

The CB₂ cannabinoid receptor agonist JWH-015 modulates human monocyte migration through defined intracellular signaling pathways

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ABSTRACT

Recruitment of leukocytes to inflammatory sites is crucial in the pathogenesis of chronic inflammatory diseases. The aim of this study was to investigate if activation of CB₂ cannabinoid receptors would modulate the chemotactic response of human monocytes. Human monocytes treated with the CB₂ agonist JWH-015 for 12 to 18h showed significantly reduced migration to chemokines CCL2 and CCL3, associated with reduced mRNA and surface expression of their receptors CCR2 and CCR1. The induction of intercellular adhesion molecule (ICAM)-1 in response to interferon (IFN)- γ was inhibited by JWH-015. Moreover, JWH-015 cross-desensitized human monocytes for migration in response to CCL2 and CCL3 by its own chemoattractant properties. The CB₂ selective antagonist SR144528, but not the CB₁ antagonist SR147778 reversed the JWH-015-induced actions, while the CB₂ agonist JWH-133 mimicked the effects of JWH-015. The investigation of underlying pathways revealed involvement of phosphatidylinositol 3 kinase (PI3K)/Akt and extracellular signal-related kinase (ERK) 1/2, but not p38 mitogen activated protein kinase (MAPK). In conclusion, selective activation of CB₂ receptors modulates chemotaxis of human monocytes, which might have crucial effects in chronic inflammatory disorders such as atherosclerosis or rheumatoid arthritis.

Key words: chemokines, chemokine receptors, ICAM-1, chronic inflammation, atherosclerosis

INTRODUCTION

Cannabinoids modulate immune functions and therefore have a therapeutic potential for the treatment of inflammatory diseases (28, 29, 40). It is thought that the immunomodulatory effects of cannabinoids are mediated by the cannabinoid receptor 2 (CB₂) expressed on immune cells. A growing body of evidence suggests that endocannabinoid signaling plays a critical role in pathophysiological conditions such as haemorrhagic or endotoxic shock (53, 56), liver cirrhosis (4), fibrosis (24, 52), and ischemic/reperfusion injury (5, 44), acute heart failure (36), osteoporosis (25, 39, 51), and cutaneous contact hypersensitivity (26). We have recently provided first experimental evidence for a possible role of CB₂ receptors in atherosclerosis progression (49).

The recruitment of inflammatory cells into the intima is crucial for the development and progression of atherosclerosis (19, 20, 57). The tethering, rolling, adhesion and trans-endothelial migration of leukocytes are triggered by local production of chemokines and chemokine receptors as well as adhesion molecules (8). Cannabinoids have been reported to modulate the migration of various cell types. Thus, it has been reported that treatment of rat macrophages with the synthetic cannabinoid CP55,940 reduced both spontaneous and formyl-metionyl-leucine-phenylalanine (fMLP)-induced chemotaxis (48). The nonpsychoactive marijuana component cannabidiol was shown to inhibit murine macrophage chemotaxis in vitro and in vivo in a CB₂ receptor-dependent manner (47). Both endogenous as well as synthetic agonists 2-AG, CP55,940 and WIN55,212-2, as well as the CB₂ selective agonists JWH-015 and JWH-133, caused a significant inhibition of the chemokine stromal cell derived factor (SDF)-1/CXCL12-induced and CXCR4-mediated chemotaxis of Jurkat or primary human T cells (11, 15). In addition, in an

experimental autoimmune encephalomyelitis (EAE) mouse model, WIN55,212-2 attenuated leukocyte rolling and adhesion on endothelial cells through the activation of CB₂ receptors (38). Furthermore, the novel CB₂ selective inverse agonist Sch.336 potently inhibited leukocyte chemotaxis to 2-AG, HU-210 or monocyte chemoattractant protein (MCP)-1/CCL2 in vitro and in vivo (33). In a mouse model of liver ischemic/reperfusion (I/R) injury, the CB₂ selective agonist JWH-133 protected against I/R damage by decreasing inflammatory cell infiltration, tissue and serum tumor necrosis factor (TNF)- α , macrophage inflammatory protein (MIP)-1 α /CCL3 and MIP-2/CXCL2 levels, and expression of adhesion molecule intercellular adhesion molecule (ICAM)-1 (5). In vitro, JWH-133 was shown to attenuate the TNF α -induced ICAM-1 and vascular cell adhesion molecule (VCAM)-1 expression in human liver sinusoidal endothelial cells (HLSECs) and the adhesion of human neutrophils to HLSECs. In a different study, anandamide dose-dependently attenuated the TNF α -induced ICAM-1 and VCAM-1 expression in human coronary artery endothelial cells (HCAECs), and the adhesion of THP-1 monocytes to HCAECs in a CB₁ and CB₂-dependent manner (6). The role of CB₂ in this process could be confirmed by additional data obtained with CB₂ agonists, which reduced ICAM-1 and VCAM-1 expression in HCAECs as well as adhesion and transendothelial migration of THP-1 monocytes (43). Finally, we have shown that THC inhibited murine peritoneal macrophage chemotaxis in response to CCL2 and reduced expression of the chemokine receptor CCR2 on splenocytes (49). These effects were blocked by the CB₂ receptor antagonist SR144528 or when cells from CB₂ knockout mice were used.

In this study, we investigated if CB₂ selective agonists would modulate the migration of human monocytes and thus may have a therapeutic potential in inflammatory conditions such as atherosclerosis.

MATERIALS AND METHODS

Reagents

The CB₂ selective agonist JWH-015 was obtained from Cayman Chemical, and JWH-133 was obtained from Tocris Bioscience. The CB₁ antagonist SR147778 and the CB₂ antagonist SR144528 were kindly provided from Sanofi-Synthelabo (France) (45, 46). Stock solutions (10 to 20 mM) of cannabinoids and antagonists were prepared in DMSO. Appropriate dilutions of DMSO were added as vehicle controls in all experiments. BSA was from Sigma-Aldrich, Inc.. CCL2, CCL3, interferon (IFN)- γ , the anti-phosphorylated extracellular signal-related kinases (ERK) 1/ERK 2 (T202/Y204; AF1018) polyclonal antibody (Ab), the anti-phosphorylated p38 (MAPK (T180/Y182; AF869) polyclonal Ab, the anti ERK 1/ERK 2 polyclonal Ab (AF1576), the phycoerythrin (PE) conjugated anti human CCR1 Ab (FAB145P) and the PE conjugated anti human CCR2 Ab (FAB151P) were all purchased from R&D Systems Europe Ltd. The PE conjugated anti human CD54/ICAM-1 Ab (555511) and the FITC conjugated anti human CD14 Ab (555397) were obtained from BD PharmingenTM. The anti human CB₂ Ab was obtained from Abcam plc (ab3561) and PE-conjugated goat anti-rabbit IgG antibody was obtained from Molecular Probes. The kinase inhibitor LY294002 (phosphatidylinositol 3 kinase (PI3K) inhibitor) was obtained from Sigma-Aldrich and PD98059 (MEK inhibitor) and SB203580 (p38 MAPK inhibitor) were obtained from Biomol Research Laboratories,

Inc.. The anti p38 (H-147; sc-7149) polyclonal Ab, the anti Akt 1/2/3 (H-136; sc-8312) polyclonal Ab and the anti-phosphorylated Akt 1/2/3 (Thr 308; sc-16646-R) polyclonal Ab were obtained from Santa Cruz Biotechnology.

Isolation of human monocytes

Human monocytes were isolated from buffy coats of healthy volunteers after informed consent. After centrifugation on Ficoll-Hypaque density gradient, mononuclear cells were collected from the interface and washed with PBS. Then, monocytes were purified from the upper interface of a hypotonic Percoll density gradient (1.129 g/ml). Purified monocytes were resuspended in RPMI 1640 with 25 mM Hepes and 500 ng/ml polymyxin B. The purity of monocytes was determined by flow cytometric analysis (CD14 staining), confirming that at least 85% purity was achieved in all experiments.

Modified Boyden chamber migration assays and checkerboard analysis

To study the effect of cannabinoid treatment on migration, cells were incubated in RPMI containing 25 mM Hepes and 500 ng/ml polymyxin B in presence or absence of different doses of JWH-015 (5, 10, 20 μ M) for 12 hours at 37 °C, in a humidified atmosphere with 5% CO₂. Then, cells were washed three times in PBS and resuspended in chemotaxis medium (RPMI containing 25 mM Hepes and 1% BSA) to test their locomotory response to medium alone, 10 nM CCL2 or 10 nM CCL3. Monocyte chemotaxis was assessed in a 48-well microchemotaxis chamber (NeuroProbe) using a 5 μ m pore size, 5- μ m-thick polyvinylpyrrolidone-free polycarbonate filter (NeuroProbe). Cells were seeded in the upper wells, while medium or chemoattractant solutions were added to the lower wells.

To test the chemotactic properties of CB₂ selective cannabinoids, untreated monocytes were tested for their ability to migrate in response to medium alone, JWH-015 (5, 10, 20 μM) or JWH-133 (10 μM). In some experiments, cells were pretreated for 10 min with the receptor antagonists SR147778 (1 μM) or SR144528 (1 μM) or for 60 minutes with the kinase inhibitors LY294002 (50 μM), PD98059 (50 μM) or SB203580 (1 μM). After the treatment with kinase inhibitors, cells were washed three times in chemotaxis medium before testing their locomotory ability. In a different set of experiments, cells and different doses (5, 10, 20 μM) of JWH-015 were seeded in the upper well, while medium alone or 10 nM CCL2 or 10 nM CCL3 were added to the lower well. Checkerboard analysis was performed by adding different doses of JWH-015 (0, 5, 10, 20 μM) both in the upper (with the cells) and in the lower well.

In all conditions, the modified Boyden chamber was incubated for 60 min, at 37 °C in a humidified atmosphere with 5% CO₂. Then, filters were removed from the chambers and stained with Diff-Quick (Baxter). Each condition was performed in duplicate. The cells of five random oil-immersion fields were counted at 1000× magnification (blinded observer) and the chemotaxis index was calculated from the number of cells migrated to the test samples divided by the number of cells migrated to the medium.

Flow cytometry

Monocytes were cultured at a concentration of 5x10⁶ cells/ml in serum-free RPMI medium containing 25 mM HEPES and 500 ng/ml polymyxin B in the presence or in absence of JWH-015 (5, 10, 20 μM) or JWH-133 (10 μM) ± receptor antagonists SR147778 (1 μM) and SR144528 (1 μM) or kinase inhibitors LY294002 (50 μM),

PD98059 (50 μM) and SB203580 (1 μM) for 18 or 24 hours, at 37 °C in a humidified atmosphere with 5% CO_2 . In some experiments, cells were stimulated with IFN- γ (100 U/ml). FITC- or PE-labeled antibodies anti human CCR1, CCR2, CD54 and CD14 as well as corresponding isotype controls were used. Surface CB₂ staining was performed by FC-blocking with human IgG and further incubation with a rabbit polyclonal antibody to CB₂, followed by incubation with PE-conjugated goat anti-rabbit IgG antibody. CellQuest software was used for acquisition and analysis on a FACSCalibur (BD Biosciences). Data were expressed as mean fluorescence intensities (MFI), compared to baseline expression (defined as 100%).

Cytotoxicity assay

Cell death was determined by quantification of lactate dehydrogenase (LDH) release (BioVision) in cell culture supernatants after 12, 18 and 24h of incubation in presence or absence of JWH-015 (20 μM), JWH-133 (10 μM), SR147778 (1 μM) or SR144528 (1 μM) and counting of trypan blue positive cells.

Apoptosis assay

Apoptosis rates of monocytes after 0, 18 and 24 h of treatment with JWH-015 (20 μM), JWH-133 (10 μM), SR147778 (1 μM) or SR144528 (1 μM) were determined via analysis of phosphatidylserine externalization using the Annexin V-FITC apoptosis detection kit (MBL).

Immunoblotting

Monocytes were cultured at a concentration of 5×10^6 cells/ml in serum-free RPMI medium containing 25 mM Hepes in presence or absence of JWH-015 (20 μ M), 10 nM CCL2 or DMSO (0.1% for various time points (between 2.5 min and 30 min)). The reaction was stopped on ice, and cells were centrifuged at 4°C to remove culture supernatants. Total protein was extracted in lysis buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM NaF, 1% NP40, 10% glycerol, 1 mM PMSF, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, 0.5 mM Na_3VO_4 . Proteins (40 μ g) were electrophoresed through polyacrylamide/SDS gels and transferred by electroblotting onto nitrocellulose membranes. Membranes were blocked for 1h in 5% (w/v) nonfat milk before incubating with appropriate dilutions of primary phospho-specific antibodies to ERK1/2, Akt or p38 MAPK as well as corresponding secondary antibodies. The blots were developed using the ECL system (ImmobilionTM Western, Millipore). Then, membranes were stripped, reblocked and reprobed to detect total ERK 1/2, Akt or p38 MAPK. Immunoblots were scanned and quantification was carried out by Image Quant software 3.3 (Molecular Dynamics). Values were normalized to total amounts of ERK 1/2, Akt or p38, respectively and expressed as percentages of medium control (defined as 100%).

RT-PCR

Total RNA from 5×10^6 human monocytes was extracted using TRI Reagent (Molecular Research Center, Inc.). Reverse Transcription and PCR were carried out with the OneStep RT-PCR Kit (Qiagen), using the following primer pairs for human CB_2 as previously described (34) : 5'-CGCCGGAAGCCCTCATACC (forward), 5'-

CCTCATTCGGGCCATTCCTG (reverse; PCR product of 522 bp). Amplification of GAPDH was used as endogenous control, using primers 5'-ACCACAGTCCATGCCATCAC (forward) and 5'-TCCACCACCCTGTTGCTGTA (reverse; PCR product of 452 bp).

Real-time RT-PCR

Monocytes were cultured at the concentration of 5×10^6 cells/ml in serum-free RPMI medium containing 25 mM HEPES and 500 ng/ml polymyxin B in the presence or in absence of JWH-015 (5, 10, 20 μ M) for 12h, at 37 °C in a humidified atmosphere with 5% CO₂. Total RNA was extracted using TRI Reagent (MRC, Inc.) and reverse transcribed using the Quantitect kit (Qiagen) according to the manufacturer's instructions. Real-time PCR was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Each measurement was done in triplicate using HPRT as endogenous control (run in separate tubes on the same plate), and relative quantification performed with the comparative method. Human CCR2 primers and probe were designed with Primer Express software (Applied Biosystems): 5' GCGTTTAATCACATTCGAGTGTTT (forward), 5' CCACTGGCAAATTAGGGA-ACAA (reverse), 5' FAM AGTGCTTCGCAGATGTCCTTGATGCTC TAMRA (probe). We used human CCR1 and HPRT primers and probes as previously described (23, 32).

Statistical analysis

All data were expressed as mean (\pm SEM). One-way ANOVA with Bonferroni's post test was performed using GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA). Differences between the *P* values below 0.05 were considered significant.

RESULTS

The cannabinoid receptor CB₂ is expressed on primary human monocytes

To obtain non-activated primary human monocytes, we performed Ficoll-Percoll density gradient centrifugation. We first confirmed the presence of CB₂ cannabinoid receptors on freshly isolated monocytes and found high expression levels on the cell surface (Fig. 1A). In addition, CB₂ mRNA expression in monocytes was detected by RT PCR (Fig. 1B). Interestingly, we observed a significant increase of CB₂ surface expression when monocytes were cultured for up to 24h (Fig. 1C). The presence of CB₂ agonist JWH-015 (20 μ M) did not change the observed modulation. To validate our experimental conditions, we tested the cell viability and extend of apoptosis in monocytes treated with cannabinoids or antagonists for up to 24h. We found an increasing percentage of dead and apoptotic cells in monocyte cultures (Fig. 2), comparable to the magnitudes previously reported (21, 37). The increase of cell death was due to the culture conditions in serum-free medium, which was performed to avoid the interference with serum factors. Importantly, treatment with cannabinoids and/or antagonists did not affect cell viability and apoptosis rates (Fig. 2). In none of the experimental conditions, we observed an

increased LDH release as compared to untreated cells after 12, 18 and 24 h of incubation (data not shown).

Treatment with the CB₂ agonist JWH-015 reduces monocyte migration to CCL2 and CCL3 by reducing chemokine receptor expression

We assessed monocyte migration in response to CCL2 and CCL3 with the modified Boyden chamber system. Pretreatment with 5 to 20 μ M of the CB₂ agonist JWH-015 for 12h significantly reduced the chemotactic response to CCL2 and CCL3 in a dose-dependent manner (Fig. 3A). The maximum inhibition was observed at 10 to 20 μ M JWH-015. We next analyzed the effect of JWH-015 on the expression of the chemokine receptors which bind CCL2 and CCL3. Treatment of monocytes with JWH-015 for 18h inhibited the surface expression of chemokine receptor CCR2 and, to a lesser extent, CCR1 at a concentration of 10 to 20 μ M (Fig. 3B, E, F). This effect was associated with significantly reduced CCR2 mRNA levels as determined after 12h of JWH-015 treatment (Fig. 3C). We also observed a marked, but not significant reduction of CCR1 mRNA levels. Pretreatment with the CB₂ antagonist SR144528 reversed the effect of JWH-015 on surface CCR2 and CCR1 expression, while the CB₁ antagonist SR147778 had no effect (Fig. 3D). It is of note that co-treatment of cells with JWH-015 and CB₂ antagonist SR144528 induced a marked, but not significant increase of chemokine receptor expression as compared to untreated cells. Treatment with CB₁ antagonist SR147778 or CB₂ antagonist SR144528 alone did not have a significant effect. Similar to JWH-015, incubation with the CB₂ agonist JWH-133 at 10 μ M also reduced CCR2 and CCR1

surface expression (Fig. 3D), while the vehicle DMSO alone did not affect mRNA or surface expression of the chemokine receptors (data not shown).

Treatment with JWH-015 modulates ICAM-1 expression on human monocytes

We further investigated the effect of JWH-015 on the expression of ICAM-1 which is implicated in monocyte recruitment. The basal expression of ICAM-1, which was strongly expressed on all monocytes, was significantly (1.44 fold) enhanced after 24h of incubation with IFN- γ (Fig. 4A, B). When JWH-015 (20 μ M) or JWH-133 (10 μ M) was added during stimulation, the IFN- γ -induced activation was significantly inhibited. The CB₂ antagonist SR144528 reversed the effect of JWH-015, while the CB₁ antagonist SR147778 did not inhibit the effect of JWH-015. Co-incubation with JWH-015 and CB₂ antagonist SR144528 induced a marked, but not significant increase of IFN- γ -induced ICAM-1 expression as compared to IFN- γ -treated cells. Similarly, the antagonists alone had a marked, but not significant effect on the IFN- γ -induced activation of ICAM-1. Neither treatment with JWH-015 nor the receptor antagonists alone reduced the basal ICAM-1 expression on the monocytes (Fig. 4A and data not shown).

JWH-015 induces chemotaxis of human monocytes

Because various cannabinoids have been reported to chemoattract different cell types, we next tested the ability of JWH-015 and JWH-133 to induce migration of freshly isolated monocytes. The number of cells migrating to the respective cannabinoid was increased as compared to the medium control, with a significant effect observed at 20 μ M JWH-015 (chemotaxis index of 1.98 ± 0.26) or 10 μ M JWH-133, respectively (chemotaxis index of

1.98 ± 0.2; Fig. 5A). Pretreatment with the CB₂ antagonist SR144528 inhibited the effect of JWH-015 and JWH-133, while the CB₁ antagonist SR147778 had no effect (Fig. 5B).

To distinguish if the migration was due to induction of chemotaxis or chemokinesis, we performed a checkerboard analysis with increasing concentrations of JWH-015 above and below the filter. We found a directed migration versus a gradient across the filter, indicating that JWH-015 induced chemotaxis rather than random chemokinesis (Fig. 5C).

JWH-015 cross-desensitizes monocytes for migration to CCL2 and CCL3

To investigate the chemotactic effect of JWH-015 in presence of a chemokine gradient, freshly isolated monocytes were seeded with increasing concentrations of JWH-015 to the upper well and chemoattracted with CCL2 or CCL3, respectively. We found that the migration to both CCL2 and CCL3 was significantly reduced in the presence of 10 to 20 μM JWH-015, suggesting that JWH-015 cross-desensitizes monocytes for migration to these chemokines (Fig. 5D).

The JWH-015 mediated modulation of monocyte migration involves PI3K/Akt and ERK 1/2, but not p38 MAPK signaling pathways

To investigate if the JWH-015 mediated effects on chemokine receptor expression and migration were dependent on distinct kinase signaling pathways, we performed experiments with selective kinase inhibitors for PI3K and MEK 1/2 (the activator of ERK 1/2) as well as p38 MAPK. Pretreatment with the PI3K inhibitor LY2940002 or the MEK 1/2 inhibitor PD98059 abolished the inhibition of CCR2 expression in response to JWH-015 (Fig. 6A). Conversely, pretreatment with the p38 MAPK inhibitor SB203580 had no

effect. Similar effects of the kinase inhibitors were observed on CCR1 expression; however, the differences were not significant. To study if JWH-015-induced migration was dependent on the same downstream pathways than the above described effect on chemokine receptor expression, we performed migration assays after pretreatment with kinase inhibitors. We found that both the PI3K inhibitor LY2940002 and the MEK 1/2 inhibitor PD98059 inhibited the chemotactic response to JWH-015, whereas the p38 MAPK inhibitor SB203580 had no effect (Fig. 6B). To confirm these findings, we determined the effect of JWH-015 on kinase activation by western blot analysis (6C, D). CCL2 was used as a positive control for kinase activation in parallel experiments. We found increased phosphorylation of the direct downstream kinases of PI3K and MEK 1/2, namely Akt and ERK 1/2. However, we also determined a significant activation of p38 MAPK, as shown on representative blots in Fig. 6C and densitometric quantification of 4 to 5 individual experiments (Fig. 6D).

DISCUSSION

Monocytes are circulating precursors of tissue macrophages and dendritic cells (DCs) (30). Monocyte-derived macrophages and DCs fulfill critical functions in innate and adaptive immunity during inflammation, and play a crucial role in various chronic diseases, such as rheumatoid arthritis and atherosclerosis (20, 50). Recruitment of monocytes from the blood stream into tissues is a complex process, in which adhesion molecules, chemokines, chemokine receptors and also intracellular proteins are involved (17, 54). Because cannabinoids have been reported to modulate the migration of various

cell types, including immune cells, we aimed to investigate if selective activation of CB₂ cannabinoid receptors would modulate human monocyte migration.

The major findings of our study are that CB₂ activation via JWH-015 modulates the recruitment of human monocytes by various mechanisms: (a) JWH-015 reduces the expression of CCR2 and CCR1, which results in reduced chemotaxis to CCL2 and CCL3; (b) JWH-015 blocks the IFN- γ induced upregulation of adhesion molecule ICAM-1; (c) JWH-015 induces monocyte migration by its own chemotactic properties and is able to cross-desensitize the migration in response to CCL2 and CCL3.

Our data confirming high levels of CB₂ expression on human monocytes (by using flow cytometry analysis and RT-PCR) suggest an important role for CB₂ in different monocyte functions. We first studied the effect of JWH-015 on CCL2 and CCL3 induced migration, two major chemokines expressed at inflammatory sites which trigger monocyte recruitment (10). The observed reduction of monocyte migration after 12h treatment with JWH-015 is explained by the reduced expression of CCR2 and, to a lesser extent, CCR1. We focused on the principal receptors involved in monocyte locomotion, such as CCR2, the corresponding receptor for CCL2, and CCR1, the receptor for CCL3. Although CCL3 is also capable of binding CCR5, this receptor is expressed at low levels on human monocytes and is considered less important for monocyte migration to CCL3 (58). We also tested the effect of JWH-015 on ICAM-1 adhesion molecule expression. ICAM-1 is a member of the IgG superfamily and represents a specific ligand for integrins, such as CD11a/CD18 (lymphocyte function associated Ag-1, abbreviated as LFA-1) and CD11b/CD18 (Mac-1) (41). ICAM-1 is expressed on both leukocytes and endothelial cells and is crucial in monocyte rolling and trans-endothelial migration (14). Furthermore,

ICAM-1 also interacts with its ligands on monocytes, thus generating homotypic aggregation of monocytes (14). In our experiments, JWH-015 was capable of inhibiting IFN- γ -induced up-regulation of ICAM-1. This is in agreement with previously published data, demonstrating that CB₂ agonists as well as the endocannabinoid anandamide are able to attenuate the TNF α -induced ICAM-1 and VCAM-1 expression in human liver sinusoidal or coronary artery endothelial cells (5, 6, 43). However, our data provide first evidence for the modulation of ICAM-1 expression on monocytes, suggesting a possible double action to reduce monocyte-endothelial cell adherence and monocyte trans-endothelial migration.

In our experiments, JWH-015 was capable of inhibiting chemokine induced migration doses of 5 to 20 μ M. These μ M concentrations have been well demonstrated as pharmacological concentrations for obtaining a functional activity of the in response to JWH-015-mediated CB₂ activation in various monocytic cells (15, 16, 27, 31, 34). JWH-015 is considered as a CB₂ selective agonist because the binding affinity to CB₁ receptors is about 30 fold lower as compared to CB₂ (K_i values of 13.8 nM and 383 nM for CB₂ and CB₁, respectively) (42). However, the effective doses for inhibition were much higher than the K_i value for CB₂, which may also lead to activation of CB₁ receptors which are also expressed at considerable levels on monocytes (13). To investigate the implication of CB₁ and CB₂ receptors in the observed modulation of migration and downregulation of chemokine receptor and adhesion molecule expression, we used selective receptor antagonists as well as a more selective CB₂ agonist, JWH-133 (K_i values of 3.4 nM and 680 nM for CB₂ and CB₁, respectively) (22). Only the CB₂ antagonist SR144528 reversed the effect of JWH-015, while the CB₁ antagonist

SR147778 had no effect. In accordance to this, the CB₂ agonist JWH-133 at a concentration of 10 μM exhibited similar effects as JWH-015. These findings suggest that the effects on migration, chemokine receptor and ICAM-1 expression were all dependent on CB₂ activation. Interestingly, our experiments performed with the CB₂ antagonist indicate that it may not only reverse the effect of JWH-015 on chemokine receptor and ICAM-1 expression, but further enhance their expression. However, neither the increase of chemokine receptor expression compared to untreated cells nor the increase of ICAM-1 compared to IFN-γ treated cells were not significant. A possible underlying mechanism for the observed effects has been previously described by Bouaboula and co-workers (7). In stably CB₂-transfected rodent cell line, the CB₂ antagonist SR144528 was shown to block not only the activity of CB₂, but also the MAPK activity of other G-protein-coupled receptors. Sustained treatment with SR 144528 induced an up-regulation of the cellular G protein level, which was associated with a concomitant loss of SR144528 ability to inhibit MAPK activation. It is conceivable that altered G protein levels in response to antagonist treatment might directly affect the expression of chemokine receptors which are G-protein-coupled receptors or downstream signals of ICAM-1 and/or chemokine receptors, which may, in turn, alter their expression level.

In addition to the long term effects (after 12-24h) of JWH-015 on monocyte migration, we also investigated short term actions (after a few minutes) of JWH-015 on the monocytes. We found that JWH-015 itself is chemoattractant for monocytes, an effect that has been previously reported for various cannabinoids on other cell types (11, 15, 33, 38, 47-49). The migration versus 20 μM JWH-015 was dependent on CB₂, as verified by experiments with selective receptor antagonists and the use of the CB₂ agonist JWH-133

which was also chemoattractant at a concentration of 10 μM . Moreover, checkerboard analysis confirmed that the locomotion was rather not due to induction of random cellular movement, but more likely a directed migration versus a JWH-015 gradient. In an attempt to understand the effect of systemic administration of the CB_2 agonist in an inflammatory condition, we further investigated the effect of JWH-015 on monocyte migration in the presence of a chemokine gradient. Increasing concentrations of JWH-015 (10-20 μM) added to the cells were able to inhibit the chemotactic response versus CCL2 and CCL3, indicating that the CB_2 agonist desensitizes monocytes for migration to other chemoattractants. A similar action has been previously reported for other chemoattractants. Indeed, certain chemoattractants desensitize the cells towards a further stimulation with other chemoattractants (1). Consequently, the systemic administration of sufficient amounts of JWH-015 may desensitize the cells towards chemokines such as CCL2 and CCL3, and thus, reduce recruitment of monocytes to inflammatory sites. Likewise, opioids have been shown to inhibit chemokine-induced migration through desensitization of chemokine receptors (18). The underlying mechanisms involved opiate-induced phosphorylation of the chemokine receptors, but not receptor internalization or altered chemokine binding capacity. It has been hypothesized that receptor dimers may exist between CB_2 receptors and chemokine receptors which may explain at least in part the observed pleiotropic effects on monocyte migration (35). It is conceivable that binding of CB_2 ligands could affect the ability of chemokine receptors to signal properly.

Various studies have investigated the role of CB_2 receptor activation on inflammatory cell migration in the past, with both increase and decrease of cell migration being

reported, depending on the agonist and cell type used (35). Thus, in different pathogenic conditions cannabinoids may exhibit either pro-or anti-inflammatory effects, which does not allow drawing general conclusions. Our data on both long and short term effects of the two CB₂ agonists JWH-015 and JWH-133 suggest an anti-inflammatory action in chronic inflammatory conditions where monocyte recruitment plays a prominent role. While reducing chemokine receptor and adhesion molecule expression, and consequently migration versus CCL2 and CCL3, JWH-015 may also recruit monocytes by its intrinsic chemotactic properties. Thus, enhanced plasma cannabinoid levels might counterbalance recruitment to local chemokine gradients at inflammatory sites.

Finally, to study the intracellular signaling pathways triggered by JWH-015, we used selective inhibitors of various kinases involved in cell migration, i.e. PI3K/Akt (LY294002) and MEK 1/2 (PD98059), which is a kinase activating ERK 1/2, as well as p38 MAPK (SB203580). The inhibitors of PI3K/Akt and MEK 1/2 reversed the JWH-015 mediated inhibition of chemokine receptor expression (long term effect) and migration versus JWH-015 (short term effect), while the inhibitor of p38 MAPK was ineffective. Conversely, the western blot analysis revealed that JWH-015 not only induced the activation of Akt and ERK 1/2, but also of p38 MAPK.

Similar discrepancies between kinase activation and their involvement in cellular functions have been previously described. Indeed, it has been shown that the PI3K/Akt and MEK1/2 pathways were not or only partially involved in monocyte chemotaxis in response to CCL2 and CCL3, although these two chemokines were capable of activating both PI3K/Akt and ERK 1/2 (2, 3, 12, 55). On the other hand, CCL2 was shown to activate p38 MAPK, and inhibitors of p38 MAPK induced a substantial inhibition of

CCL2 induced migration (2, 3, 9). Our data suggest two potential targets, namely PI3K/Akt and ERK 1/2, selectively involved in JWH-015 mediated short term and long term effects on monocyte migration, without involvement of classical p38 MAPK-dependent intracellular pathways (Fig. 6). However, while JWH-015 induces p38 MAPK phosphorylation, the potential downstream targets of p38 signaling in our model remain unclear.

In conclusion, we have shown that the cannabinoid JWH-015 modulates the recruitment of human monocytes by various immediate and delayed effects in a CB₂ dependent manner. Both short and long term effects depend on PI3K/Akt and ERK 1/2 signaling: the immediate effect is the induction of monocyte migration by its own potent chemotactic properties, which might inhibit the recruitment to local inflammatory sites by desensitizing cells to chemokine gradients; the delayed effects are reduced monocyte migration versus CCL2 and CCL3 via downregulation of CCR2 and CCR1, and inhibition of IFN- γ -induced ICAM-1 upregulation. Altogether, these anti-inflammatory properties might have crucial effects in chronic inflammatory disorders such as atherosclerosis and rheumatoid arthritis.

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DISCLOSURES

None.

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Figure Legends

Fig. 1. Cannabinoid receptor CB₂ expression in primary human monocytes. *A*: Representative histogram of flow cytometric analysis, demonstrating high CB₂ surface expression on monocytes with (black line) or without CD32 blocking (dashed line) versus isotype control (solid fill in grey). *B*: Representative RT PCR results for CB₂ mRNA expression of 2 different monocyte donors. No PCR product was detected in the control w/o reverse transcriptase (no RT control), confirming that the amplification product was cDNA specific. *C*: Quantification of surface CB₂ expression (flow cytometric analysis) on human monocytes cultured for the indicated time points in presence or absence of 20 μM JWH-015 (n=4). **P*<0.05 for medium alone (w/o) 24h versus time 0 or 18h; #*P*<0.05 for JWH-015 12h versus time 0; ##*P*<0.05 for JWH-015 24h versus time 0 and 18h.

Fig. 2. Analysis of cell viability and apoptosis rate in cultured human monocytes. Monocytes were cultured in serum-free medium ± 0.1% DMSO (v, vehicle), 20 μM JWH-015, 10 μM JWH-133, 1 μM SR144 528 or 1 μM SR147778 for the indicated time points. *A, B*: Cell death was determined as percentage of Trypan blue positive cells in a total of 500 counted cells (n=2). *C-D*: The apoptosis rate was determined by flow cytometric analysis of Annexin V-FITC and Propidium Iodide (PI) staining. Early apoptotic cells are Annexin V single positive cells, whereas late apoptotic/necrotic cells are Annexin V-/PI-double positive cells (n=4). *E*: Representative histograms of flow cytometric analysis of Annexin V staining after 12h (cells were gated to exclude PI positive cells).

Fig. 3. Pretreatment with JWH-015 reduces monocyte migration to CCL2 and CCL3 via inhibition of CCR1 and CCR2 expression in a CB₂ dependent manner. *A*: Monocytes were treated with JWH-015 for 12h, washed and tested for their ability to migrate to 10 nM CCL2 or CCL3 (n=6), ***P*<0.01, ****P*<0.001 versus CCL2 alone; #*P*<0.05, ##*P*<0.01 versus CCL3 alone. *B*: FACS analysis of CCR2 and CCR1 expression on monocytes after 18h of JWH-015 treatment, ***P*<0.01, ****P*<0.001 for CCR2 versus untreated (n=3); #*P*<0.05 for CCR1 versus untreated (n=4). *C*: Real time RT PCR analysis of CCR2 and CCR1 mRNA levels (normalized to HPRT control) in monocytes after 12h of JWH-015 treatment, ****P*<0.001 for CCR2 versus untreated (n=5); CCR1 not significant (n=4). *D*: FACS analysis after 18h ± 20 μM JWH-015 alone (n=11) or in presence of CB₁ antagonist SR147778 (aCB₁; 1 μM) or CB₂ antagonist SR144528 (aCB₂; 1 μM; n=9 for antagonists + JWH-015; n=2 for antagonists alone), or the CB₂ agonist JWH-133 (10 μM) alone (n=5), ****P*<0.001 versus untreated, ‡*P*<0.001 versus JWH-015, #*P*<0.05 versus untreated, ##*P*<0.01 versus JWH-015. *E*, *F*: Representative histograms of CCR2 and CCR1 expression of untreated cells (solid fill in grey) or treated with 20 μM JWH-015 (black line).

Fig. 4. *A*: JWH-015 inhibits the IFN-γ-induced ICAM-1 expression on monocytes. Monocytes were stimulated with 100 U/ml IFN-γ for 24h alone or in the presence of 20 μM JWH-015 or 10 μM JWH-133 ± CB₁ antagonist SR147778 (aCB₁; 1 μM) or CB₂ antagonist SR144528 (aCB₂; 1 μM; n=2 for aCB₁ and aCB₂ alone, n=4 for aCB₁ and aCB₂ + IFN-γ ± JWH-015, n=6 for all other conditions), **P*<0.05, ****P*<0.001. *B*: Representative histogram of ICAM-1 expression (flow cytometric analysis) of

unstimulated cells (solid fill in grey), stimulated with IFN- γ alone (black line) or in presence of 20 μ M JWH-015 (dashed line).

Figure 5. JWH-015 and JWH-133 induce chemotaxis of human monocytes in a CB₂ dependent manner. *A*: Untreated monocytes were tested for their ability to migrate to increasing concentrations of JWH-015 (n=6) or JWH-133 (n=3). ** P <0.01, * P <0.05 versus w/o JWH-015. *B*: Migration versus 20 μ M JWH-015 or 10 μ M JWH-133 in the presence of CB₁ antagonist SR147778 (aCB₁; 1 μ M) or CB₂ antagonist SR144528 (aCB₂; 1 μ M); n=8 for JWH-015, n=4 for JWH-133; *** P <0.001 versus JWH-015, ** P <0.01 versus JWH-133. *C*: Checkerboard analysis, showing that monocyte migration depended on a JWH-015 gradient across the filter. Untreated monocytes were seeded with increasing concentrations of JWH-015 to the upper well, and increasing concentrations of JWH-015 were added to the lower well. Bars represent means of n=10. *D*: JWH-015 desensitizes monocytes for migration to CCL2 and CCL3 gradients. Untreated monocytes were seeded with increasing concentrations of JWH-015 to the upper well, and 10 nM CCL2 or CCL3 were added to the lower well (n=6), * P <0.05 versus w/o JWH-015; # P <0.05 versus w/o JWH-015.

Fig. 6. JWH-015 induced Akt and ERK 1/2, but not p38 MAPK signaling pathways are involved in the modulation of chemokine receptors and migration. *A*: FACS analysis of monocyte CCR1 and CCR2 expression after 18h of treatment with JWH-015 (20 μ M) in the presence of LY294002 (LY; 50 μ M; inhibitor of PI3K, a kinase directly activating Akt), PD98059 (PD; 50 μ M; inhibitor of MEK 1/2, a kinase directly activating ERK 1/2)

and SB203580 (SB; 1 μ M; inhibitor of p38 MAPK). $**P<0.01$ versus control; $^{##}P<0.01$, $^{\#}P<0.05$ versus JWH-015; $n=5$ for CCR2 and $n=6$ for CCR1. *B*: Untreated monocytes were tested for their ability to migrate to JWH-015 (20 μ M) in the presence of LY294002 (50 μ M), PD98059 (50 μ M) and SB203580 (1 μ M) kinase inhibitors ($n=4$), $*P<0.05$, $**P<0.01$ versus JWH-015. *C-D*: Monocytes were treated with 20 μ M JWH-015, 10 nM CCL2 (positive control) or vehicle (v; 0.1% DMSO) for the indicated time points, and whole cell lysates were subjected to western blot analysis for detection of phosphorylated and total Akt, ERK 1/2 and p38 MAPK. Representative western blots and quantification of 5 (Akt, p38) or 4 (ERK1/2) different experiments are shown. $*P<0.05$ versus untreated.

Fig. 7. Schematic representation of JWH-015-induced modulation of monocyte migration (left panel) and underlying molecular mechanisms (right panels). The immediate action of JWH-015 binding to CB₂ is to chemoattract monocytes, which is dependent on PI3K/Akt and ERK 1/2 kinase phosphorylation (1). The delayed effect of JWH-015 binding to CB₂ is inhibition of CCR2 and CCR1 mRNA and, consequently, surface expression (2). This effect, which is also dependent on PI3K/Akt and ERK 1/2 signaling, leads to reduced chemotaxis versus CCL2 and CCL3, the corresponding chemokines which bind to CCR2 and CCR1. Conversely, p38 MAPK which is also phosphorylated by JWH-015, is neither required for direct chemoattraction nor for inhibition of chemokine receptor expression.

Figure 1

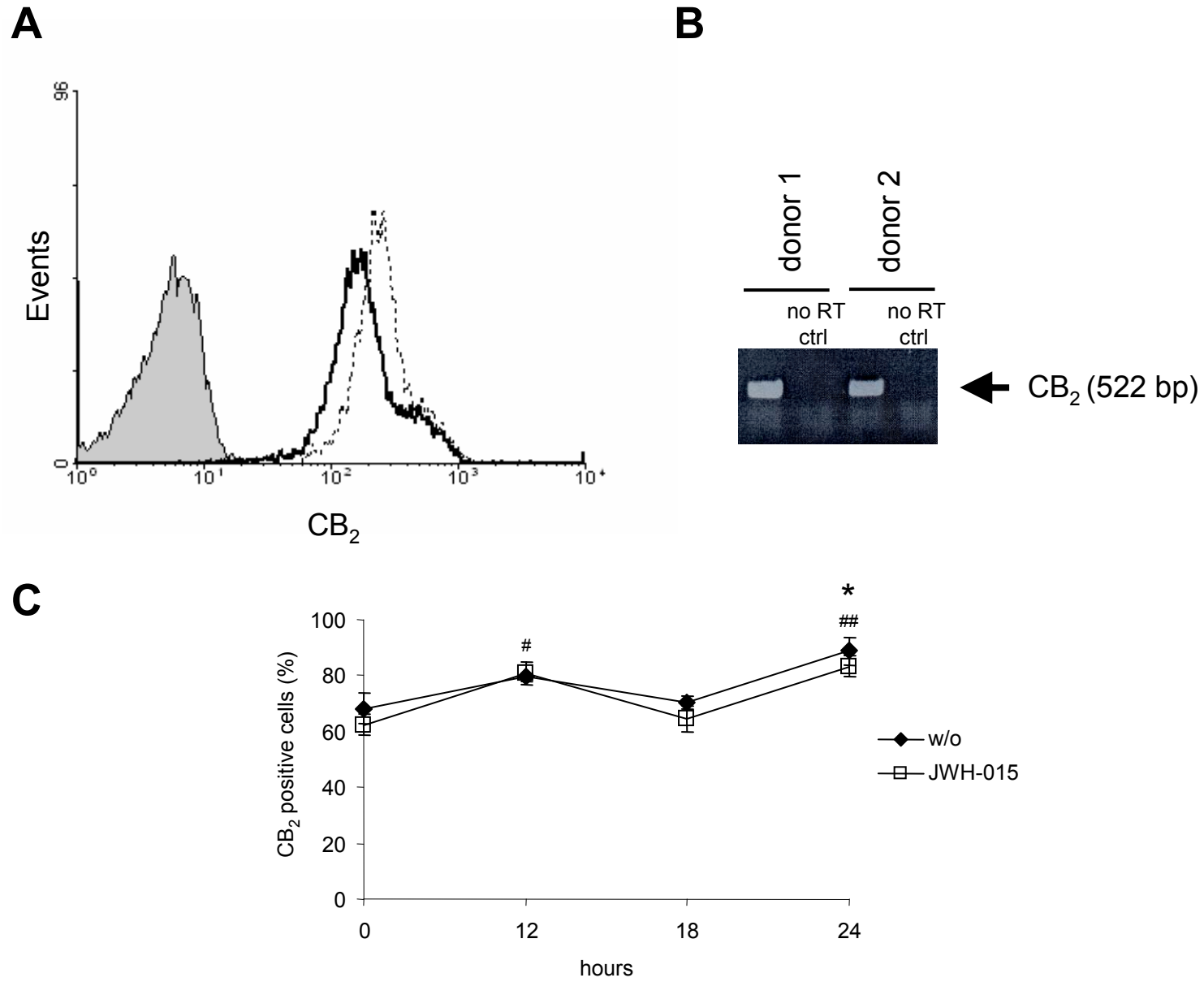


Figure 2

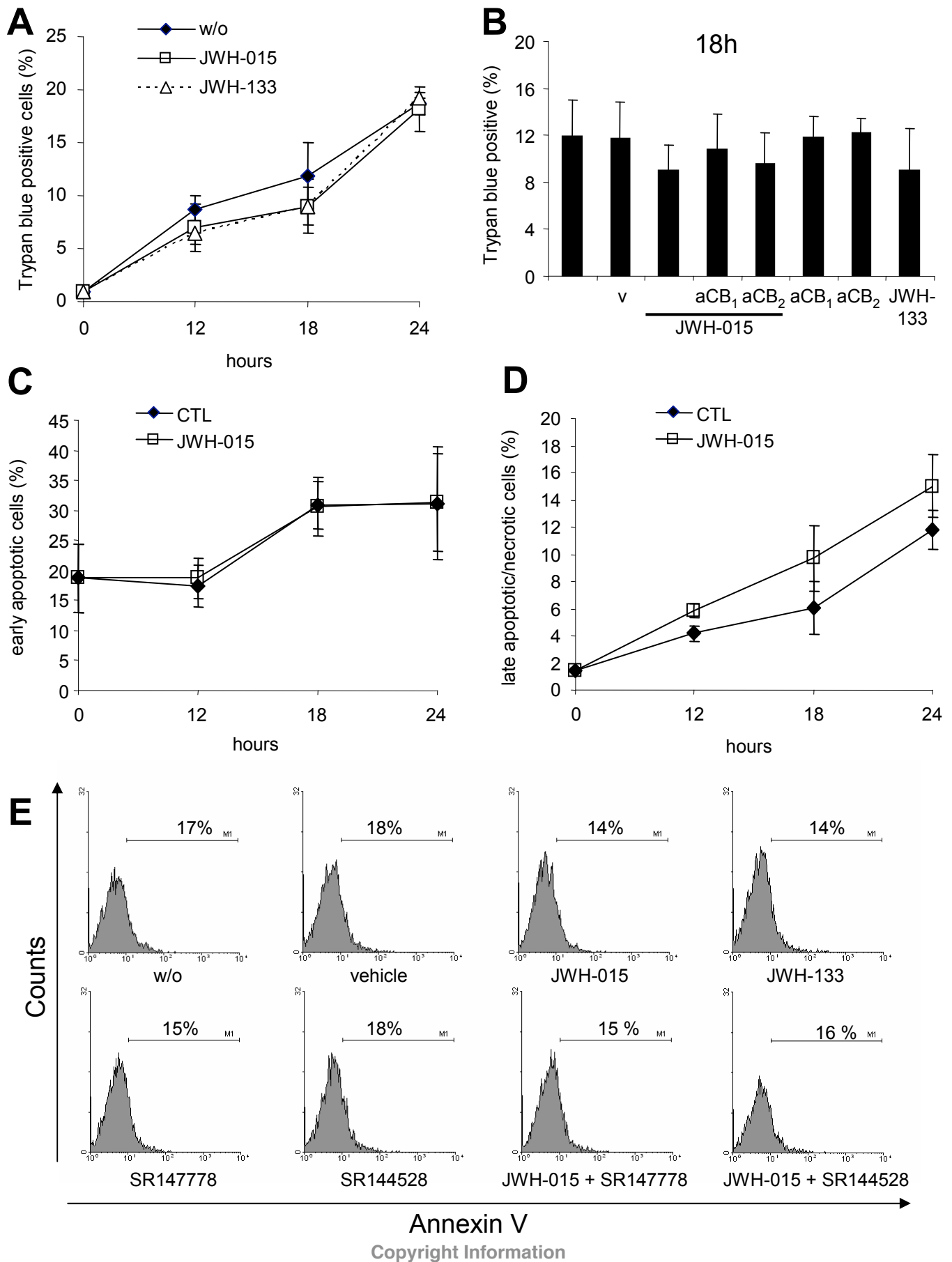


Figure 3

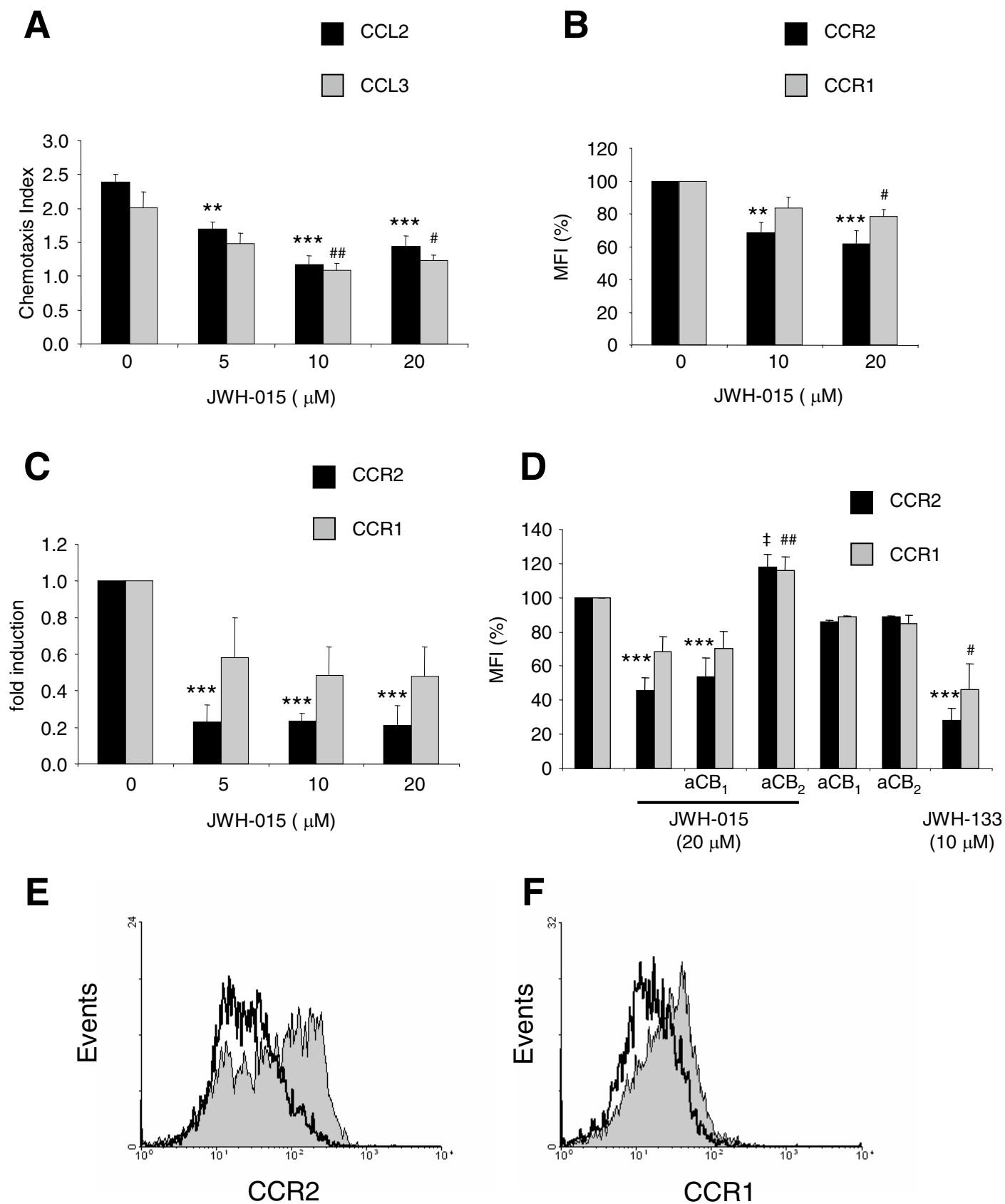
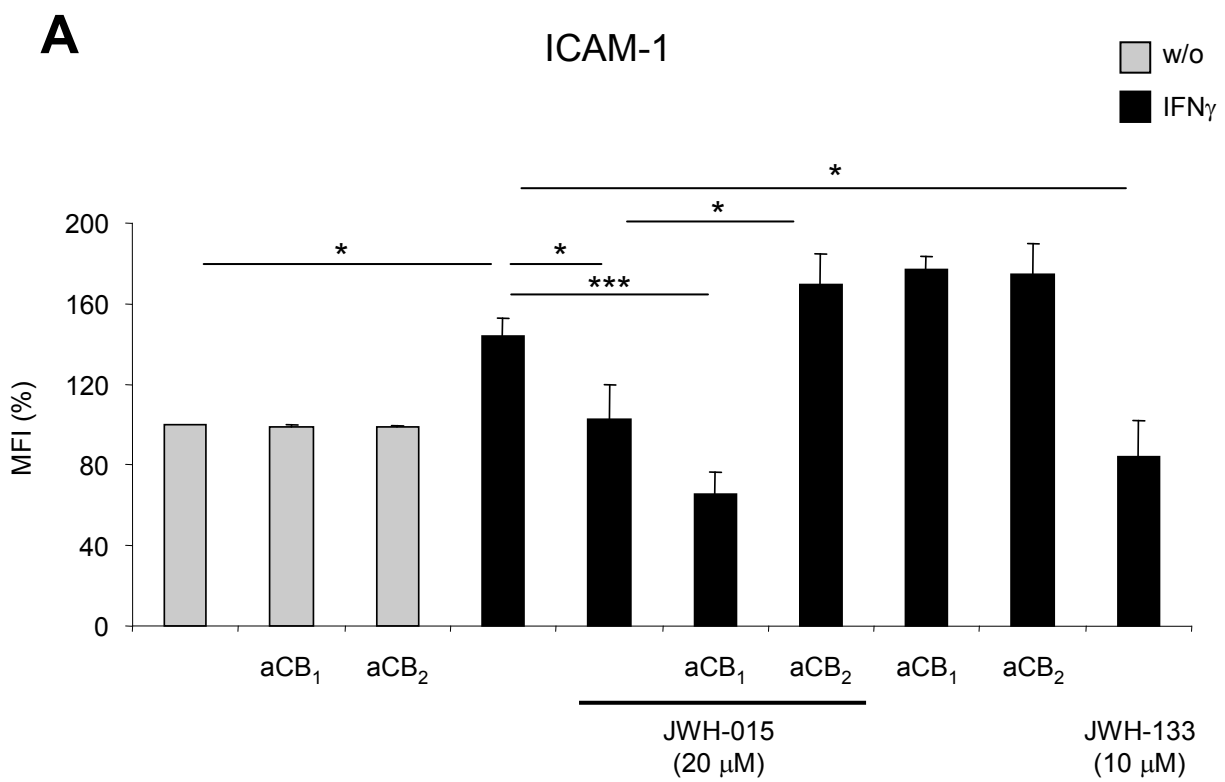


Figure 4



B

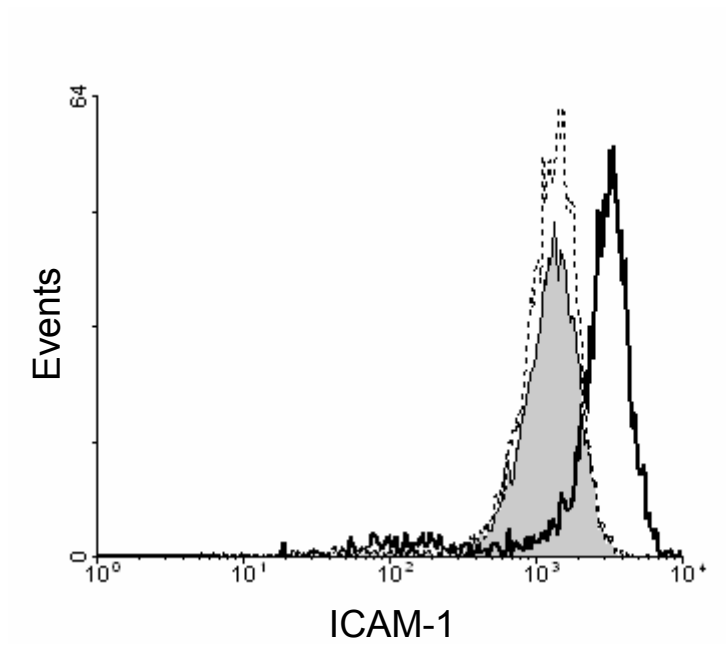


Figure 5

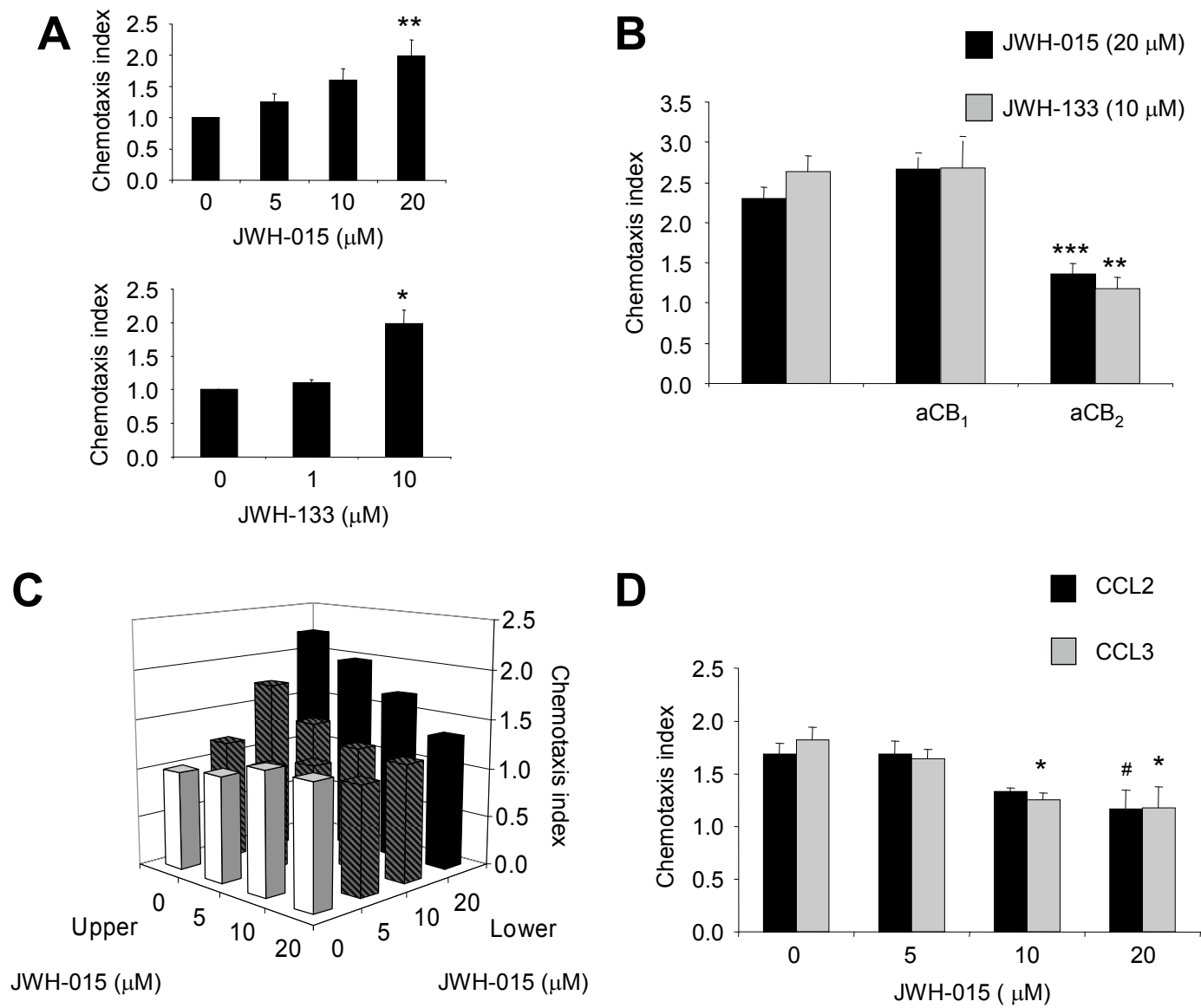


Figure 6

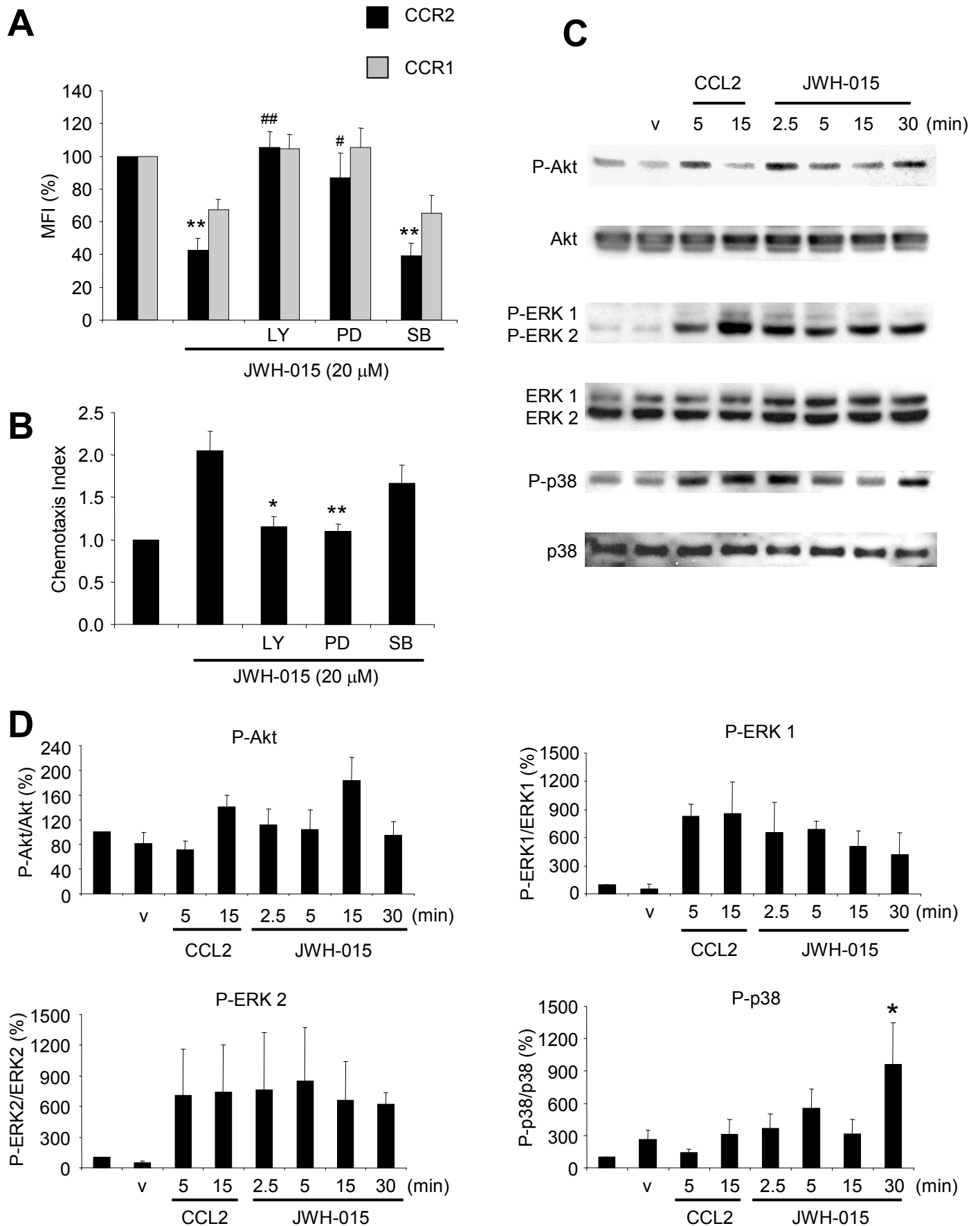


Figure 7

