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

Short Title: CB₂ aggravates allergic inflammation

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Abstract

Background: Accumulation of activated eosinophils in tissue is a hallmark of allergic inflammation. The endocannabinoid 2-arachidonoylglycerol (2-AG) has been proposed to elicit eosinophil migration in a CB₂ receptor/G_{i/o}-dependent manner. However, it has been claimed recently that this process may also involve other mechanisms such as cytokine priming and the metabolism of 2-AG into eicosanoids. Here we explored the direct contribution of specific CB₂ receptor activation to human and mouse eosinophil effector function *in vitro* and *in vivo*.

Methods: *In vitro* studies including CB₂ expression, adhesion and migratory responsiveness, respiratory burst, degranulation and calcium mobilization, were conducted in human peripheral blood eosinophils and mouse bone marrow-derived eosinophils. Allergic airway inflammation was assessed in mouse models of acute OVA-induced asthma and directed eosinophil migration.

Results: CB₂ expression was significantly higher in eosinophils from symptomatic allergic donors. The selective CB₂ receptor agonist JWH-133 induced a moderate migratory response in eosinophils. However, short-term exposure to JWH-133 potently enhanced chemoattractant-induced eosinophil shape change, chemotaxis, CD11b surface expression

and adhesion as well as production of reactive oxygen species. Receptor specificity of the observed effects was confirmed in eosinophils from CB₂ knockout mice and by using the selective CB₂ antagonist SR144528. Of note, systemic application of JWH-133 clearly primed eosinophil directed migration *in vivo* and aggravated both AHR and eosinophil influx into the airways in a CB₂-specific manner. This effect was completely absent in eosinophil-deficient Δ dblGATA mice.

Conclusion: Our data indicate that CB₂ may directly contribute to the pathogenesis of eosinophil-driven diseases. Moreover, we provide new insights into the molecular mechanisms underlying the CB₂-mediated priming of eosinophils. Hence, antagonism of CB₂ receptors may represent a novel pharmacological approach for the treatment of allergic inflammation and other eosinophilic disorders.

Keywords: AHR, Cannabinoid receptor 2, Eosinophils, Ovalbumin induced asthma

Priming

Abbreviations:

Ab, antibody; **AF**, Alexa Fluor; **2-AG**, 2-arachidonoylglycerol; **AHR**, airway hyperresponsiveness; **bmEos**, mouse bone marrow-derived eosinophils; **CI**, chemotactic or chemokinetic index; **CysLTs**, Cysteinyl-Leukotrienes; **Cy5**, Cyanine 5; **DCF**, 2'7'-dichlorofluorescein, **DCFDA**, 2',7'-dichlorofluorescein diacetate, **ERK**, extracellular signal-regulated kinase; **FSC**, forward scatter; **IL**, interleukin; **LC-MS**, Liquid chromatography-mass spectrometry; **LT(x)**, Leukotriene; **MAPK**, mitogen-activated protein kinase; **MEK**, mitogen/extracellular signal-regulated kinase; **OVA**, ovalbumin; **PAF**, platelet activating factor; **PBMC**, peripheral blood mononuclear cells; **PE**, phycoerythrin; **PI3K**, phosphoinositide 3-kinase; **PG**, prostaglandin; **PMNL**, polymorphonuclear leukocytes; **PTX**, pertussis toxin; **ROCK**, rho-associated protein kinase; **SSC**, side scatter;

Introduction

Eosinophils are potent effector cells in the pathogenesis of many disorders, ranging from allergy and bronchial asthma (1), to eosinophilic esophagitis (2), colitis ulcerosa (3), hypereosinophilic syndrome (4) and renal disease (5). Activated eosinophils are one of the primary sources of cytotoxic proteins, proinflammatory cytokines and growth factors, such as IL-4, IL-5, IL-10 and IL-13 (6) and promote numerous immunoregulatory functions that lead to the progression of inflammation, mucus secretion, tissue remodeling and angiogenesis (7).

Regarding asthma, levels of eosinophil granule proteins such as major basic protein (MBP) or eosinophil peroxidase (EPO) broadly correlate with disease severity (8). Moreover, it has been shown that patients who receive treatment based on eosinophil counts in sputum have significantly fewer severe asthma exacerbations than patients treated according to standard management therapy (9).

Corticosteroids are currently the most effective treatment to reduce eosinophil numbers in the blood and tissue. However, the pleiotropic effects, especially of orally applied corticosteroids to control severe asthma can result in potentially harmful side effects and thus limit their therapeutic use (10). Therefore, considerable effort has been invested in the development of drugs that can effectively control the trafficking and activation of eosinophils in order to ameliorate the inflammatory response. Current clinical trials with the eosinophil-targeted mepolizumab revealed an oral glucocorticoid-sparing effect and significantly reduced exacerbation rates in well-selected patients with persistent eosinophilic asthma; however FEV₁ seemed to remain largely unaffected (11,12). Of note, asthma is a heterogeneous condition with diverse characteristics and disease variants. Thus, stratification of patients by clinical characteristics (phenotypes) and pathogenetic mechanisms (endotypes) should lead to more targeted and personalized approaches to asthma therapy. Endocannabinoids are bioactive lipids released from the cell membrane upon cell activation. The two main endocannabinoids are the arachidonic acid (AA) derivatives 2-

arachidonoylglycerol (2-AG) (13) and *N*-arachidonylethanolamine (anandamide, AEA) (14). Most of their actions are mediated by two G-protein-coupled receptors, the cannabinoid receptors 1 and 2 (CB₁ and CB₂). In contrast to CB₁, which is abundantly expressed in the nervous system (15), CB₂ is mainly found in lymphoid organs and cells of the immune system (16). Amongst others, CB₂ expression has been reported for B cells, monocytes/macrophages and eosinophils, indicating a crucial immunoregulatory role for CB₂ and its ligands (17).

Up to now, only three studies have examined the direct contribution of the endocannabinoid 2-AG on eosinophil migration *in vitro*. Oka *et al.* demonstrated that 2-AG induces the chemotaxis of EoL-1 cells and human peripheral blood eosinophils in a CB₂-dependent manner, although the order of the pharmacologically effective concentration of 2-AG was significantly higher than that of other chemoattractants (18). Interestingly, another group confirmed this minimal CB₂-mediated effect of 2-AG and suggested that 2-AG-induced migration is a complex process that may involve other mechanisms, such as cytokine priming, rapid 2-AG metabolism into eicosanoids, and further generation of 15-lipoxygenase metabolites (19).

Moreover, due to the use of different rodent models and varying pharmacological approaches, the *in vivo* role of CB₂ in eosinophilic disorders is still uncertain. Indeed, a CB₂ inverse agonist was shown to block ovalbumin (OVA) -induced lung eosinophilia in mice (20). Accordingly, the CB₂ antagonist SR144528A mediated beneficial effects in oxazolone-induced contact dermatitis (21). In contrast, Giannini *et al.* evaluated the effects of the CB₁/CB₂ receptor agonist CP55,940 on OVA-induced asthma in guinea pigs and concluded that both CB₁ and CB₂ receptors are involved in lung protection (22). Similarly, THC reduced cytokine and IgE level as well as mucus production in OVA-challenged mice (23) however the use of CB₁/CB₂ knockout mice revealed that the effects of THC were cannabinoid receptor independent (24).

Because of the conflicting reports on the pathophysiological role of CB₂ receptors, we set out to explore the direct contribution of specific CB₂ activation on eosinophil effector function. To this end,

the effects of the endocannabinoid 2-AG and the potent and selective CB₂ receptor agonist JWH-133 were studied *in vitro* and in mouse models of acute OVA-induced asthma and directed eosinophil migration. Our results provide clear evidence for a novel CB₂-induced eosinophil-specific “priming mechanism” that potentiates eosinophil effector function *in vitro* and enhances bronchial inflammation *in vivo*, reflected by both increased airway resistance and eosinophil influx into the airways.

Materials and Methods:

Detailed materials and methods are provided in the supplement.

Preparation of mouse eosinophils:

Bone marrow-derived eosinophils (bmEos) were differentiated *ex vivo* from unselected bone marrow progenitors using a well-defined cytokine regimen (25).

Flow cytometric staining of CB₂ receptors on eosinophils

Purified human eosinophils were stained with a polyclonal rabbit anti-human CB₂ primary Ab or isotype control, followed by a goat anti-rabbit secondary Ab (AF-647). CB₂ expression was quantified by flow cytometry.

Shape change assay:

Isolated eosinophils, PMNL or PBMC were pre-treated as indicated and stimulated. Shape change was estimated by flow cytometry as the increase of forward scatter.

Migration:

Chemotaxis and Chemokinesis: Purified human eosinophils or bmEos were pre-treated as indicated, placed into the top of a 48-well micro-Boyden chamber (human) or a 96-well chemotaxis plate (bmEos) and were allowed to migrate towards the indicated chemoattractant or vehicle.

Calcium flux:

Isolated human eosinophils or bmEos were loaded with Fluo-3-AM in the presence of 0.02% pluronic F-127 (26). Changes in intracellular Ca^{2+} were detected as the increase of the fluorescence in the FL1-(530/30nm) channel.

Adhesion assay under flow:

Vena8™ biochips (Cellix Ltd., Dublin, Ireland) were coated with ICAM-1 and coated channels were superfused with purified. Adhesion was monitored using a Hamamatsu ORCA-03G digital camera and CellixVenaFlux software.

CD11b-upregulation:

Whole blood samples or PMNL were pre-treated as indicated and incubated with agonists for 30 min at 37 °C (27). Samples were stained with anti-CD16-PE-Cy5 and anti-CD11b-PE (ICRF44) Ab. CD11b upregulation was analyzed by flow cytometry.

***In vivo* chemotaxis:**

Eight week-old IL-5Tg mice were treated i.p. with JWH-133 (5 mg/kg/d) or vehicle for three consecutive days. *In vivo* chemotaxis of eosinophils was induced by intranasal instillation of 4μg eotaxin-2/CCL24. Bronchoalveolar lavage fluid (BALF) was collected 5 h post-instillation, and migration was evaluated by flow cytometric counting of highly granular (high side scatter) CD11c⁻/Siglec F⁺ cells.

Mouse model of allergic lung inflammation:

Eight week old female C57Bl6/N mice were immunized by i.p. injections of 10 µg of OVA adsorbed to Al(OH)₃ on days 0 and 7. Mice were challenged by an aerosol of OVA in saline on days 14 and 16. Additionally, mice received a daily i.p. injection of CB₂ agonist/antagonist (10 mg/kg) or vehicle on day 9 to day 16. On day 17 either airway hyperresponsiveness to methacholine was recorded with the FlexiVent system (Scireq, Montreal,CA) or BALF was taken and analyzed by flow cytometry.

Results

CB₂ receptor expression is enhanced on eosinophils from allergic donors

CB₂ expression has been reported for B cells, monocytes/macrophages, NK cells, basophils and eosinophils, and at lower levels, for neutrophils and T cells (16,28,29). In this study, CB₂ surface expression was confirmed by flow cytometric staining in- and off-season in human peripheral blood eosinophils from allergic volunteers with seasonal respiratory symptoms and healthy subjects. Of note, quantification revealed ~3.4 fold higher expression of CB₂ on eosinophils from symptomatic allergic donors compared to healthy controls. Interestingly no CB₂ upregulation was observed off-season in eosinophils from asymptomatic allergic donors (**Fig. 1**).

The CB₂ receptor agonist JWH-133 specifically enhances human and mouse eosinophil function

Conflicting data exist regarding the effect of CB₂ activation in eosinophils and neutrophils, and little is known about its impact on basophil function (30,31). Thus, here we explored the effect of the selective CB₂ agonist JWH-133 on human eosinophils, neutrophils and basophils by means of shape change. Having encountered a chemotactic factor *in vivo*, leukocytes immediately begin to rearrange their cytoskeleton and change their shape to facilitate their attachment to microvascular endothelial

cells. Such morphological changes can be detected by flow cytometry as changes in the forward scatter properties of the cells (32). Representative plots are provided in Figure E5. First, purified eosinophils were pre-treated with JWH-133 (100 nM) or vehicle, cells were stimulated with serial dilutions of eotaxin-2/CCL24 and shape change was assessed by flow cytometry. As shown in Fig. 2A, JWH-133 led to a significant increase of eosinophil responses; particularly, the sensitivity to eotaxin-2/CCL24 was increased up to three-fold. To verify that this effect was mediated through selective CB₂ activation, eosinophils were exposed to the CB₂ antagonist SR144528 (1 μM, 10 min at RT) prior to the assay. As illustrated in Fig. 2B, SR144528 totally prevented the enhancing effect of JWH-133. Similarly, the endocannabinoid 2-AG concentration-dependently increased eotaxin-2/CCL24-induced shape change and elicited a weak, but dose-dependent shape change by itself with a maximum response observed at 500 nM (Fig. 2C), similar to JWH-133 (Fig. 2D). Emphasizing the cell type-specific impact of selective CB₂ activation, pretreatment with JWH-133 only slightly enhanced basophil shape change as induced by eotaxin-2/CCL24 (Fig. 2E) and did not affect neutrophil responses to IL-8 (Fig. 2F).

Besides shape change, the chemoattractant-induced up-regulation of adhesion molecules such as α_mβ₂ integrins (CD11b/CD18; Mac-1) is another prerequisite for eosinophil effector functions (33). Thus, human eosinophils in whole blood and mouse bmEos were pre-treated with JWH-133 (1 μM and 250 nM respectively) or vehicle and stimulated with eotaxin-2/CCL24. Interestingly, JWH-133 clearly potentiated the ability of eotaxin-2/CCL24 to upregulate CD11b in human eosinophils, whereas in mouse eosinophils, JWH-133 directly induced CD11b surface expression. At lower concentrations of eotaxin-2/CCL24, JWH-133 led to a ~50% increase of CD11b in human eosinophils (Fig. 3A) and to a ~25% increase in bmEos (Fig. 3B). Similar modulatory effects were observed with 2-AG (250 nM) (Fig. E1).

The same pattern of CB₂-mediated priming was observed in adhesion assays under flow conditions. Pretreatment of purified human eosinophils with 100 nM JWH-133 led to a significant two-fold increase of firm adherent cells on ICAM-1 coated channels compared to eotaxin-2/CCL24 alone. Again, this effect was completely prevented with the CB₂ antagonist SR144528 (1 μM) (**Fig. 3C**). Representative pictures are provided in **Fig. 3D** and **E**.

To explore the direct contribution of CB₂ activation to eosinophil recruitment, *in vitro* and *in vivo* migration experiments were performed. As shown in **Fig. 4A**, JWH-133 itself elicited a moderate chemotactic response in human eosinophils but not in mouse eosinophils (**Fig. E2**), similar to the previously noted effect of the endogenous CB₂ agonist 2-AG (19). The maximal chemotactic response (CI ~ 3.9) was observed at ~100 nM of JWH-133. At micromolar levels JWH-133 evoked chemokinesis, with a maximal response (CI ~1.8) observed at 3 μM. Moreover, at low nanomolar concentrations JWH-133 (5 nM) significantly increased the migratory capacity of human eosinophils (**Fig. 4B**) and bmEos (**Fig. 4C**) towards eotaxin-2/CCL24 by ~80% and 28% respectively. Emphasizing the physiological importance of these observations, systemic administration of JWH-133 (5 mg/kg/d) for three consecutive days significantly enhanced the eotaxin-2/CCL24-directed accumulation of eosinophils in the airways of IL-5 transgenic mice by ~29% (**Fig. 4D**). To further confirm the direct involvement of CB₂ in the recruitment of mouse eosinophils, bmEos were isolated from WT and CB₂-KO mice, treated with vehicle or JWH-133 (250 nM) and were then allowed to migrate towards serial dilutions of eotaxin-2. **Fig. 4E** shows that the priming effect of the cannabinoid agonist is not present in CB₂-KO mice, whereas JWH-133 treatment resulted in a significantly increased migratory response of bmEos from WT mice.

CB₂ receptor activation enhances respiratory burst but not degranulation in human eosinophils

At sites of allergic inflammation, activated eosinophils cause tissue damage by the production of reactive oxygen species (ROS) and the release of toxic granule proteins (34). To explore the impact of selective CB₂ activation on eosinophil respiratory burst, purified human eosinophils were applied to 250 nM JWH-133 for 5 min at 37 °C and respiratory burst was induced with eotaxin-2/CCL24. As shown in **Fig. 5A**, JWH-133 itself failed to induce respiratory burst, but significantly increased the eotaxin-2/CCL24 induced ROS production up to ~50%.

Interestingly, although having significant effects on eosinophil recruitment and ROS production, JWH-133 neither induced nor primed human eosinophil degranulation as assessed by means of CD63 upregulation and EPO release (**Fig. 5B and C**).

JWH-133 modulates eosinophil responses in a pertussis toxin (PTX) -insensitive manner

CB₂ receptors are known to activate heterotrimeric G_{i/o} type G proteins leading to the inhibition of adenylyl cyclase (AC). Furthermore, CB₂ signaling was shown to involve MAPK (p38 and p42/44) and PI3K activity (35). To explore whether the observed effects depend on G_{α_i} activation, purified human eosinophils were incubated in the presence of pertussis toxin (PTX; 5 µg/ml for 20 min at 37 °C) and shape change was induced with PGD₂ (PTX-insensitive) or eotaxin-2/CCL24 (PTX-sensitive). Notably, pretreatment with PTX did not affect the modulatory effects on PGD₂-induced shape change (**Fig. 6A and B**). As expected, PTX pretreatment almost completely blocked the G_{α_i}-dependent CCR3/CCL24 response but not the priming properties of JWH-133 (**Fig. 6C and D**).

JWH-133 induces calcium release in human and mouse eosinophils

Having confirmed that CB₂ signaling in human eosinophils is transmitted in a G_{αi} independent manner, we next aimed to explore the role of Ca²⁺ in the CB₂ pathway. To this end, responses of the endogenous ligand 2-AG were compared with those of the synthetic agonist JWH-133. At 100 nM, JWH-133 only induced a weak Ca²⁺ signal in human eosinophils, whereas 2-AG exhibited a robust five-fold stronger response (**Fig. 6G**). Consistent with JWH-133 and 2-AG sharing the same receptor, JWH-133 (100 nM) desensitized the calcium response induced by 2-AG (100 nM) (**Fig. E3A**). However, 100 nM of JWH-133 or 2-AG, concentrations that were effective in enhancing shape change responses in human eosinophils, did not increase eotaxin-2/CCL24-induced Ca²⁺ flux (**Fig. E3B**), neither did 200 nM of JWH-133 (**Fig. E3C**), although at this concentration a robust and CB₂ dependent Ca²⁺ flux was found in human (**Fig. 6E**) and mouse eosinophils (**Fig. 6F**). To confirm the observed PTX insensibility of CB₂ signaling, human eosinophils were treated with PTX (5 μg/ml, 20 min 37 °C) and Ca²⁺ flux was induced with JWH-133 (200 nM), 2-AG (200 nM) or eotaxin-2/CCL24 (1 nM), respectively. As expected, Ca²⁺ responses to eotaxin-2/CCL24 were almost completely blocked, whereas JWH-133- and 2-AG-induced Ca²⁺ responses remained unaffected (**Fig. 6H**). In contrast, pretreatment with the PLC inhibitor U-73122 (but not its inactive form U73343), as well as the IP₃ receptor antagonist 2-APB abolished Ca²⁺ responses induced by JWH-133 and 2-AG indicating that CB₂ interacts with G_{αq} rather than G_{αi}. 2-APB is also capable of inhibiting TRP channels. Thus, to exclude the involvement of TRP, control experiments in the presence of 3 mM EGTA were conducted, which showed that Ca²⁺ influx from the extracellular space, and hence TRP channels, are not involved in the Ca²⁺ response to CB₂ receptor activation (**Fig. 6H**).

Effects of JWH-133 on human eosinophils are mediated via MEK1/2 and p160 ROCK

Human eosinophil migration is regulated by multiple signaling pathways involving PI3K, ROCK, ERK and p38 MAPK (36). To assess the down-stream components of the CB₂ pathway in human eosinophils, cells were pre-treated with LY-294002 (PI3K, 10 μM), U-0126 (MEK1/2, 10 μM), PD-184161 (MEK1/2, 18 μM), SB202190 (p38 MAPK, 50 μM, Y-27632 (ROCK, 200 nM) or vehicle, respectively. Concentrations were chosen according to literature and pre-treatment was performed for 20 min at 37 °C (36). Thereafter, eosinophil shape change was induced with eotaxin-2/CCL24. As illustrated in **Fig. E4**, we found that the MEK1/2 inhibitors U-0126 (**B**) and PD-184161 (**C**) as well as the p160 ROCK inhibitor Y-27632 (**D**) significantly reduced the priming effect of JWH-133 for eotaxin-2/CCL24 responses, whereas blockade of PI3K (**E**) and p38 MAP kinase (**F**) had no effect.

Having confirmed that JWH-133 augments eosinophil responsiveness to other chemoattractants via MEK1/2 and ROCK signaling in shape change, we investigated the selective contribution of MEK1/2 and ROCK to JWH-133-induced chemotaxis. Similarly, pretreatment with U-0126 and Y-27632 prior to the chemotaxis assay, effectively inhibited eosinophil migration towards serial dilutions of JWH-133 (**Fig. E4 G and H**).

Systemic application of JWH-133 worsens airway hyperreactivity (AHR) in mice

Our *in vitro* data clearly shows that CB₂ ligands significantly contribute to eosinophil activation and responsiveness. To prove the *in vivo* relevance of these observations we used an acute model of OVA-induced asthma in mice. In brief, mice were immunized to OVA on days 0 and 7, were treated i.p. with JWH-133 (10mg/kg), SR144528 (10mg/kg), a combination of both or vehicle from day 9 to day 16 and were challenged with inhaled OVA aerosol on days 14 and 16. On day 17, either airway hyperresponsiveness to methacholine was recorded or BALF was taken. As illustrated in **Fig. 7**, JWH-133 significantly impaired airway resistance and compliance compared to the vehicle group (**Fig. 7A and B**) whereas SR144528 pretreatment fully prevented these effects of JWH-133 (**Fig. 7C and D**). Of

the potent inflammatory lipid mediators comprising the cysteinyl leukotrienes (CysLTs: LTC₄, LTD₄, and LTE₄), only LTE₄ is stable and abundant *in vivo*. Besides mast cells, eosinophils are the main source of CysLTs which contribute not only to bronchoconstriction and airway hyperreactivity (37), but as in the case of LTE₄ also to eosinophil recruitment (38). Increased levels of LTE₄ can be detected in urine (39), BALF (40) and exhaled breath condensate (41) of patients after allergen challenge. Accordingly, mass spectrometric analysis of BALF from OVA-challenged mice revealed slightly increased LTC₄ levels (**Fig. 7 E**) and significantly elevated concentrations of LTD₄ and LTE₄ in JWH-133-treated mice compared to vehicle-treated controls. Again, the effect of JWH-133 could be abolished with SR144528 pretreatment (**Fig. 7 F and G**). We furthermore determined eosinophil counts in the BALF of JWH-133- and vehicle-treated mice. Consistent with our results from the *in vitro* and *in vivo* migration assays, flow cytometric analysis revealed 3-fold higher eosinophil counts in the JWH-133-treated group compared to controls (**Fig. 7H**). Representative histology images of lung sections are provided in **Fig. E6**. To confirm the eosinophil-specific effect of the CB₂ agonist to the pathogenesis of allergic asthma, further experiments in eosinophil-deficient Δ dblGATA mice were conducted. Treatment with JWH-133 worsened airway resistance in WT mice, but mediated beneficial effects on lung parameters in the Δ dblGATA group (**Fig. E7 A/B**). Thus, our results show unequivocally, that systemic CB₂ activation directly contributes to the pathophysiology of asthma in mice by enhancing eosinophil migration and effector function.

Discussion

In this study, we propose a novel mechanism of CB₂-induced priming of eosinophils that may directly contribute to the pathogenesis of eosinophilic diseases. This notion is supported by the observation that pre-treatment with the selective and stable CB₂ receptor agonist JWH-133 profoundly increased eosinophil responsiveness towards chemoattractants such as eotaxin-2/CCL24 and PGD₂, with respect to shape change, integrin expression, adhesion, chemotaxis, and ROS production. The magnitude of primed responses was dependent on the concentration of JWH-133

and of the chemoattractant. In line with these *in vitro* data, systemic application of JWH-133 amplified the eotaxin-2/CCL24-directed recruitment of eosinophils into the airways of IL-5Tg mice and exacerbated OVA-induced asthma-like inflammation by increasing eosinophil influx into lungs and worsening of AHR. Oka *et al.* were the first who demonstrated that EoL-1 cells and human peripheral blood eosinophils express CB₂ but not CB₁ receptors (28). Consistent with these data, we found that purified human eosinophils from healthy subjects express an appreciable amount of CB₂ on mRNA (data not shown) and protein levels. Enhanced gene expression of CB₂ has been shown for lung eosinophils in allergic patients after allergen challenge (42). Accordingly, we could demonstrate that also CB₂ protein expression is significantly increased on the cell surface of peripheral blood eosinophils from symptomatic allergic donors, supporting a role for CB₂ and its ligands in the regulation of allergen-induced eosinophilic inflammation.

Activation with the selective agonist JWH-133 at nanomolar levels only induced a slight chemotactic response in human eosinophils, but amplified eotaxin-2/CCL24 induced eosinophil migration, especially at low concentrations. In contrast, IL-8-induced neutrophil activation remained unaffected by JWH-133, which is in line with previous reports demonstrating that the effects of 2-AG on neutrophils are mainly caused by the degradation of 2-AG to AA and subsequent *de novo* synthesis of LTB₄ (30).

Pre-activation or priming of eosinophils by proinflammatory cytokines in the peripheral blood is a crucial step in the pathogenesis of allergic diseases. Several priming-dependent eosinophil responses, such as migration (43), adhesion (44) and degranulation (45) have been shown to be amplified in allergic patients. Moreover, a “hyperadhesive” eosinophil phenotype, characterized by increased levels of the adhesion molecule integrin $\alpha_M\beta_2$ (CD11b/CD18) has been described in allergic patients after allergen challenge (46). Here we could show that exposure to the selective CB₂ receptor agonist JWH-133 prior to stimulation with chemoattractants yields significantly upregulated amounts of CD11b on the surface of both human and mouse eosinophils. Accordingly, JWH-133 also

primes eosinophils for an enhanced capacity to adhere to ICAM-1 under physiological flow conditions, an important prerequisite of endothelial transmigration.

To further prove the *in vivo* relevance of the observed modulating activities of CB₂, well-established mouse models of directed eosinophil migration and OVA-induced asthma were performed. Of note, we could show that daily treatment with JWH-133 not only aggravated lung parameters, but also led to increased eosinophil counts in the airways of OVA-challenged mice. Interestingly, these effects were completely absent in eosinophil-deficient Δ dblGATA mice, indicating that eosinophils are the major target of JWH-133 in allergic inflammation. Moreover, BALF of JWH-133-treated animals contained significantly higher CysLT levels compared to control animals; again, pointing to a more severe disease state due to systemic CB₂ activation. Noteworthy, Larose et al. demonstrated that 2-AG alone or in combination with platelet activating factor (PAF) induced CysLT biosynthesis in human eosinophils *in vitro*. However, 2-AG/PAF-induced synthesis was blocked by MAG lipase inhibitors, indicating that this effect is more related to 2-AG degradation and metabolic transformation into eicosanoids than to selective CB₂ activation³⁵. In contrast to 2-AG, JWH-133 is not degradable to AA, and therefore can't be further used for CysLT synthesis indicating a direct correlation exists between the observed enhanced CysLT levels and activation of CB₂. In accordance with our findings, recent work provided further evidence that CB₂ receptor activation is capable of enhancing inflammatory processes. Treatment with the CB₂ agonist JWH-133 potentiated adipose tissue inflammation in mice on high fat diet (47). Notably, 2-AG plasma levels were also found to positively correlate with the body mass index (BMI) in humans (48). Interestingly, the prevalence of asthma, its severity and reduced responsiveness to standard medication seem to be associated with obesity and high BMI (49). However, the molecular mechanism leading to these derangements are still poorly understood, but it is tempting to speculate that CB₂ activation by systemically elevated 2-AG levels and facilitation of eosinophil recruitment into the airways might be involved.

In both, human eosinophils and monocytes, 2-AG-induced CB₂ signaling was reported to be G_{α_{i/o}}-dependent as pretreatment with PTX abrogated the chemotactic responses of these cells (28). Conversely, our data strongly suggest a G_{α_{i/o}}/adenylyl cyclase-independent pathway, substantiated by the fact that PTX was unable to prevent eosinophil priming and Ca²⁺ flux following CB₂ activation. Furthermore, we observed that the PLC inhibitor U-73122 and the IP₃ receptor antagonist 2-APB were capable of reducing JWH-133 and 2-AG induced Ca²⁺ release. Thus, eosinophil CB₂ receptors seem to interact with G_{α_q} rather than, or in addition to, G_{α_i}-proteins. Of note, Shoemaker *et al.* showed previously that cannabinoid agonists display different rank orders of potencies and receptor occupancies for regulation of intracellular effectors. Endogenous ligands such as 2-AG are more “efficient” agonists requiring only half the receptor occupancy to elicit same effects as synthetic agonists. Accordingly, we found that 2-AG induced a much stronger Ca²⁺ response compared to JWH-133 (50). CB₂ receptors have previously been shown to induce a G_{β_γ}-dependent MAPK/ERK signaling cascade (51). Here we found that the induction of MEK 1/2 and ROCK activity is likely to be a part of the signaling mechanism accounting for the priming effect of JWH-133. Our observations are in line with previous findings indicating the involvement of MEK1/2-ERK activation in IL-5 and GM-CSF induced priming of human eosinophils (52). Moreover, MEK1/2-ERK and ROCK signaling regulates a variety of pro-inflammatory cellular processes such as eosinophil migration, degranulation and respiratory burst (36,53) which afford the progression of eosinophilic inflammation.

In summary, the results of the present study demonstrate for the first time that specific CB₂ activation represents a novel priming process leading to enhanced migratory responsiveness of human and mouse eosinophils *in vitro* and *in vivo*. This CB₂-mediated amplification of eosinophil migration seems to occur G_{α_{i/o}}/adenylyl cyclase-independent, but involves G_{α_q}/MEK/ROCK signaling. Previous studies in asthmatic patients showed a beneficial effect of inhaled or orally taken cannabinoids by dilating bronchial smooth muscles (54,55). In contrast, our data provides evidence for the involvement of the endocannabinoid/CB₂ axis in the progression of allergic inflammatory processes and indicates possible undesirable proinflammatory effects of long-term cannabinoid use.

Thus, specific CB₂ receptor antagonism may open a new therapeutic approach for allergic disorders and other eosinophil-driven diseases.

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Figure Legends

Figure 1. CB₂ expression is enhanced on eosinophils from symptomatic allergic donors. Purified human eosinophils were stained with a polyclonal rabbit anti-human CB₂ primary Ab or isotype control (1:50), followed by a goat anti-rabbit secondary Ab (AF-647; 1:500).

Figure 2. JWH-133 differentially modulates shape change in human eosinophils, basophils and neutrophils. **A:** Eosinophils were pre-treated with JWH-133 (100 nM) or vehicle and stimulated with eotaxin-2/CCL24 (Eot-2). **B:** Prior to JWH-133 treatment, eosinophils were incubated with SR144528 (1 μM) and then treated as described in **A**. **C and D:** Eosinophils were pre-treated with 2-AG or JWH-133 and stimulated with eotaxin-2/CCL24 (0.6 nM) or vehicle. **E:** Basophils (HLA-DR⁺/CD123⁺ PBMC) were treated as described in **A**. **F:** Neutrophils (CD16⁺ PMNL) were pre-treated with JWH-133 (100 nM) or vehicle and stimulated with IL-8. Data are shown as mean ± SEM. * p<0.05 versus vehicle n=4-7.

Figure 3. JWH-133 primes human and mouse eosinophils for an enhanced CD11b expression and adhesion. **A:** Human whole blood samples were stained with anti-CD11b-PE and anti-CD16-PE-Cy5 Ab and pretreated with JWH-133 (1 μM) or vehicle and then stimulated with eotaxin-2/CCL24. **B:** bmEos were stained with anti-mouse CD11b-PE Ab, pre-treated with JWH-133 (250 nM) or vehicle and stimulated with eotaxin-2/CCL24. **C:** Eosinophils were pretreated with SR144528 (1 μM) or vehicle, treated with JWH-133 (250 nM) or vehicle and stimulation with eotaxin-2/CCL24 (0.6 nM). ICAM-1 coated channels were superfused with human eosinophils and adhesion was monitored. Data are shown as mean ± SEM, * p<0.05 n=4-8.

Figure 4. JWH-133 enhances the migratory responsiveness of human and mouse eosinophils. A:

Chemotaxis: eosinophils were allowed to migrate towards serial dilutions of JWH-133, vehicle, or eotaxin-2/CCL24. Chemokinesis: Eosinophils were pretreated with serial dilutions of JWH-133 or vehicle and were allowed to migrate towards assay buffer only. **B:** eosinophils were pretreated with JWH-133 (5 nM) and were allowed to migrate towards eotaxin-2 (1nM). **C:** bmEos were pretreated with vehicle or SR144528 (1 μ M), mixed with JWH-133 (250 nM) or vehicle and cells were allowed to migrate towards eotaxin-2/CCL24 (100 nM). **D:** IL-5Tg BALB/c mice were treated i.p. with JWH-133 (5mg/kg/d) or vehicle for 3 days. Five hours after intranasal application of 4 μ g eotaxin-2, BALF was collected and eosinophils were counted as Siglec F⁺/CD11c⁻ cells. **E:** bmEos were isolated from WT or CB₂-KO mice, treated with JWH-133 (250 nM) or vehicle and were allowed to migrate towards serial dilutions of eotaxin-2/CCL24. Data are shown as mean \pm SEM, * p< 0.05, n=4-7.

Figure 5. JWH-133 enhances eosinophil respiratory burst but not degranulation. A: Eosinophils

were pre-treated with JWH-133 (250 nM) or vehicle, DCFDA (50 μ M) was added and cells were stimulated with eotaxin-2/CCL24 for 30 min at 37 °C. Data are shown as mean \pm SEM, n=6. **B and C:** Eosinophils were pre-treated with JWH-133 (250 nM) or vehicle and CD63 expression and EPO release was induced with serial dilutions of C5a. Data are shown as mean \pm SEM, n= 5.

Figure 6. JWH-133 modulates eosinophil function in a pertussis toxin (PTX)-insensitive manner.

Isolated human eosinophils were pre-treated with vehicle (**A** and **C**) or PTX (5 μ g/ml) (**B** and **D**) for 20 min at 37 °C, and exposed to JWH-133 (100 nM) or vehicle for 5 min at room temperature. Shape change was stimulated for 4 min at 37 °C with serial dilutions of PGD₂ (**A** and **B**) or eotaxin-2/CCL24 (**C** and **D**). Isolated human (**E**) or mouse (**F**) eosinophils were loaded with Fluo3-AM, incubated with 1 μ M SR144528 or vehicle, mixed with 200 nM JWH-133 and Ca²⁺ flux was measured by FACS. **G:** Ca²⁺ mobilization was elicited by 100 nM of JWH-133 and 2-AG. Data are shown as representative of 3-5 independent experiments. **H:** Eosinophil were pretreated with 5 μ g/ml PTX for 20 min, with 6 μ M U-73122 or its inactive form U73343, 50 μ M 2-APB or vehicle, and Ca²⁺ flux was induced by 200 nM

JWH, 200 nM 2-AG or 1 nM eotaxin-2/CCL24. In some experiments EGTA (3 mM) was added prior to stimulation. Data are shown as mean \pm SEM, * $p < 0.05$, $n = 5-10$.

Figure 7. JWH-133 deteriorates lung function and increases eosinophil counts in BALF. Eight-week-old female C57BL6 mice were sensitized and exposed to ovalbumin and their lung function was assessed while applying increasing doses of methacholine (MCH) by a FlexiVent system. Mice were given either JWH-133 (10 mg/kg) or vehicle from day 10 to 17 and airway resistance (**A**) and compliance (**B**) were measured. For **C** and **D**, SR144528 (10 mg/kg) was applied alone or in combination with JWH-133. **E**, **F** and **G**: 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. **H**: Flow cytometric analysis of cells in the BALF of mice treated as in (**A**). Data are shown as mean \pm SEM, * $p < 0.05$, $n = 6 - 9$.

Supplementary Figure Legends

Figure E1. The endocannabinoid 2-AG enhances eotaxin-2/CCL24 induced CD11b upregulation. **A:** Human PMNL were stained with anti-CD11b-PE Ab and anti-CD16-PE-Cy5 Ab and were pretreated with 2-AG (250 nM) or vehicle and then stimulated with eotaxin-2/CCL24. Data are shown as mean \pm SEM, $n = 5$.

Figure E2. JWH-133 does not induce chemotaxis in mouse eosinophils. BmEos were allowed to migrate towards serial dilutions of JWH-133 in a microBoyden chamber. Data are shown as mean \pm SEM, $n = 3$.

Figure E3. CB₂ desensitization and the effect on eotaxin-2/CCL24 induced Ca²⁺ flux.

A: Stimulation with JWH-133 (100 nM) desensitizes the CB₂ receptor for further 2-AG (100 nM)

responses. **B:** Pre-treatment with 100 nM JWH-133 or 100 nM 2-AG does not affect responses induced by eotaxin-2/CCL24 (1.5 nM). Data are shown as a representative of 3-5 independent experiments. **C:** Pre-treatment with 200 nM JWH-133 does not affect eotaxin-2/CCL24 induced responses. Data are shown as mean \pm SEM, n= 5.

Figure E4. MEK1/2 and p160 ROCK are involved in the modulating effect of JWH-133. Eosinophils were pre-treated with the protein kinase inhibitors LY-294002 (PI3K, 10 μ M, **B**), U-0126 (MEK1/2, 10 μ M, **C**), PD-184161 (MEK1/2, 18 μ M, **D**), SB202190 (p38 MAPK, 50 μ M, **E**) and Y-27632 (ROCK, 200 nM, **F**) or vehicle (**A**). Following incubation with JWH-133 (100 nM) or vehicle, eosinophils were stimulated with eotaxin-2/CCL24. 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. Data are shown as mean \pm SEM, * p<0.05, n= 5-14.

Figure E5. Representative images of eosinophil shape change assessed by flow cytometry. **A:** FSC/SSC plot of unstimulated isolated human eosinophils. **B:** FSC/SSC plot of isolated human eosinophils stimulated with 0.6 nM 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. **C:** Same as in **B**, but eosinophils were pretreated with 100 nM JWH-133, leading to a further increase of the FSC parameter.

Figure E6. Representative histological pictures of paraffin sections of lungs from OVA-challenged mice. Mice were treated as in **Fig. 7A** 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.

Figure E7. Eosinophils are required for JWH-133 induced aggravation of lung parameters. Mice were treated as in (Fig 7A). **A:** BALB/c WT mice. **B:** Δ dblGATA mice. Data are shown as mean \pm SEM, * $p < 0.05$, $n = 3 - 6$.

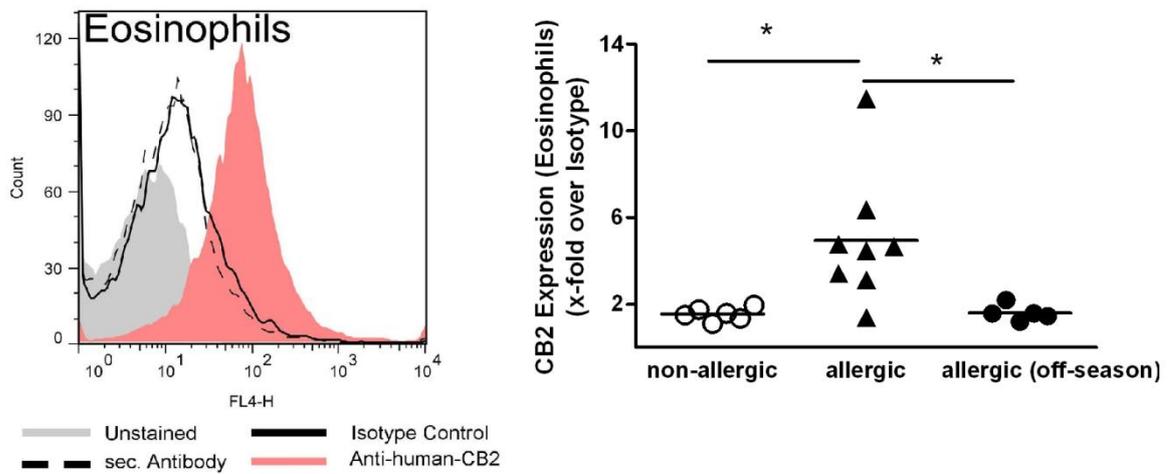


Figure 1.

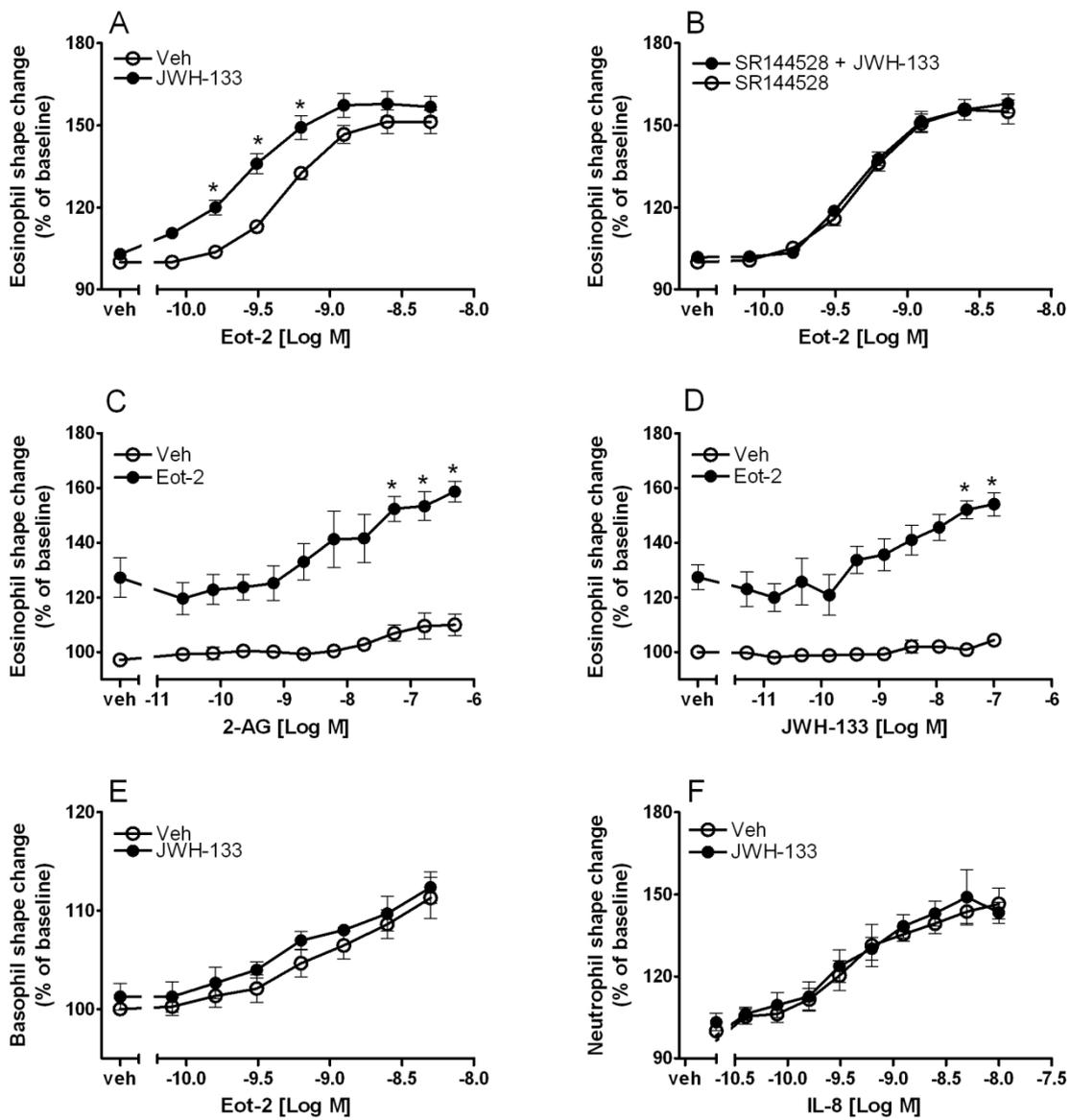


Figure 2.

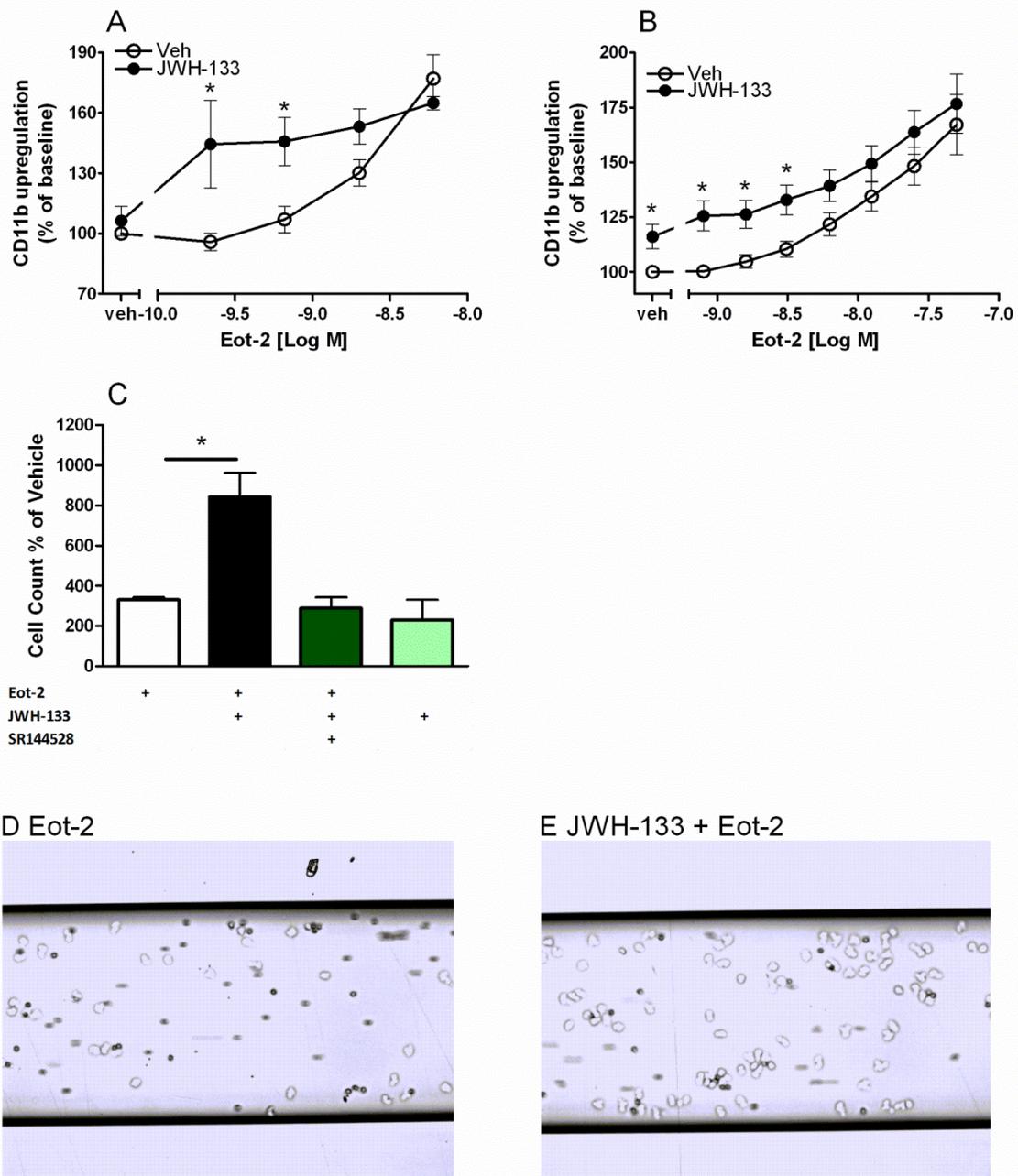


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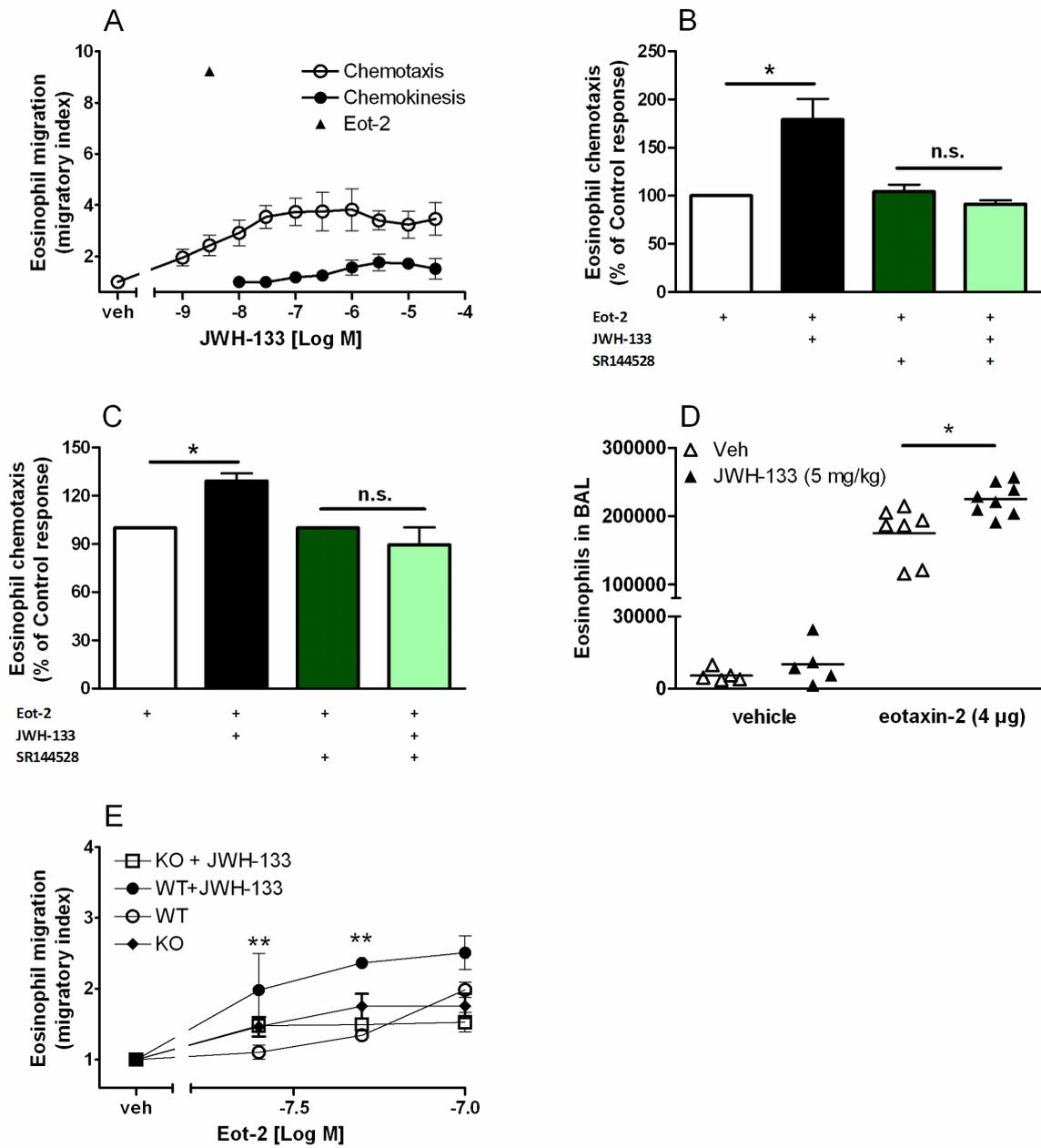


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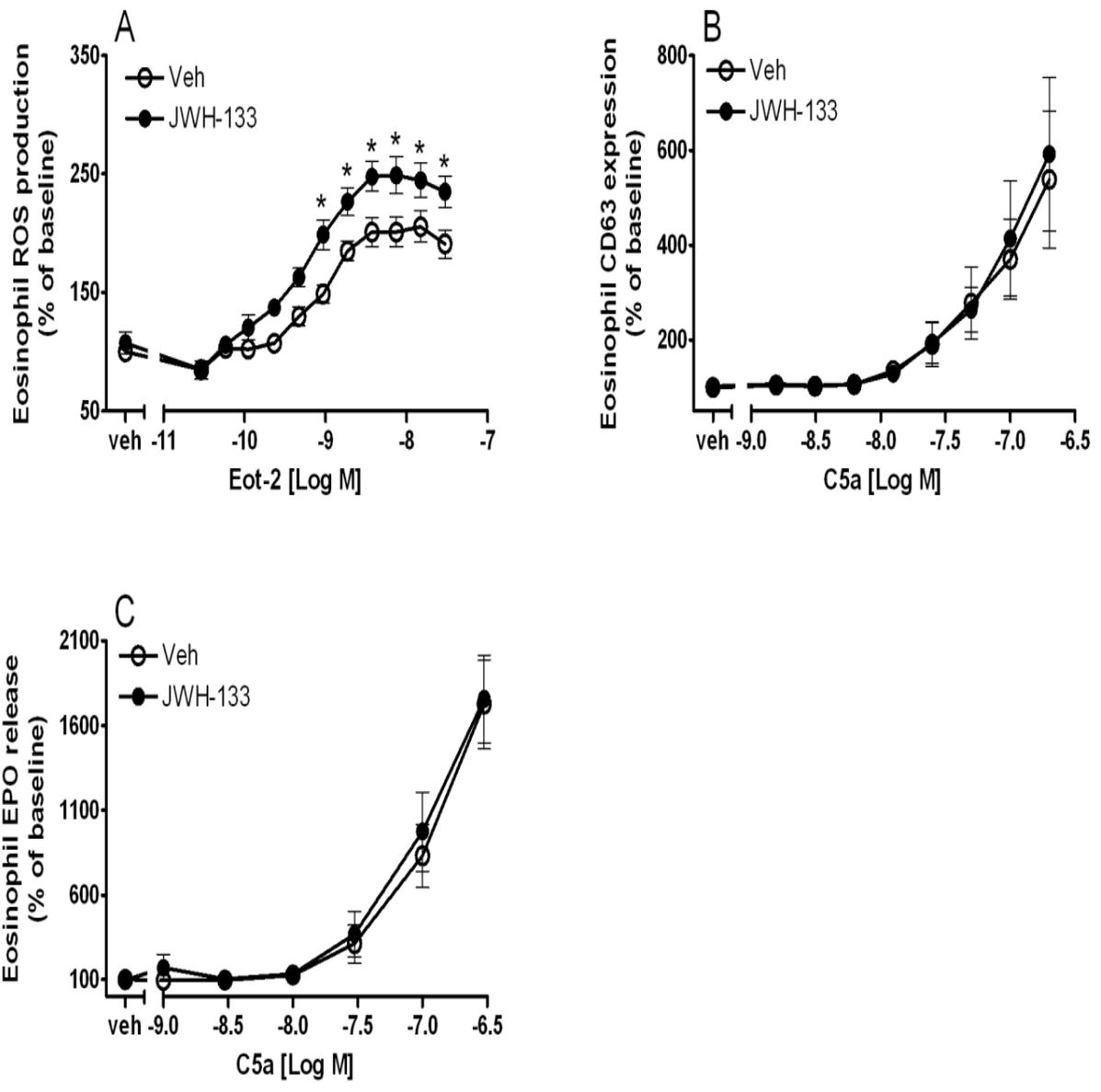


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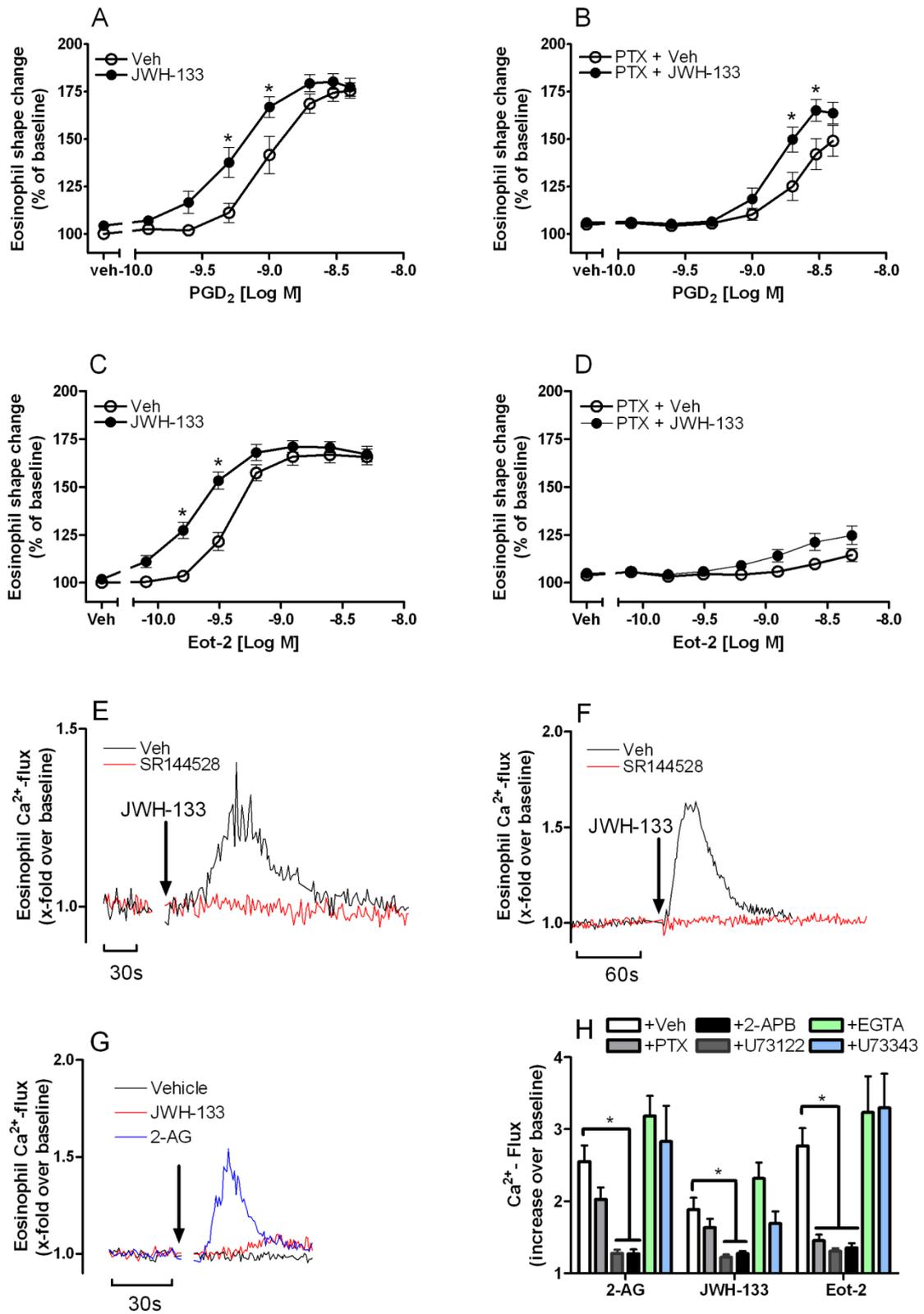


Figure 6.

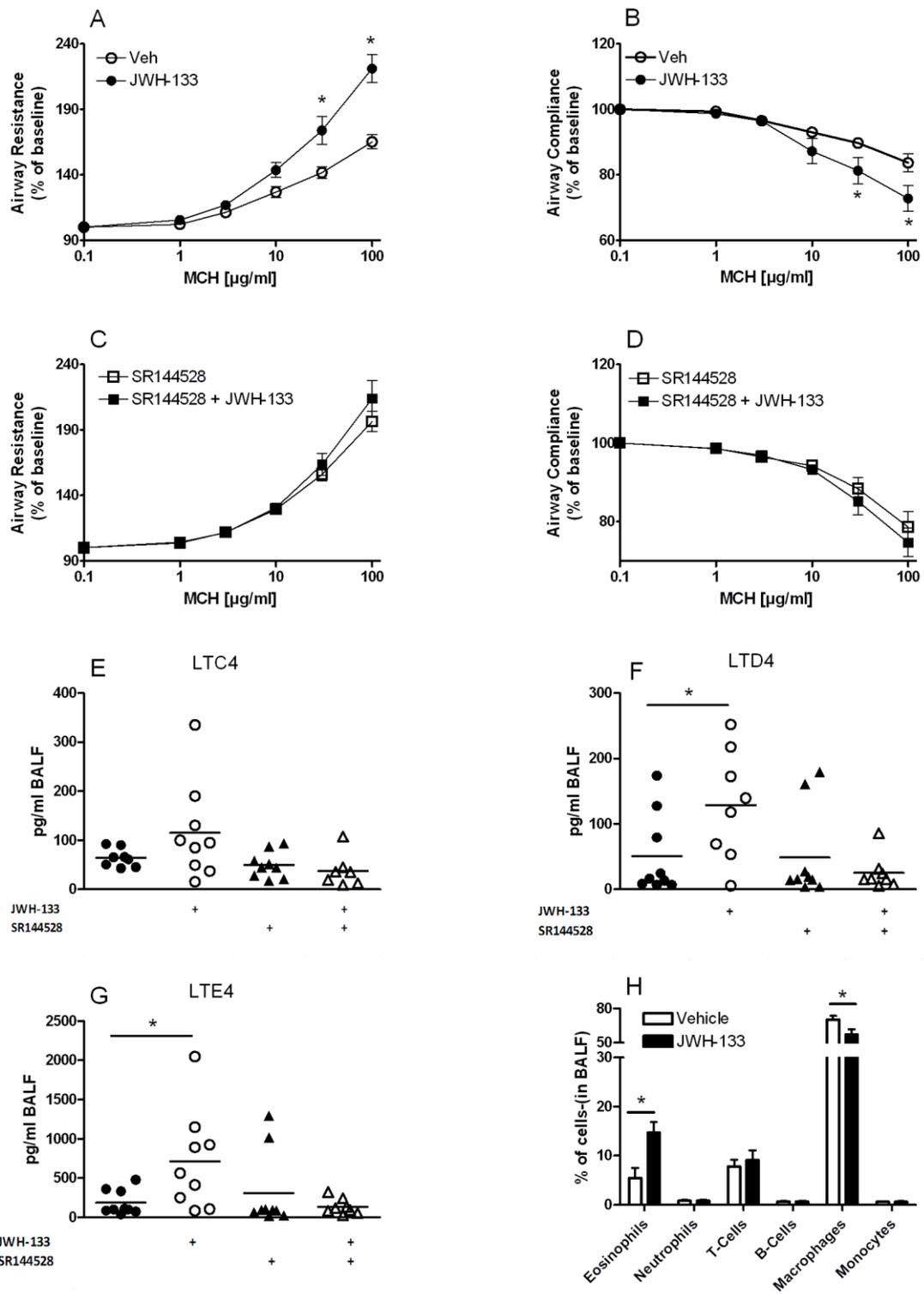


Figure 7.