.05$ versus vehicle $n=6$.

V.IX. CB₂ stimulation induces intracellular Ca²⁺ flux in eosinophils

The previous experiment revealed a potential role of a G_q mediated signaling cascade induced by CB₂ stimulation with JWH-133. In general, stimulation of G_{q/11} coupled receptors leads to activation of phospholipase C (PLC). PLC hydrolyses phosphatidylinositol 4,5-bisphosphate and is thereby generating diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP₃) resulting in protein kinase C (PKC) activation and Ca²⁺ release, respectively (Berridge, 1993).

Intracellular Ca²⁺ release in eosinophils can easily be monitored with Ca²⁺ sensitive dyes which increase their fluorescence upon Ca²⁺ binding. The dye that I used for Ca²⁺ flux assays, is Fluo-3-AM which is the acetoxymethylester form of Fluo-3. Due to its lipophilicity it can pass through the cell membrane and is cleaved by unspecific esterases in the cytosol, thus trapping the dye inside the cell.

Stimulation of eosinophils with 200nM JWH-133 led to Ca²⁺ increase up to 1.4 fold over baseline which I could inhibit by pre-treating the cells with SR144528 (1μM) (**Fig. V-10A**). At 100nM JWH-133 induced only a weak Ca²⁺ signal compared to the endocannabinoid 2-AG which was about five-fold stronger (**Fig. V-10B**).

To confirm the PTX insensitivity of CB₂ signaling, eosinophils were again treated with 5μg/ml PTX and Ca²⁺ release was either induced by JWH-133 (200nM), 2-AG (200nM) or eotaxin-2 (1nM). As anticipated PTX treatment completely blocked eotaxin-2 induced Ca²⁺ release but neither 2-AG nor JWH-133 (**Fig. V-10C**). As opposed to PTX, the PLC inhibitor U-73122 (but not the inactive form U-73343) and the IP₃ receptor antagonist 2-APB were capable of inhibiting CB₂-induced responses. 2-APB is also known to inhibit TRP channels; however, control experiments in the presence of 3mM EGTA showed that the observed Ca²⁺ signal is not due to Ca²⁺ influx from the extracellular space and, therefore, not TRP mediated (**Fig. V-10C**).

According to the data obtained, CB₂ is suggested to signal via a Gα_{i/o}/adenylyl cyclase-independent pathway by interacting with Gα_{q/11} resulting in PLC mediated IP₃ generation and subsequent Ca²⁺ release.

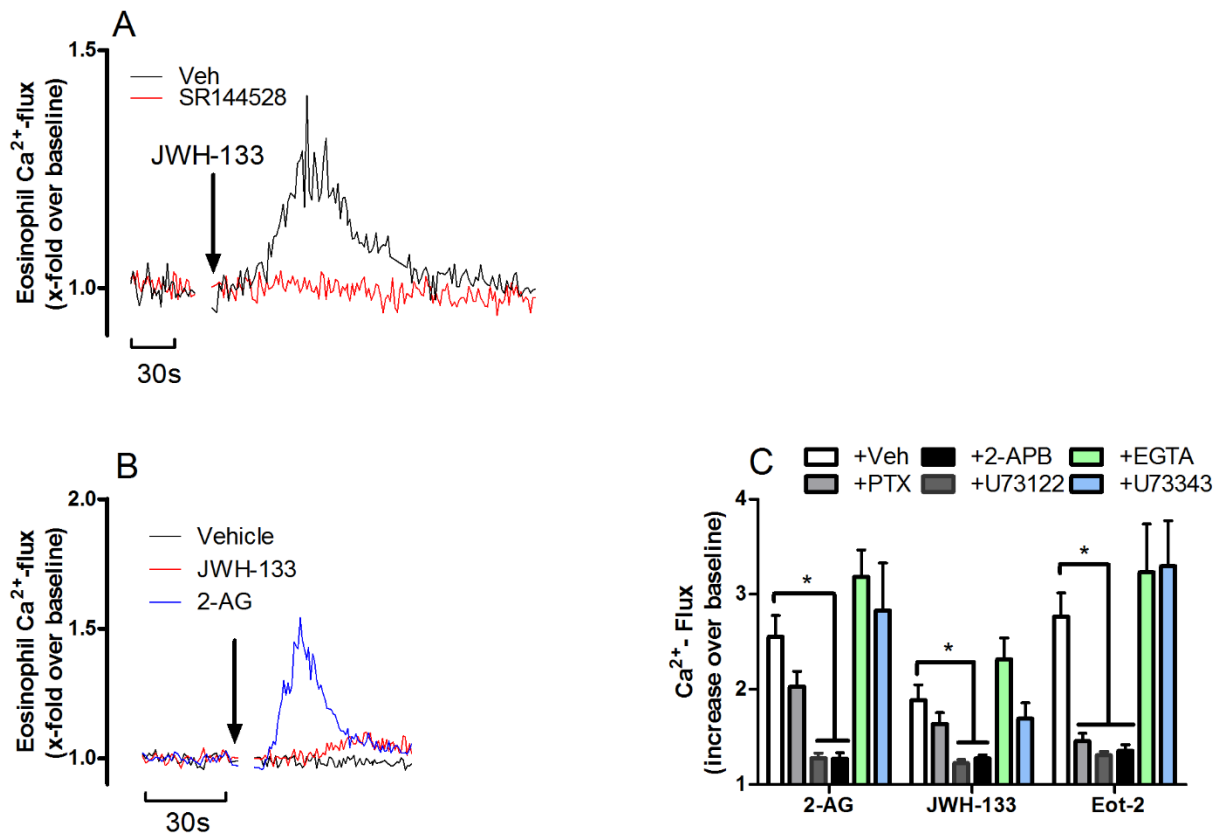


Figure V-10 JWH-133 and 2-AG are inducing eosinophil Ca^{2+} flux via CB_2 receptor activation

Human peripheral blood eosinophils or PMNL were resuspended in 1ml assay buffer (without $\text{Ca}^{2+}/\text{Mg}^{2+}$) to a concentration of 10×10^6 cells/ml. The Ca^{2+} sensitive dye Fluo-3 AM was added to a final concentration of $2 \mu\text{M}$ in the presence of 0.02% pluronic F-127 and cells were incubated for 60min at room temperature in the dark.

A: Eosinophils were pre-treated with SR144528 or vehicle for 10min at room temperature. Baseline fluorescence was acquired for 30sec and then Ca^{2+} flux was induced by 200nM JWH-133. **B:** Ca^{2+} flux was induced by 100nM JWH-133 or 2-AG. **C:** Eosinophils were pretreated with $5 \mu\text{g/ml}$ PTX for 20min, with $6 \mu\text{M}$ U-73122 or its inactive form U73343, $50 \mu\text{M}$ 2-APB or vehicle, and Ca^{2+} flux was induced by 200nM JWH, 200 nM 2-AG or 1nM eotaxin-2. In some experiments EGTA (3mM) was added prior to stimulation. Data are shown as mean $\pm$ SEM, * $p < 0.05$, $n=5-10$

V.X. Desensitization of CB₂ and the effect on eotaxin-2 induced Ca²⁺ flux

In the previous chapter I showed that the same concentrations of 2-AG and JWH-133 are having significantly different effects on the magnitude of released Ca²⁺. This may suggest that their pathways slightly differ or might involve the activation of a different receptor. Previous studies reported human eosinophils to express the main 2-AG degrading enzyme, MAGL. Furthermore Ca²⁺ release studies in human neutrophils revealed that 2-AG is rapidly converted to AA and LTB₄ is generated which was then inducing Ca²⁺ release (Chouinard et al., 2011). To exclude possible involvement of LOX or COX products on the observed Ca²⁺ flux, desensitization experiments were conducted. In **Fig. V-11A** it is shown that stimulation of eosinophils with 100nM JWH-133 is inhibiting a subsequent response induced by 2-AG. It can be concluded, hence, that JWH-133 and 2-AG are inducing intracellular Ca²⁺ release indeed via the same receptor.

Next, I was interested to elucidate whether the priming effect of CB₂ activation is due to enhanced Ca²⁺ release. Therefore, eosinophils were stimulated with 2-AG or JWH-133 (100nM) and after 60 sec cells were again stimulated by eotaxin-2. As clearly apparent from **Fig. V-11C**, opposed to the enhancing effects of JWH-133 on shape change, CD11b and chemotaxis, Ca²⁺ release by eotaxin-2 was unaffected by prior CB₂ activation. In contrast, it rather seems to lower the amount of released Ca²⁺ by eotaxin-2, probably due to the already released Ca²⁺ by JWH-133.

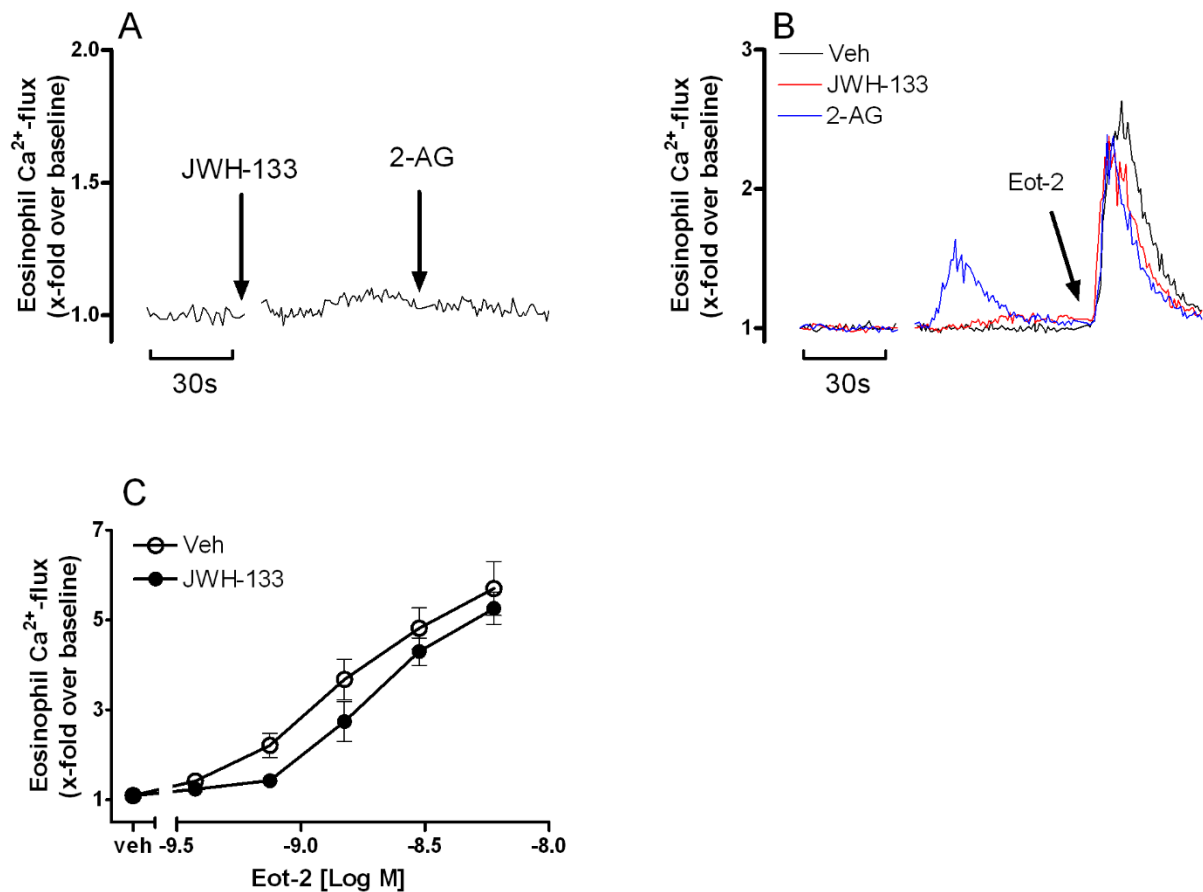


Figure V-11 Desensitization of CB₂ and the effect of JWH-133 pre-treatment on eotaxin-2 induced Ca²⁺ release

Human peripheral blood eosinophils or PMNL were resuspended in 1ml assay buffer (without Ca²⁺/Mg²⁺) to a concentration of 10x10⁶ cells/ml. The Ca²⁺ sensitive dye Fluo-3 AM was added to a final concentration of 2μM in the presence of 0.02% pluronic F-127 and cells were incubated for 60min at room temperature in the dark. **A:** Baseline fluorescence was acquired for 30sec and then 100nM JWH-133 was added. After measuring intracellular Ca²⁺ for another 60sec, 100nM 2-AG was added. **B:** After acquiring baseline fluorescence for 30sec 100nM JWH-133 or 2-AG were added. The second stimulus is set by 1.5nM eotaxin-2 60sec after the first stimulation. **C:** Same as in **B** but with serial dilutions of eotaxin-2. Data are shown as mean ± SEM, * *p* < 0.05, *n*=5-10

V.XI. Signaling pathways activated by CB₂ stimulation

Calcium release via IP₃ generation is not the only possible signaling pathway of G_q coupled GPCRs. Accordingly, CB₂ has been shown to induce a MAPK/ERK signaling cascade dependent on the G_{βγ} subunit of the GPCR.

In order to detect down-stream targets of the CB₂ pathway in human eosinophils, various kinases also involved in the migration of eosinophils including PI3K, ROCK, ERK and p38 MAPK (Adachi et al., 2001) were pharmacologically inhibited. For this purpose, I used the validated kinase inhibitors U-0126 (MEK1/2, 10μM), PD-184161 (MEK1/2, 18μM), LY-294002 (PI3K, 10μM), SB-202190 (p38 MAPK, 50μM) and Y-27632 (ROCK, 200nM). Concentrations were chosen according to literature and cells were pre-treated for 20min at 37°C. Following a 5min incubation with JWH-133 (100nM), eosinophils were stimulated with serial dilutions of eotaxin-2.

As shown in **Fig. V-12**, I discovered that the MEK1/2 inhibitors U-0126 (**B**) and PD-184161 (**C**) as well as the p160 ROCK inhibitor Y-27632 (**D**) effectively reduced the priming effect of JWH-133 for eotaxin-2 induced responses, whereas blockade of PI3K (**E**) and p38 MAP kinase (**F**) showed no effect.

After confirming that JWH-133 augments eosinophil responsiveness via MEK1/2 and ROCK signaling in shape change, I studied the selective contribution of MEK1/2 and ROCK to JWH-133-induced chemotaxis. Similarly, pre-treatment with U-0126 and Y-27632 prior to the induction of chemotaxis, significantly inhibited eosinophil migration towards serial dilutions of JWH-133 (**Fig. V-12 G and H**).

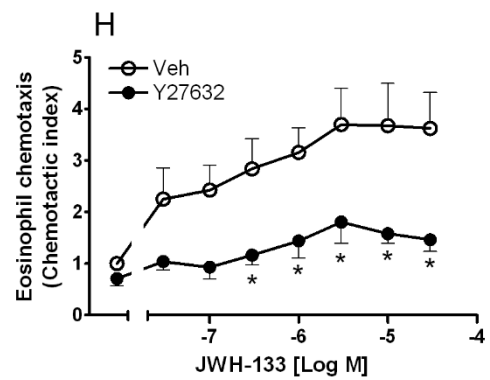
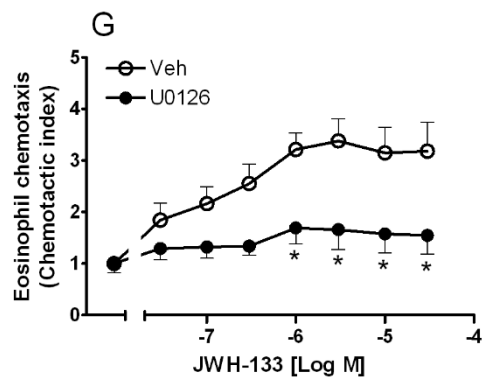
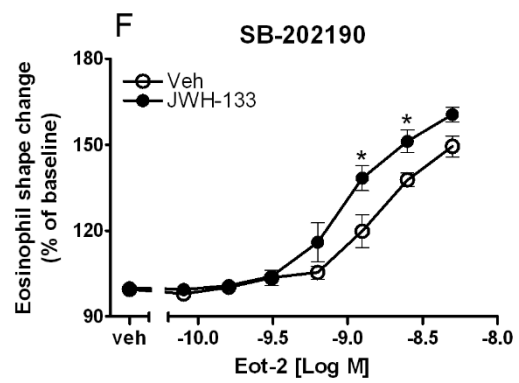
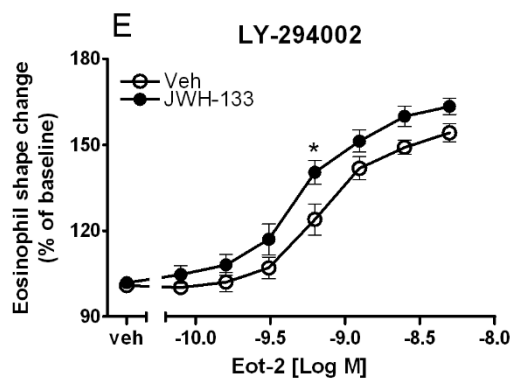
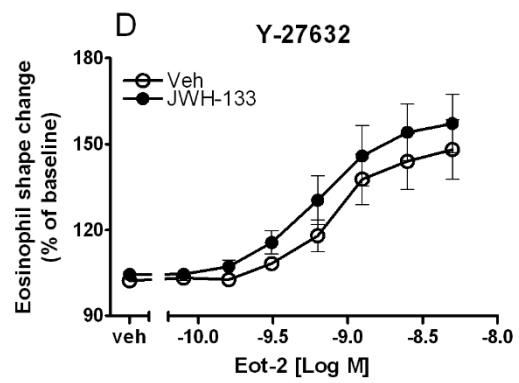
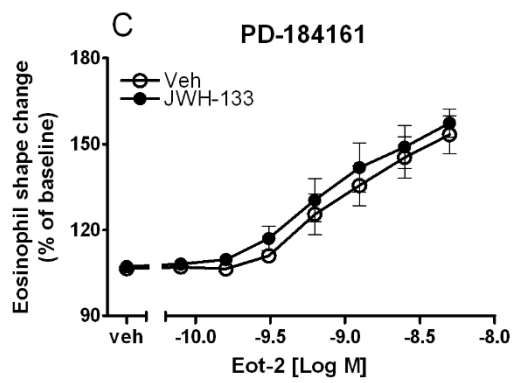
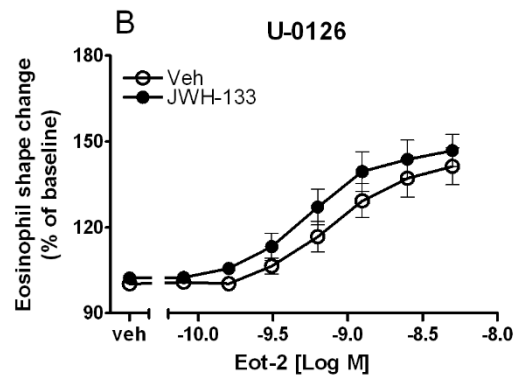
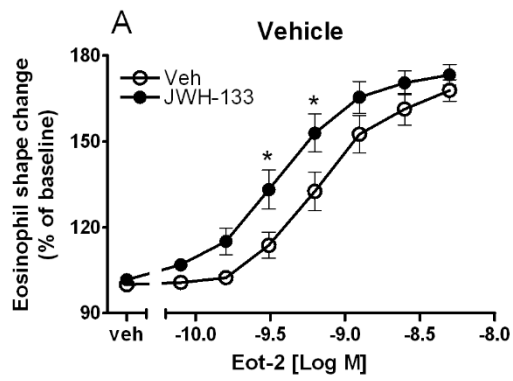


Figure V-12 MEK1/2 and p160 ROCK are involved in the modulating effect of JWH-133.

*Isolated peripheral blood eosinophils were resuspended in assay buffer (with $\text{Ca}^{2+}/\text{Mg}^{2+}$) at a concentration of 2×10^6 cells/ml. Eosinophils were pre-treated with the protein kinase inhibitors LY-294002 (PI3K, 10 μM , **B**), U-0126 (MEK1/2, 10 μM , **C**), PD-184161 (MEK1/2, 18 μM , **D**), SB202190 (p38 MAPK, 50 μM , **E**) and Y-27632 (ROCK, 200 nM, **F**) or vehicle (**A**). Following incubation with JWH-133 (100 nM) or vehicle, eosinophils were stimulated with serial dilutions of eotaxin-2 for 4min at 37°C. Immediately thereafter, samples were transferred to ice and 150 μl fixative solution was added to preserve cell structure until analysis by flow cytometry. In **G** and **H** eosinophils were pretreated with U-0126 and Y-27632 or vehicle and were allowed to migrate towards serial dilutions of JWH-133 for 1h at 37°C. After the incubation, cells that have migrated to the bottom wells of the micro-Boyden chamber were transferred to small FACS tubes and 150 μl fixative solution was added. Analysis was done by flow cytometry. Data are shown as mean $\pm$ SEM. * $p < 0.05$ versus vehicle $n=5-12$*

VI Results Part II (Cannabinoids *in vivo* + mouse *in vitro*)

In the first part of the results section, I characterized the regulatory role of the endocannabinoid/CB₂ axis in the priming and activation of human peripheral blood eosinophils. Furthermore CB₂ receptor expression was higher in eosinophils isolated from allergic donors compared to healthy control, implying a potential role in the pathogenesis of eosinophil-driven allergic diseases such as asthma.

The next step of this study focuses on the involvement of the endocannabinoid system in the biology of mouse eosinophils *in vitro* to later address the contribution of the CB₂ receptor to eosinophil function in an *in vivo* mouse model of allergic asthma.

VI.I. CB₂ activation on mouse bmEos leads to Ca²⁺ flux

The isolation of peripheral blood eosinophils from mice only yields a limited amount of cells, thus eosinophils were differentiated from mouse bone marrow. To this end, unselected bone marrow-derived progenitors cells were cultivated in the presence of rmFLT3-L, rmSCF and rmIL-5 according to a well-established protocol (Dyer et al., 2008). After about 10-12 days of culturing, more than 90% of the cells were eosinophils according to visual inspection of Diff-Quik stained preparations.

To quickly assess if mouse eosinophils respond to the selective cannabinoid receptor 2 agonist JWH-133, I incubated bmEos with the Ca²⁺ sensitive dye Fluo-3-AM and monitored intracellular Ca²⁺ releases upon JWH-133 stimulation. In **Fig VI.I.** it can be seen that JWH-133 (200nM) induced Ca²⁺ release up to 1.6 fold over baseline which could be completely blocked with the specific CB₂ antagonist SR144528.

Here, I could show that mouse bmEos indeed express a functional CB₂ receptor and its stimulation leads to a similar Ca²⁺ release observed in human eosinophils.

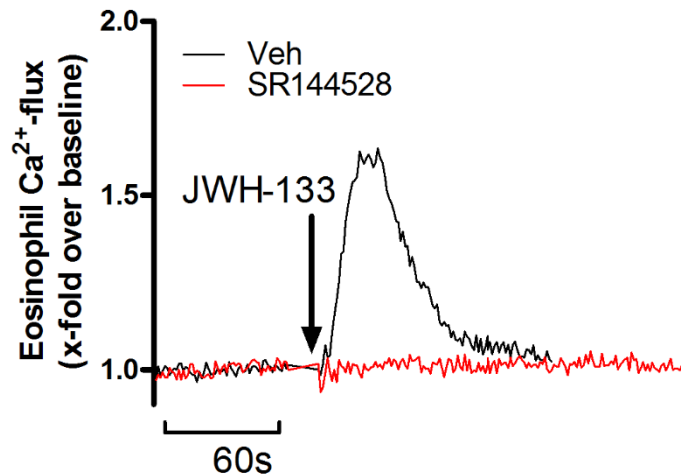


Figure VI-1 JWH-133 induces intracellular Ca^{2+} releases in bmEos

BmEos were resuspended in 1ml assay buffer (without $\text{Ca}^{2+}/\text{Mg}^{2+}$) to a concentration of 10×10^6 cells/ml. The Ca^{2+} sensitive dye Fluo-3 AM was added to give a final concentration of $2 \mu\text{M}$ in the presence of 0.02% pluronic F-127 and bmEos were incubated for 60min at room temperature in the dark. Cells were pre-treated with SR144528 ($1 \mu\text{M}$) or vehicle for 10min at room temperature. Baseline fluorescence was acquired for 60sec and then 200nM JWH-133 was added and Ca^{2+} release was monitored. Data are shown as a representative of 5 independent experiments.

VI.II. CD11b is upregulated by JWH-133

In chapter **V.IV**, I could show that human peripheral blood eosinophils express more CD11b (α_m subunit of the integrin Mac-1) when they were pre-treated with JWH-133 prior to eotaxin-2 stimulation. In mouse eosinophils, Mac-1 is involved in the migration of eosinophils to eotaxin-2 (Das et al., 1997) and has furthermore been shown to be important in an ovalbumin induced allergic asthma model, where application of an antibody to CD11b greatly inhibited eosinophil recruitment (Kanwar et al., 2001).

Accordingly, **Fig. VI.** shows that pre-treatment of bmEos with JWH-133 (200nM) led to up-regulation of CD11b independent of eotaxin-2 stimulation as already bmEos

treated with JWH-133 only showed ~20% more CD11b expression as compared to vehicle. Interestingly, the effects of JWH-133 and eotaxin-2 were additive reaching a maximum of ~ 1.75 fold over baseline at 30nM eotaxin-2.

Therefore, JWH-133 shows no priming effect on eotaxin-2 induced CD11b upregulation in mouse bmEos, but directly affects the amount of CD11b on the cell surface. The final outcome might be the same as in human eosinophils as more CD11b allows enhanced interaction with its ligands *in vivo*.

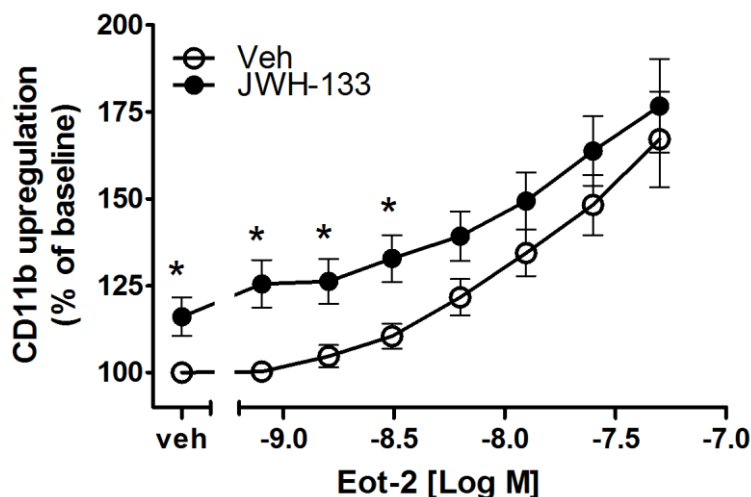


Figure VI-2 JWH-133 is upregulating CD11b on bmEos

*BmEos were pre-treated with JWH-133 (200nM) or vehicle for 10 min at room temperature. CD11b upregulation was induced by incubating the cells in the presence of serial eotaxin-2 concentrations for 30 min at 37 °C. Samples were stained with an anti-mouse-CD11b-PE antibody. CD11b upregulation was analyzed by flow cytometry and expressed as percent of the vehicle response. Data are shown as mean $\pm$ SEM. * $p < 0.05$ versus vehicle $n = 6$.*

VI.II. JWH-133 enhances the chemotactic behavior of bmEos

In the previous experiments I could demonstrate that activation of CB₂ elicits similar effects in mouse bmEos and human peripheral blood eosinophils. To further explore the possible involvement of the cannabinoid system in the recruitment of mouse eosinophils, I focused on the chemotactic behavior of bmEos after JWH-133 stimulation *in vitro*, as well as in a mouse model of directed leukocyte recruitment.

Fig. **VI-3A** displays the effect of JWH-133 (250nM) pre-treatment on the eotaxin-2 induced chemotaxis. CB₂ stimulation significantly enhanced the migratory capacity of mouse eosinophils towards increasing concentrations of eotaxin-2 up to 28%. Of note, bmEos isolated from CB₂-knockout (KO) mice did not respond to JWH-133 treatment in comparison to wild type mice, clearly showing that the observed effects are indeed mediated by CB₂ receptors (**VI-3B**).

Next, I confirmed the observed effects seen on mouse eosinophils by using IL-5 transgenic animals in a model of directed migration. In brief, JWH-133 was administrated to mice via intraperitoneal injection at a dose of 5mg/kg/day for 3 consecutive days. Thereafter, eotaxin-2 was instilled via intranasal application and the BAL fluid was taken after 5 hours.

In **Fig.VI-3C** you can see that systemic JWH-133 application clearly primed mouse eosinophils for enhanced chemotactic responses towards eotaxin-2, as the total eosinophil counts in the lungs of treated animals were significantly elevated up to 1.3 fold over eotaxin-2 alone.

Although IL-5 transgenic animals overexpress IL-5 which is a well-known priming factor for eosinophils, JWH-133 still elevated the recruitment into the lungs indicating that IL-5 and CB₂ activation might even show additive effects.

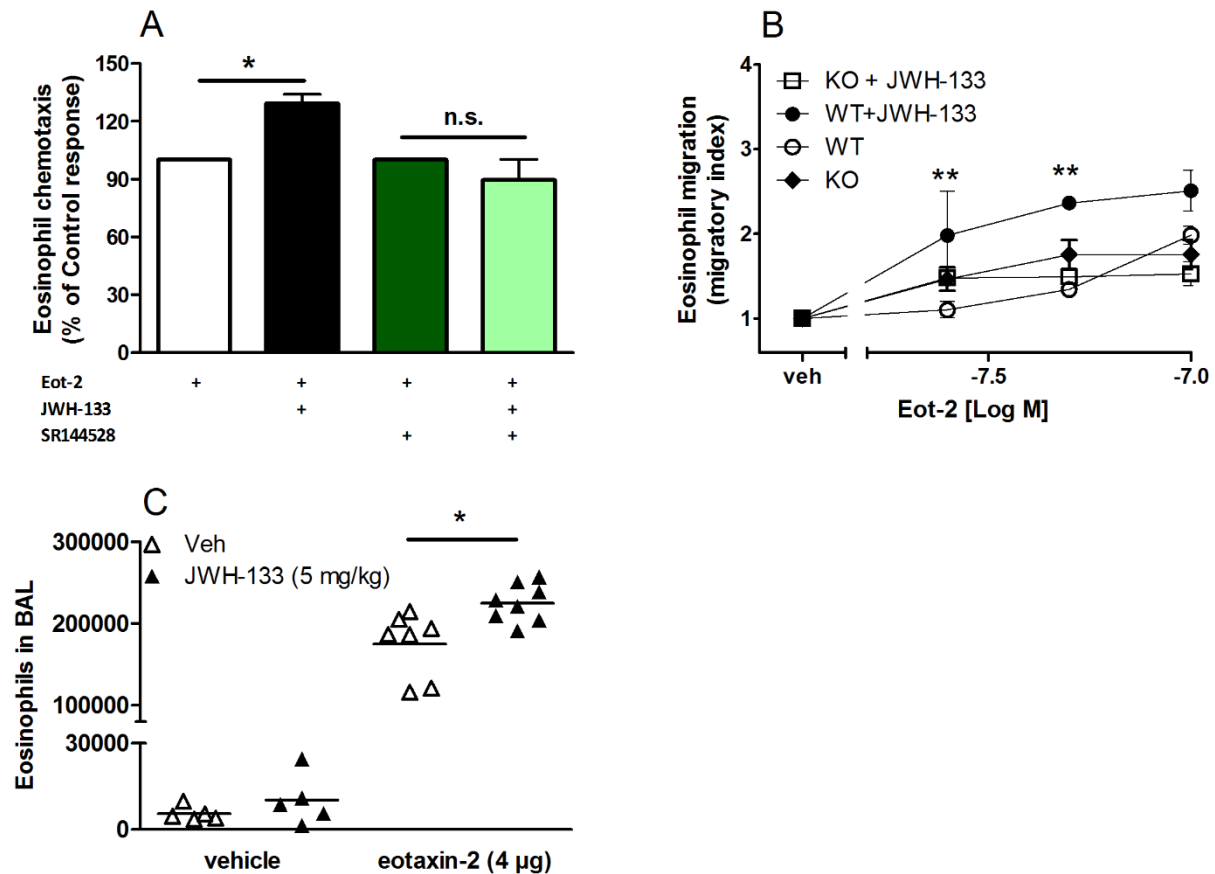


Figure VI-3 JWH-133 is enhancing the migratory capacity of bmEos

A: *bmEos* were resuspended at a concentration of 2×10^6 cells/ml. Cells were pre-treated with JWH-133 (250nM) or vehicle and 50µl were placed into each of the top wells of a 96-well chemotaxis plate where they were allowed to migrate towards the indicated chemoattractant for 3 hours at 37 °C. After 3h, cells that have migrated to the bottom wells of the micro-Boyden chamber were transferred to small FACS tubes and 150µl fixative solution was added. Analysis was done by flow cytometry. **B:** Same as in **A** but *bmEos* were isolated from CB₂-KO mice or WT. **C:** Interleukin-5 transgenic (IL-5Tg) mice were treated with JWH-133 (5 mg/kg/d) or vehicle intraperitoneally (i.p.) for three days. Finally 4µg eotaxin-2 was applied via intranasal instillation and bronchoalveolar lavage fluid (BALF) was collected after 5h. Samples were stained with an anti-mouse Siglec-F antibody conjugated to AF647 and an anti-mouse CD11c antibody conjugated to FITC. Eosinophils were identified as Siglec-F⁺/CD11c⁻ cells and migration of mouse eosinophils into the lung was assessed by flow cytometric counting.

VI.III. Systemic application of JWH-133 aggravates AHR in mice

The *in vitro* data shown in the last chapters strongly suggests that CB₂ ligands contribute to both human and mouse eosinophil activation and responsiveness. To further link the obtained data to the pathophysiological processes of allergic diseases, I used an acute model of ovalbumin (OVA) induced asthma in mice. In brief, 8 week old female C57BL6/J mice were immunized to OVA by injecting 10mg together with the Th₂ adjuvant Al(OH)₃ on day 0 and 7. Mice received a daily intraperitoneal dose of either JWH-133 (10mg/kg) alone or in combination with the CB₂ antagonist SR144528 (10mg/kg) from day 9 till day 16. Finally, mice received an OVA challenge via inhalation of a 0.5% OVA aerosol on day 14 and 16. On day 17, both airways resistance and compliance was measured or BAL fluid was taken for analyses of cellular content and lipid mediators.

As shown in **Fig. VI-4A** and **B**, systemic application of JWH-133 significantly impaired airway resistance and compliance compared to the untreated control group. Simultaneous application of SR144528 was able to completely prevent these effects (**Fig. VI-4C** and **D**).

Apart from mast cells, eosinophils are potent producers of lipid mediators, especially cysteinyl leukotrienes (CysLTs) (Eum et al., 2003). Cys-Leukotrienes are potent inflammatory mediators and comprise LTC₄, LTD₄ and LTE₄ and only the latter one is stable whereas LTC₄ is rapidly metabolized to LTD₄ and finally to LTE₄ (Haeggström and Funk, 2011). Previous studies connected CysLTs to bronchoconstriction and airway hyperresponsiveness, but also in the case of LTE₄ to eosinophil recruitment (Eum et al., 2003; Imai et al., 1997). Moreover, LTE₄ was shown to be increased in urine and BALF of patients after allergen challenge (Drazen et al., 1992; Lam et al., 1988).

Accordingly, using mass spectrometry analysis, which was done by Alexander Fauland at Karolinska Institutet, slightly elevated levels of LTC₄ (**Fig. VI-5A**) and significantly increased amounts of LTD₄ and LTE₄ (**Fig. VI-5B** and **C**) were found in the BALF of JWH-133 treated animals.

Flow cytometric analysis of BALF leukocyte content confirmed my previously shown chemotaxis *in vitro* data, as eosinophil counts in JWH-133 treated mice were ~2.5 times higher than vehicle control.

Finally H&E staining of paraffin embedded lung tissue from both vehicle and JWH-133 treated mice showed enhanced tissue destruction and elevated inflammatory cell infiltrates confirming the flow cytometry data (**Fig. VI-6**).

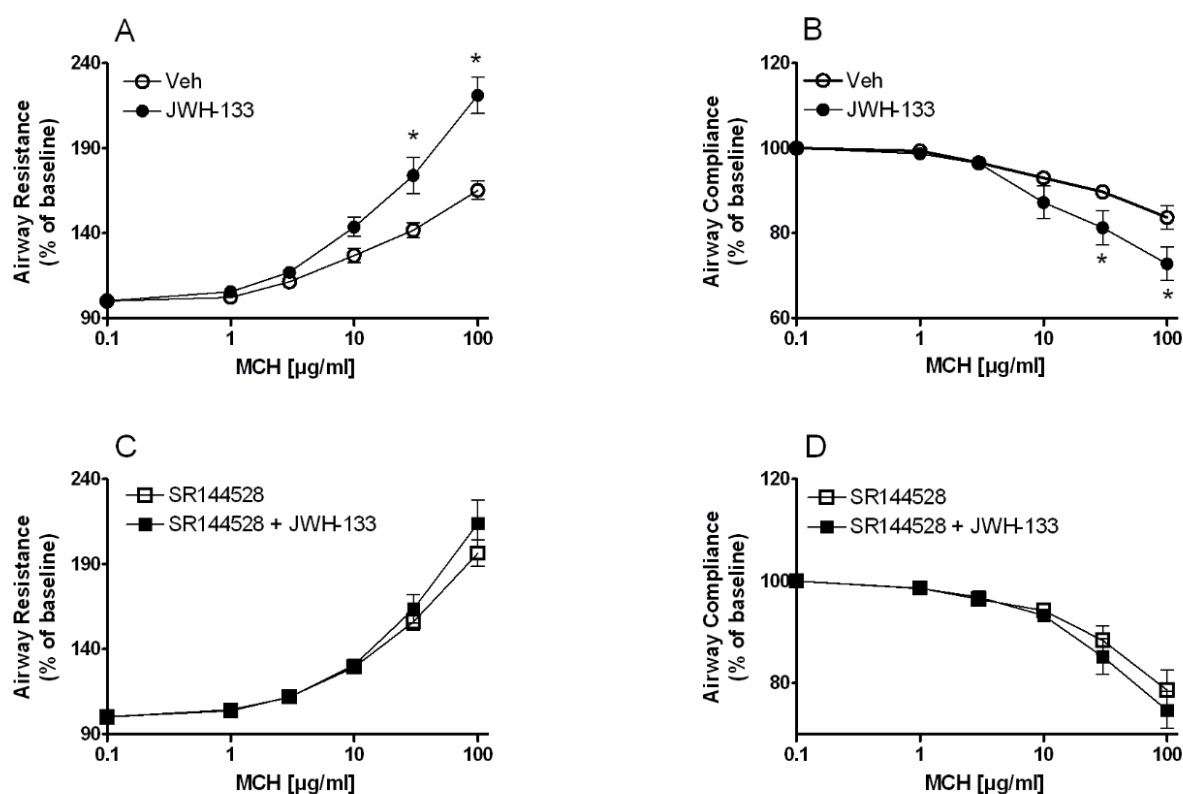


Figure VI-4 JWH-133 deteriorates airway resistance and compliance

Eight week-old female C57Bl6/N mice were immunized by *i.p.* injections of 10µg of OVA adsorbed to Al(OH)₃ on days 0 and 7. Mice were challenged by an aerosol of 0.5% (w/v) of OVA in saline for 30min on days 14 and 16. In between, mice received a daily *i.p.* injection of a combination of JWH-133 and/or SR144528 (10 mg/kg) or vehicle from day 9 onwards till day 16. On day 17 airway resistance (**A + C**) and compliance (**B + D**) were measured.). Data are shown as mean ± SEM, * $p < 0.05$, $n = 6 - 9$.

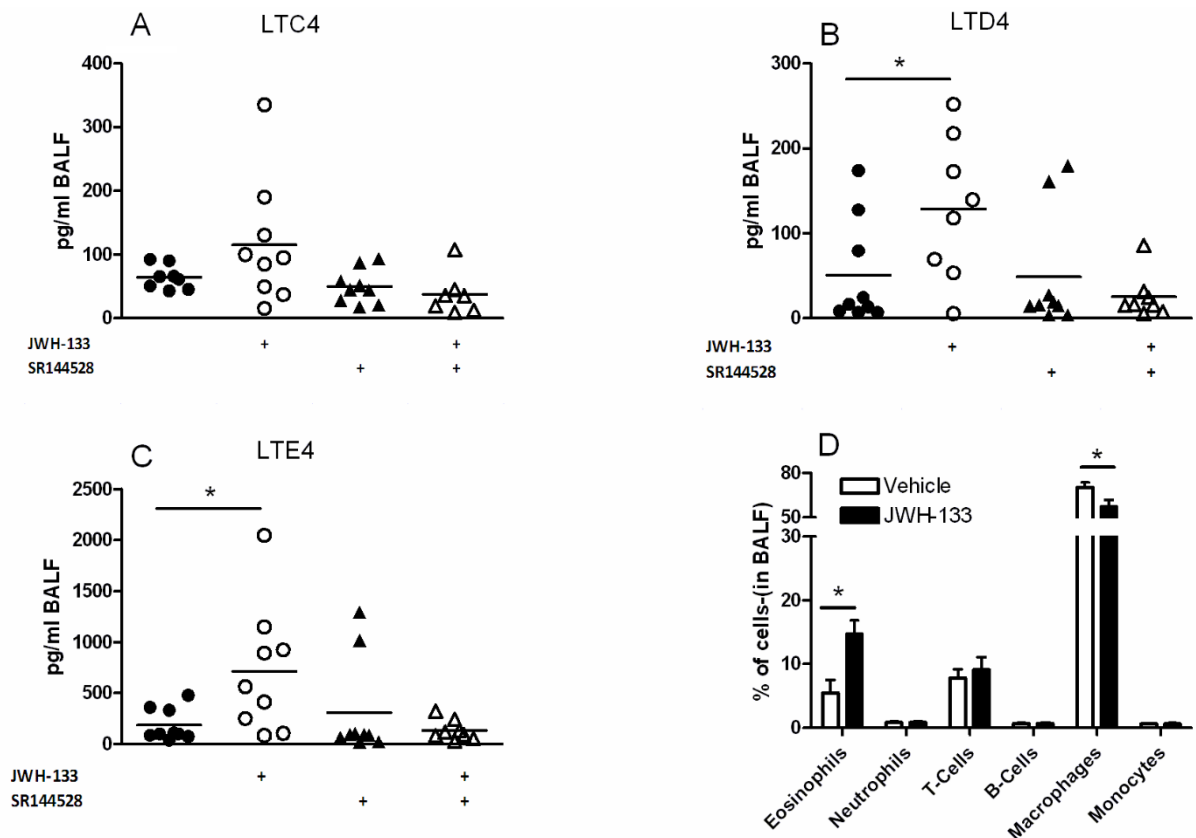


Figure VI-5 JWH-133 increases eosinophil counts and leukotriene levels in BALF

*Eight week-old female C57Bl6/N mice were immunized by i.p. injections of 10 μ g of OVA adsorbed to Al(OH)₃ on days 0 and 7. Mice were challenged by an aerosol of 0.5% (w/v) of OVA in saline for 30min on days 14 and 16. In between, mice received a daily i.p. injection of a combination of JWH-133 and/or SR144528 (10 mg/kg) or vehicle from day 9 onwards till day 16. On day 17 BAL fluid was taken. **A, B** and **C**: cysLT analysis in the BALF by mass spectrometry and revealed slightly increased LTC₄ but significantly enhanced LTD₄ as well as LTE₄ levels in the JWH-133 treated animals which could be reversed by SR144528. **D**: Flow cytometric analysis of cells in the BALF of mice. (Mass spectrometry was kindly carried out by Alexander Fauland in the laboratory of Craig Wheelock, Stockholm, SE) Data are shown as mean $\pm$ SEM, * $p < 0.05$, $n = 6 - 9$.*

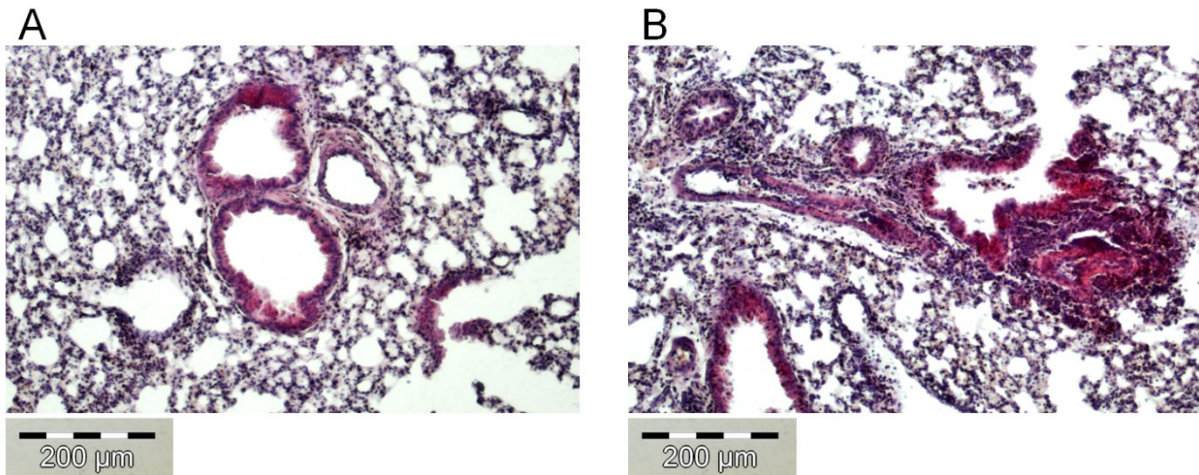


Figure VI-6. Representative histological pictures of paraffin sections of lungs from OVA-challenged mice.

*Mice were treated as in **Fig. VI-5** and lung sections were fixed with 4% formaldehyde and embedded in paraffin. Lung histopathology was evaluated by H&E and visually examined with an Olympus IX70 microscope; photographs were taken with an Olympus DP50 camera. **A**: vehicle treated mice. **B**: JWH-133 treated mice. Representative images of 4 independent experiments are shown.*

VI.IV. Eosinophils are required for the detrimental effects of JWH-133 in allergic pulmonary inflammation

Systemic application of JWH-133 may activate CB₂ not only on eosinophils but also on a variety of other cells including T and B cells, monocytes, macrophages and also smooth muscle cells (Klein et al., 2003).

Thus, to examine whether the observed effects are in fact eosinophil-specific, the same model of ovalbumin-induced pulmonary inflammation was performed in eosinophil-deficient $\Delta db/GATA$ mice on the BALB/c background. Those mice were engineered by deletion of a palindromic GATA-binding site (dbl-GATA) in the hematopoietic promotor resulting in selective loss of the eosinophil lineage (Yu et al., 2002)

In **Fig. VI-7** it can be observed that JWH-133 still increases airway resistance in BALB/c WT mice (**Fig. VI-7A**), whereas this effect is even reversed in eosinophil-deficient $\Delta dbfGATA$ mice (**Fig. VI-7B**), confirming that eosinophils are indeed the major target of JWH-133 in allergic inflammation

Concluding, the results show clearly that systemic CB₂ activation directly contributes to the pathophysiology of the used asthma model in mice by enhancing eosinophil chemotaxis and function.

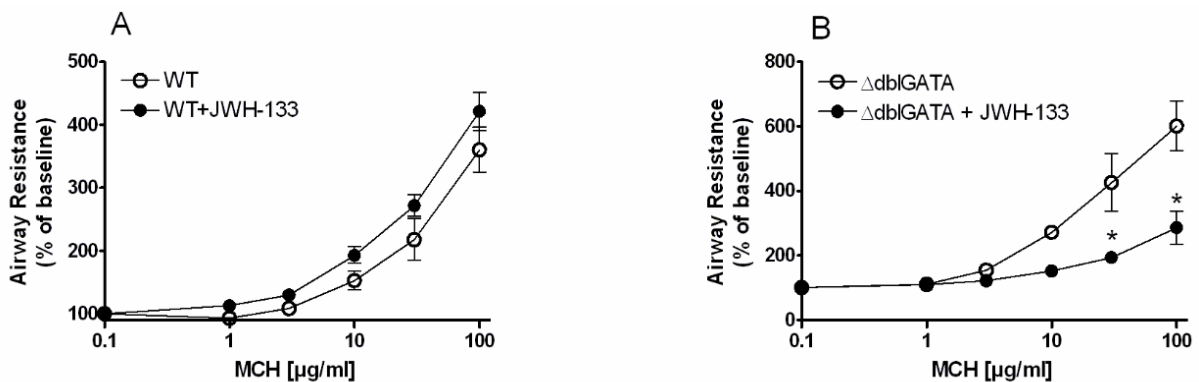


Figure VI-7. Eosinophils are required for the JWH-133 induced aggravation of lung parameters

*Eight week-old female WT BALB/c mice or eosinophil deficient $\Delta dbfGATA$ mice were immunized by i.p. injections of 10 μg of OVA adsorbed to $\text{Al}(\text{OH})_3$ on days 0 and 7. Mice were challenged by an aerosol of 0.5% (w/v) of OVA in saline for 30 min on days 14 and 16. In between, mice received a daily i.p. injection of a combination of JWH-133 and/or SR144528 (10 mg/kg) or vehicle from day 9 onwards till day 16. On day 17 airway resistance was measured. **A:** Airway resistance of WT mice either treated with or without JWH-133. **B:** Same as in **A**, but with $\Delta dbfGATA$ mice. Data are shown as mean $\pm$ SEM, * $p < 0.05$, $n = 3 - 6$.*

VII. Discussion

Up to now, reports on the effects of cannabinoids on eosinophils, especially in allergy and asthma have been conflicting, showing both pro- and anti-inflammatory results depending on the model and agonist used.

In this study I could characterize a novel mechanism involving the selective activation of the cannabinoid receptor, CB₂, resulting in a primed state of eosinophils. Primed cells display enhanced migratory and effector functions and may thus be directly related to the pathogenesis of eosinophil-associated diseases.

In the first part of the study I analyzed whether human peripheral blood eosinophils express appreciable amounts of cannabinoid receptor protein on their cell surface. Previously, CB₂ receptor was found to be the main CB receptor expressed on cells of the immune system and its expression was reported on eosinophils, monocytes, macrophages, NK cells, B- and T-cells. Interestingly, CB₂ expression on human neutrophils, was reported on mRNA basis but later analysis done by Western blotting revealed negligible expression (Oka et al., 2004). It was suggested that over-amplification of contaminating mRNA from eosinophils may have led to the positive results (Turcotte et al., 2015). Concerning eosinophils, the first study revealing significant amounts of CB₂ but not CB₁ expression on human eosinophils was done by Oka et al. in 2004. A subsequent study investigated gene expression after an *in vivo* allergen challenge of asthmatic patients and revealed that expression of CNR2 mRNA, the corresponding gene of the CB₂ receptor, was greatly enhanced (Esnault et al., 2013).

Accordingly, I could demonstrate that eosinophils isolated from allergic donors with acute respiratory symptoms, express more than 3 times more cannabinoid receptor 2 protein than healthy controls. Moreover, protein upregulation seems also be dependent on a previous allergen challenge, as eosinophils isolated from allergic donors during off-season of the respective allergen, showed no enhanced CB₂ expression.

Thus, it seems that activation of the CB₂ receptor contributes to the pathogenesis of allergic diseases, as 2-AG, the main CB₂ agonist was reported to be a chemoattractant for human eosinophils and was further shown to be enhanced in

allergic inflammation (Mimura et al., 2012). Upregulation of the corresponding receptor in allergic donors might therefore represent the result of effective priming and prepares the eosinophil for an enhanced migration into the affected tissue.

Accordingly, my next aim was to explore the effects of selective CB₂ receptor stimulation on the effector function of human eosinophils. I could show that activation of CB₂ by the selective agonist JWH-133 induced a small migratory response in human eosinophils but enhanced eotaxin-2/CCL24 induced migration, especially at low nanomolar concentrations. Moreover, I could detect enhanced non-directional movement of eosinophils, so called chemokinesis, at higher concentrations of JWH-133. Interestingly, the effects of JWH-133 on eosinophil migration resemble the actions of the major priming cytokines for eosinophils, IL-5, IL-3 and GM-CSF, which are all reported to induce chemokinetic activity and show a low chemotactic effectiveness (Simson and Foster, 2000).

Neutrophil chemotaxis to 2-AG, in contrast to eosinophils, was shown to be dependent on the hydrolysis of 2-AG to arachidonic acid and subsequent *de novo* LTB₄ synthesis and successive stimulation of its receptor BLT₁ (Chouinard et al., 2011). Correspondingly, the synthetic cannabinoid JWH-133, which is not degradable by MAGL, thus no free arachidonic acid can be generated, was not affecting IL-8 induced neutrophil activation nor able to induce chemotaxis on its own.

So far little is known about the effects of cannabinoids on basophils, exempt from one study showing reduced CD63 expression upon 2-AG stimulation. Although the CB₂ antagonist SR144528 returned CD63 expression back to control values, they also found the cannabimimetic eicosanoid PEA, which is not activating CB receptors, to be as efficient as 2-AG in reducing CD63 expression, which casted doubts on CB₂ involvement (Vannacci et al., 2002). The data obtained during my PhD studies showed that basophils display slightly enhanced, but negligible, responses towards eotaxin-2 when pre-treated with JWH-133 indicating insignificant interaction of CB₂ with basophil function.

Concluding from the first results, CB₂ activation seems to be a novel priming factor for human eosinophils. Interestingly, priming of eosinophils by cytokines or other stimuli like adhesion to extracellular matrix (ECM) proteins or endothelial cells is an important step in the pathogenesis of allergic diseases. Previous studies revealed several priming-dependent responses in eosinophils such as migration, adhesion and

degranulation. Furthermore, these responses were shown to be greatly enhanced in peripheral blood eosinophils of allergic patients, highlighting the potential importance of CB₂-mediated eosinophil priming (Carlson et al., 1992; Griffin et al., 1991; Håkansson et al., 1997).

Based on these observations, adhesion experiments were conducted and I could produce evidence that activation of CB₂ by the selective CB₂ receptor agonist JWH-133 prior to stimulation with chemoattractants significantly enhanced the amount of CD11b on the surface of both human and mouse eosinophils. Upregulation of the integrin CD11b/CD18 by eotaxin is also a feature of IL-5 mediated priming of human eosinophils and is thereby regulating homing and tissue accumulation (Simson and Foster, 2000). CD11b is the α M subunit of the integrin α M β ₂, and is one of seven integrin molecules expressed by eosinophils. Priming of eosinophils through binding to activated endothelial cells via ICAM-1 α M β ₂ interaction was previously shown to increase LTC₄ synthesis and EPO release. Moreover, the released amount of LTC₄ was reported to be substantially greater than those required for bronchoconstriction in airway explants (Muñoz et al., 1999).

α M β ₂ is rapidly increased after exposure to cytokines or inflammatory mediators and is further associated with both atopic dermatitis and allergic asthma (Matsumoto and Bochner, 2013b; Walker et al., 1993). Regarding allergic asthma, a “hyperadhesive” eosinophil phenotype, identified by greatly enhanced α M β ₂ levels has been described in allergic patients after allergen challenge. Interestingly, of all integrins analysed only α M β ₂ levels were enhanced after segmental allergen challenge, indicating that α M β ₂ is probably the major integrin responsible for eosinophil extravasation in allergic diseases (Barthel et al., 2006).

Accordingly, the observed enhanced CD11b levels induced by JWH-133 in my experiments correlated with an increased capability to adhere to ICAM-1, the natural ligand of CD11b, under conditions of shear stress. Of note, binding of eosinophils and subsequent firm adhesion to endothelial cells via α M β ₂-ICAM-1 interaction was shown to be crucial in the transendothelial migration (Ebisawa et al., 1992).

In the first part of the study I described the contribution of selective CB₂ receptor agonism to the priming state of eosinophils. My next aim was to assess the obtained *in vitro* data in established models of eosinophil migration *in vivo*. First, I investigated the effects of CB₂ activation in a model of directed eosinophil migration using IL-5

transgenic BALB/c mice. These mice were genetically modified by using regulatory elements from the CD3 δ gene to drive T-cells to express IL-5, resulting in greatly elevated eosinophil counts in order to mimic eosinophilic disease states (Lee et al., 1997). The systemic treatment of mice with JWH-133 and subsequent intranasal instillation of the eosinophil specific cytokine eotaxin-2, led to an enhanced eosinophil migration into the lungs of treated mice.

Having confirmed the *in vitro* data, the next step was to verify the *in vivo* relevance in a mouse model of allergic inflammatory asthma. In short, C57BL6/J mice were sensitized to ovalbumin and JWH-133 was given daily for 7 consecutive days starting five days prior to the first OVA challenge. Using this model I could detect increased counts of eosinophils in the airways of JWH-133 treated mice as compared to controls. Furthermore, airway resistance and compliance were measured and were found to be significantly aggravated in the JWH-133-treated animals. The main immune cell types involved in the OVA-induced asthma model in mice apart from eosinophils are macrophages and T-cells which both express detectable amounts of CB₂ on their cell surface (Galiègue et al., 1995).

To definitely prove that indeed CB₂ mediated priming of eosinophils and not potentially altered cytokine production of T-cells or macrophages is responsible for the observed decrease in lung function, I conducted the same experiment in eosinophil deficient $\Delta db/GATA$ mice. These mice were engineered to have a deletion of a high-affinity GATA binding site in the GATA-1 promotor which leads to the selective loss of eosinophils whereas other hematopoietic lineages are not affected (Yu et al., 2002). Interestingly, the JWH-133 mediated effects on lung parameters were completely absent in $\Delta db/GATA$ mice, suggesting that eosinophils are indeed the major target of JWH-133 in this model of allergic inflammation.

In a previous study, enhanced CysLT biosynthesis was observed in human eosinophils exposed to 2-AG. This effect was further amplified when cells were additionally treated with platelet activating factor (PAF). MAG lipase inhibitors were shown to completely block CysLT synthesis, indicating that metabolism of 2-AG to arachidonic acid and subsequent eicosanoid synthesis, rather than enhanced CB₂ activation led to the observed increase in CysLT levels (Larose et al., 2014b).

Interestingly I could detect significantly higher LTD₄ and LTE₄ levels in the BAL fluid of JWH-133 treated animals, which was entirely blocked when the specific CB₂

antagonist SR144528 was co-administered. JWH-133 does not contain an arachidonic acid domain and thus cannot serve as a substrate for MAGL, indicating a direct correlation between CysLT biosynthesis and CB₂ activation *in vivo*. Of note, CysLT were shown to play important roles in allergic asthma, contributing not only to bronchoconstriction and airway hyperresponsiveness (Eum et al., 2003) but also eosinophil recruitment (Imai et al., 1997). Correspondingly, the observed enhanced LTE₄ levels might be involved in the further recruitment of eosinophils to the lungs of JWH-133 treated animals, thereby promoting tissue inflammation and damage. Moreover LTE₄ levels were shown to be increased in patients after allergen challenge and could be detected in exhaled breath and urine (Csoma et al., 2002; Drazen et al., 1992).

In accordance with our findings, a recent work in mice on high fat diet demonstrates that JWH-133 is potentiating adipose tissue inflammation, thus suggesting a role in obesity induced inflammation. By using CB₂-KO mice, they demonstrated that the JWH-133 induced effects were abrogated upon genetic deletion of CB₂ (Deveaux et al., 2009). Recent studies performed in obese patients, showed increased production of the endocannabinoid 2-AG by adipocytes associated with enhanced circulating levels (Gonthier et al., 2007). 2-AG levels were also shown to be linked to the grade of obesity by a study from 2008, which pointed out that 2-AG plasma levels positively correlate with body mass index (BMI) in humans (Côté et al., 2007). Notably, obesity and a high BMI seem to be connected to the prevalence of asthma, its severity and the reduced responses to standard management therapy resulting in an increased risk of asthma-related hospitalizations (Mosen et al., 2008).

The exact molecular mechanism linking obesity to asthma are still poorly understood but enhanced plasma 2-AG levels associated with increased priming and hence more efficient recruitment of eosinophils to the airways, represents a conclusive hypothesis remaining to be verified in future studies.

Finally, I assessed the potential signalling pathways in eosinophils activated by CB₂ stimulation. Previous data on CB₂ signalling in monocytes and eosinophils reported a Gα_{i/o} dependent pathway, as treatment with pertussis toxin (PTX) abolished the 2-AG induced effects (Oka et al., 2004). Contrarily, the data obtained during this study is suggesting that eosinophil CB₂ receptors seem to activate Gα_q- rather than, or in addition, Gα_i-proteins. This observation is based on Ca²⁺ flux and shape change

experiments which revealed insensitivity of the induced effects to PTX. On the other hand, the established PLC inhibitor U-73122 and the IP₃ receptor antagonist 2-APB were capable of diminishing JWH-133 and 2-AG induced Ca²⁺ release. Interestingly, the same concentration of 2-AG induced a much higher release in intracellular Ca²⁺ compared to JWH.-133. This discrepancy can be readily explained by a study done by Shoemaker et al., who showed that cannabinoid agonists have different rank orders of potencies and receptor occupancies. The endocannabinoid 2-AG was shown to be a more efficient ligand and requires only half the receptor occupancy to cause the same effects as synthetic agonists like JWH-133 (Shoemaker et al., 2005).

Downstream analysis of the activated pathway revealed induction of MEK1/2 and ROCK might be the responsible signalling mechanism leading to the priming effect of JWH-133 on human eosinophils. Interestingly, previous data also suggest MEK1/2-ERK activation to be responsible for IL-5 or GM-CSF induced priming effects (Bates et al., 2000). Moreover, both kinases are regulating several pro-inflammatory processes like migration, degranulation and respiratory burst of eosinophils (Adachi et al., 2001; Henry et al., 2005) facilitating eosinophilic inflammation. Especially stimulation of eosinophils with eotaxins, leading to CCR3 activation, is activating RAS-MAPK and subsequently ERK-1/2 dependent pathways involved in the regulation of eosinophil migration (El-Shazly et al., 1999). Not only eosinophil activation depends on ERK but also survival. Both the IL-5 and GM-CSF pro-survival stimuli were shown to be diminished by ERK inhibition, highlighting the importance of this pathway in allergic diseases where eosinophil apoptosis is greatly reduced (Shen et al., 2009). Interestingly IL-5 and eotaxin exhibit different amplitudes and kinetics of MAPK activation, which might account for their different biological functions (Alam and Gorska, 2011).

Generally ERK1/2 belongs to the mitogen-activated kinases which are included in the proline-directed serine-threonine protein kinases. MAPK, which integrate the signals from a variety of receptors and translate them to cellular functions, are essential for immune cell migration, metabolism, production of pro-inflammatory mediators, differentiation and survival. Regarding asthma, it has been shown that genetic or pharmacological targeting of MAPK is capable of diminishing allergic inflammation in the airways. Moreover, there is a strong correlation between phosphorylated ERK1/2 and clinical severity of asthma and the number of eosinophils in the airways (Alam and Gorska, 2011). Furthermore MAPKs are involved in the production of the

eosinophil chemoattractants eotaxin-3 and RANTES by epithelial cells resulting in a chemokine gradient attracting peripheral blood eosinophils. During their extravasation, the p38 pathway is regulating the expression of adhesion molecules including ICAM-1 and VCAM-1 on endothelial cells. Overall, the MAPK-dependent pathways play a pivotal role in the initiation and perpetuation of allergic inflammation (Alam and Gorska, 2011).

Interestingly there's little data about the direct effects of marijuana smoking and its impact on lung function in health and disease. Two studies done 30 years ago addressed the effects of inhaled or orally taken Δ 9-Tetrahydrocannabinol on human airway function in healthy controls and asthmatics. Δ 9-THC was found to induce mild but significant bronchodilatation in healthy controls and asthmatics and was generally well tolerated aside from slight cough and chest discomfort in 3 out of 11 normal subjects. Interestingly, Δ 9-THC inhalation or oral administration induced moderate to severe bronchoconstriction in some asthmatic patients. After testing for a potential allergic reaction to Δ 9-THC, which was negative, they attributed the seen adverse reaction to a local irritating effect (Abboud and Sanders, 1976; Tashkin et al., 1977). A potential mechanism explaining the reaction in asthmatics may be the upregulation of endocannabinoid receptors on eosinophils of asthmatics addressed in the study by Esnault et al. and my own data (Esnault et al., 2013). Normal healthy human airways are devoid of eosinophils but they can be found in rich numbers in asthmatic patients. Inhaled or orally taken Δ 9-THC may act directly on the upregulated CB2 receptors on eosinophils in the airways of asthmatics and can thereby induce enhanced activation.

After major policy changes on cannabis legalization in some countries including the United States, there is increasing interest whether recreational marijuana use is associated with physical health problems (Meier MH et al., 2016). A recent study done by Meier et al. followed 1037 individuals over a time period of 20 years and analysed health decline connected with marijuana and/or tobacco use. Unsurprisingly tobacco pack-years were associated with worse health outcomes in lung function, periodontal health, systemic inflammation, metabolic syndrome, HDL-cholesterol level, triglyceride levels, glycated hemoglobin and self-reported health (Meier MH et al., 2016). All of those remained significantly altered even after controlling for cannabis joint-years and childhood health.

Cannabis joint-years on the other hand were associated with poorer periodontal health, lung function and self-reported health. Interestingly after controlling for tobacco pack-years and childhood health only associations with poor periodontal health and altered lung-function as reflected by a decrease in the ratio of air volume during the first second of expiration (FEV₁) / forced vital capacity (FVC) remained significant. The latter, however, was probably not due to airway obstruction as no decreases in FEV₁ were observed. Instead, cannabis users showed higher FVC values, which had also been reported previously (Meier MH et al., 2016; Pletcher MJ et al., 2012). Some researchers have proposed that the higher FVC value is due to deep breathing maneuvers which could stretch the lungs, thereby resulting in a higher vital capacity.

Although this study showed no effects of long-term cannabis use on lung health, it should be kept in mind that natural marijuana contains a variety of substances which can activate or inhibit both cannabinoid receptors. Moreover it contains substances which have already been shown to have beneficial effects on human health (Pacher and Kunos, 2013) and cannot be compared to synthetic substances used in synthetic marijuana blends. Especially the synthetic cannabinoid substances used in synthetic marijuana like JWH-018, JWH-073, JWH-200, CP-47,497, which are mostly potent selective agonists of the CB1 receptor (de Havenon et al., 2011), are connected to serious illness and death after smoking or ingestion (Amitpal S. Nat et al., 2013; Derungs et al., 2012; Kemp et al., 2016).

Concluding, the data obtained during my PhD studies revealed for the first time that selective CB2 receptor activation is a novel priming factor for both human and mouse eosinophils leading to enhanced migratory responses *in vitro* and *in vivo*. The priming process is induced by a Gα_q/PLC mediated Ca²⁺ release followed by activation of MEK/ROCK activation. In contrast to previous studies showing beneficial bronchodilatory effects of orally taken or inhaled cannabinoids, I could show contribution of the endocannabinoid system to the progression of allergic inflammatory responses suggesting possible undesirable effects of prolonged cannabinoid use.

As a result, selective CB₂ receptor antagonism may provide a new therapeutic approach for allergic disorders and eosinophil-associated diseases.

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