Role of cannabinoid receptor 2 in mediating interleukin-1β-induced inflammation in rheumatoid arthritis synovial fibroblasts

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Abstract

Objective

Recent studies showed that the expression of cannabinoid receptor 2 (CB2), not CB1, is upregulated at both the mRNA and protein levels in rheumatoid arthritis synovial fibroblasts (RASFs), however, little is known about its endogenous role in pro-inflammatory cytokine signalling in RASFs. Our aim was to investigate the role of CB2 receptor in mediating IL-1β-induced inflammation in human RASFs.

Methods

Human RASFs were pretreated with CB2 selective agonist (JWH-133), followed by stimulation with interleukin-1β (IL-1β, 10 ng/mL). The role of CB2 in IL-1β signalling was examined using small interfering RNA (siRNA) or an overexpression plasmid specific for CB2.

Results

Pretreatment with JWH-133 did not reduce IL-1β-induced IL-6 and IL-8 production and amplified the cellular expression of cyclooxygenase-2 (COX-2) by >2-fold in human RASFs. Furthermore, the knockdown of CB2 using siRNA markedly inhibited IL-1β-induced IL-6, IL-8, ENA-78, and RANTES production by more than 50% and completely abrogated COX-2 expression in human RASFs. MMP-2 and MMP-9 activity was also reduced by 50% with CB2 knockdown. On the contrary, overexpression of CB2 in human RASFs further increased IL-1β-induced IL-6, IL-8, and RANTES by approximately 3-fold whereas ENA-78 expression increased by 1.5-fold. Immunoprecipitation analysis to study the protein-protein interactions revealed that JWH-133 coordinates CB2 association with TGFβ-activated kinase 1 (TAK1), a key signalling molecule, to increase IL-1β-induced nuclear translocation of transcription factors nuclear factor-κBp65 (NF-κBp65) and activation protein-1 (AP-1).

Conclusion

Overall, our results indicate for the first time that CB2 mediates IL-1β-induced signalling pathways in RASFs and may serve as a potential target to manage pain and inflammation in RA.

Key words

inflammation, endocannabinoids, arthritis, rheumatoid, fibroblasts, interleukin-1β

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterised by inflammation and joint degradation. Rheumatoid arthritis synovial fibroblasts (RASFs) are active contributors of RA by producing inflammatory mediators, chemotactic factors, and matrix metalloproteinases (MMPs) that degrade bone and joints. Current therapies for RA alleviate symptoms (non-steroidal anti-inflammatory drugs, NSAIDs) and slow down disease progression (disease-modifying anti-rheumatic drugs, DMARDs). However, these therapies do not fully manage pain as suggested by the studies showing that disease activity score-28 (DAS-28) scores do not reflect a reduction in pain in RA patients on current treatment options (1).

The endocannabinoid system (ECS) is made up two evolutionally conserved receptors, cannabinoid receptor 1 and 2 (CB1 and CB2), both G_i protein-coupled receptors. The ECS regulate several physiological and pathological conditions, including immunomodulation, inflammation, analgesia, and addiction (2-4). The two thoroughly studied endogenous ligands to the ECS are anandamide (AEA) and 2-arachagonlyglycerol (2-AG). Binding of these ligands to CB receptors releases $G_{\alpha i}$ which reduces adenylyl cyclase activity. This causes a reduction in cyclic AMP (cAMP) levels, which results in analgesic and anti-inflammatory effects (5). CB1 is mainly expressed in the central nervous system and primarily responsible for the psychoactive effects of cannabinoids concomitant to the neuroprotective effects (6). CB2 is mainly expressed peripherally with its highest expression being on immune cells, where it is thereby associated with the anti-inflammatory effect of cannabinoids (5, 6). Currently, only two FDA approved xenobiotics target the ECS. Dronabinol (Marinol®), a synthetic THC analogue, has been FDA approved for HIV/AIDS patients as an appetite stimulating agent (7). Sativex, a combination of tetrahydrocannabinol (THC) and cannabidiol (CBD), is approved in Europe and Canada as a treatment for pain in cancer and multiple sclerosis patients (8).

Recent studies suggest that the ECS may reduce both pain and inflammation in RA (9, 10). Both CB1 and CB2 expression have been characterised in RASFs, where CB2 was shown to be upregulated in RA synovial tissue and fibroblasts compared to osteoarthritis samples. The endogenous ligands AEA and 2-AG are found at detectable levels in the synovial fluid of RA patients, whereas they are undetectable in healthy joints suggesting the ECS is more active in RA. However, the reason for CB2 upregulation and the effects of CB2-targeted therapies remains unknown. The effects of CB2 specific agonists seems to be dependent on the cell type. JWH-133 is a specific CB2 agonist which has over 200 times more affinity for CB2 than CB1, therefore is a useful agonist to study the role of CB2 in various diseases (11). Interestingly, JWH-133 administration reduced pain-related behaviour in a rat monosodium iodate-induced osteoarthritis model (12). JWH-133 also showed anti-inflammatory and bone-protective effects in a mouse collagen induced arthritis model (13). However, JWH-133 administration in retinal pigment epithelium (RPE) cells is pro-inflammatory (14). This suggests that endocannabinoid signalling via CB2 can be either beneficial or harmful depending on the system. Therefore, in order to determine how beneficial ECS targeted therapies will be for RA, it is essential to understand how endocannabinoid signalling via CB2, which is expressed peripherally and specifically upregulated in the RA synovium, may modulate the production of inflammatory mediators by resident synovial fibroblasts in RA joints.

In this study, we characterised the role of the CB2 receptor in inflammatory signalling in RASFs, using both siRNA knockdown and plasmid-based overexpression of CB2, as well as direct activation of CB2 by its specific agonist JWH-133. By modulating its expression levels, we demonstrated that CB2 may enhance inflammatory signals associated with pain in RASFs. Using the CB2-specific agonist JWH-133, we showed that CB2 signalling contributes to the production of IL-1β-induced pro-inflammatory molecules, and propose a

possible mechanism through which it exacerbates IL-1β-induced signalling in RASFs.

Materials and methods

Chemicals and reagents

Antibodies used in this this study are as follows: MyD88, TRAF6, p-TAK-$^{Thr184/187}$, p-IRAK4$^{Thr345/Ser346}$, p-IRAK-1^{Thr209}, IRAK4, p65-NFκB, p-cJun^{Ser73} and anti-DYKDDDDK (FLAG) Tag were purchased from Cell Signaling Technologies (Danvers, MA; cat. no. 4283, 8028S, 90C7, D6D7, T209, 4363P, 8242S, D47G9, and 2368S, respectively). TAK1 and CB2 antibodies were purchased from Abcam (Cambridge, MA; cat. no. ab109526, ab3561) IRAK1 and β-Actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA; sc-47778; H273). All antibodies were diluted in 5% BSA/TBST according to the manufacturers' recommendation. JWH-133 was purchased from Tocris (Cat: 1343; ≥98% HPLC) and dissolved in DMSO at a stock concentration of 10 mM. Recombinant human IL-1β and TNF-α were purchased from R&D systems (cat. no. 201-LB and 210-TA).

Culturing of human RASFs

De-identified human synovial tissues from patients diagnosed with RA according to the American College of Rheumatology (ACR) guidelines were obtained from Cooperative Human Tissue Network (CTHN; Columbus, OH) and National Disease Research Interchange (NDRI; Philadelphia, PA). Tissues used in this study were collected from 8 females and 2 males with an average age of 50±5.7 years. Samples were acquired according to an Institutional Review Board (IRB) approved protocol in compliance with the Declaration of Helsinki. To isolate fibroblasts, synovial tissue was digested in Dispase, collagenase, and DNAse before being seeded in 72 cm^2 flasks. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 5000 U/ml penicillin, 5 mg/ml streptomycin, and 10 μg/ml gentamicin. Upon confluency (>85%) cells were passaged with brief trypsinisation as previously described (15, 16). Experiments were done using cells that were passed at least 4 to 5 times to ensure pure fibroblast population by confirming presence of RASF specific markers such as Cadherin-11, CD55, and CD106 (17). For experimental purpose, RASFs between passages 5–10 were used. All treatments were done in serum free media. Each experiment was performed on cell lines established from at least 3 or more different RA patients in this study.

Treatment of RASFs

RASFs were seeded in 6-well plates and grown to >85% confluency. Cells were pretreated with 10 or 20 μM of JWH-133 for 10 minutes prior to the addition of IL-1β (10 ng/mL) for 30 minutes for signalling studies or 24 hours to evaluate the production of IL-6 and IL-8 and examine COX-1 and COX-2 expression. Conditioned media was used for the quantification of IL-6, IL-8, and PGE_2 by ELISA and cell lysates were prepared for the analysis of IL-1β signalling proteins like p-P38, p-JNK, p-ERK, and p-TAK-1(Thr$^{184/187}$) using Western immunoblotting.

qRT-PCR

RASFs were treated with IL-1β (10 ng/mL) or TNF-α (20ng/mL) or non-stimulated (NS) for 8 hours prior to termination. Cells were collected in 1mL of TRIzol Reagent (ThermoFisher Scientific, cat. no. 15596026). RNA was extracted using the company provided protocol. 150 ng of RNA was used to make cDNA using Superscript II cDNA kit (ThermoFisher, cat. no. 11904018). SYBR Green quantitative real-time PCR was used for analysis of CB1 (PPH01504A) and CB2 (PPH02723A) expression using Qiagen RT^2 primers with GAPDH (QT00079247) as a control. Quantification of the relative expression was done using the ΔΔCt method.

Western immunoblotting

Whole cell extract was prepared using RIPA buffer (50 mM Tris pH 7.6, 150 mM CaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease and phosphatase inhibitors (Roche Basel, Switzerland). Protein was measured using DC Protein Assay ™ (Bio-Rad, Hercules, CA). An equal amount of protein (25 μg) for each sample was loaded and separated on a 10% acrylamide gel and transferred onto PVDF membrane (EMD Millipore, Billerica, MA). Blots were then blocked in TBST containing 5% nonfat dry milk for two hours prior to overnight incubation with respective primary antibody with dilution according to manufacturer. Protein bands were visualised using chemiluminescence and analysed using Image Lab software (Bio Rad) for band intensity. Blots were probed for β-actin to ensure equal loading.

Nuclear and cytosolic fractions

Nuclear and cytosolic fractions were isolated as previously described (18). Briefly, cells were treated with JWH-133 and/or IL-1β for 30 minutes prior to the addition of buffer containing HEPES (10 mM pH 7.9), EDTA (0.1 mM), EGTA (0.1 mM), DTT (1 mM), and PMSF (0.5 mM). Cells were incubated in buffer for 30 minutes, then 10% Triton-X 100 was added. Cells were collected and spun at 12,700 rpm for 5 minutes, after which the supernatant was collected as cytosolic fraction. Nuclear extraction buffer containing HEPES (10 mM), NaCl (0.4 M) and 1 mM of EDTA, EGTA, DTT, and PMSF was added to the remaining cell pellet and vortexed at 4ºC for an hour. The supernatant was collected as nuclear fraction after centrifugation at 10,000 rpm for 15 minutes.

Assay for IL-6, IL-8, ENA-78, and RANTES production

The conditioned media was collected from IL-1β stimulated samples with or without JWH-133, spun down at 10,000 rpm for 10 minutes at 4ºC to remove particulate matter, and collected in fresh Eppendorf tubes. The collected supernatants were analysed for human IL-6 (DY206), IL-8 (DY208), ENA-78 (DY254), and RANTES (DY278) levels using colorimetric sandwich ELISA kits (R&D Systems, Minneapolis, MN) as per manufacturer's instructions.

Insect cell expression system

TAK1-TAB2 fusion plasmid was purchased from University of Dundee

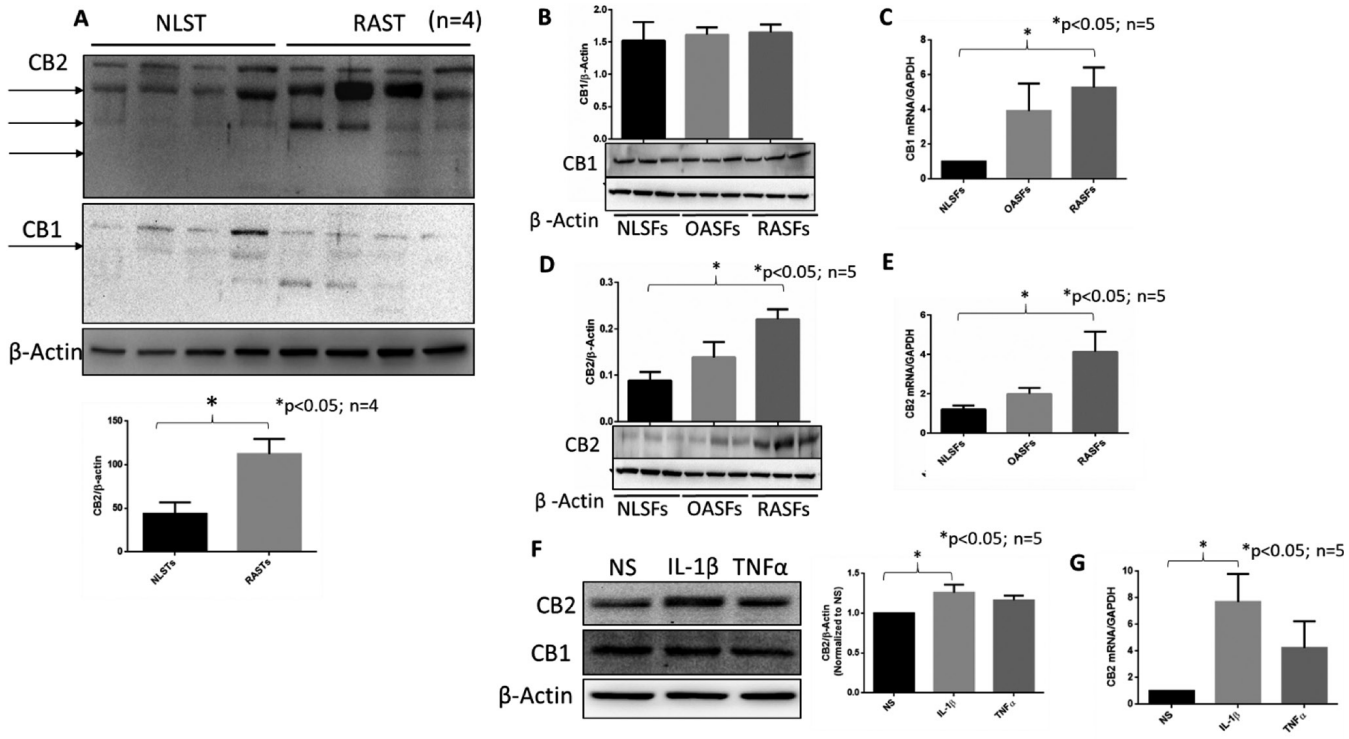

Fig. 1. Charactering CB1 and CB2 Expression in RA.
(A) Normal and RA synovial tissues (NLST and RAST) were digested and evaluated for CB1 and CB2 expression. RASTs had higher CB2 expression that normal tissue. * $p<0.05$, Student's *t*-test, n=4 of RA and normal tissue. Normal and RA synovial fibroblasts were isolated from tissue and grown to confluency before being serum starved overnight. **(B** and **D)** Cell lysates were collected and probed for CB1 (**B**) and CB2 (**D**) expression. Representative blot of 3 RASFs, OASFs, and NLSFs is shown, however, analysis was performed on SF cell lines obtained from 5 donors for each condition. **(C** and **E)** RNA was isolated from RASFs, OASFs, and NLSFs and subjected to mRNA analysis of CB1 **(C)** and CB2 **(E)** using SYBR green qRT-PCR. **(F)** RASFs were then stimulated with IL-1β (10 ng/ml) and TNF-α (20 ng/ml) for 24 hours then lysates were assayed for CB1 and CB2 expression or **(G)** for 8 hours for mRNA analysis. *$p<0.05$ n=5, one-way ANOVA. NS = non-stimulated.

(United Kingdom). V5 tag was inserted into the multiple cloning site of the plasmid using *BamH1*. Plasmid was transformed into DH10Bac™ competent cells (ThermoFisher Scientific) and selected for using kanamycin and ampicillin resistance. Bacmid was purified and sequenced using PCR before being transfected into Sf9 cells with Cellfectin (ThermoFisher Scientific). Ten days after transfection, recombinant baculovirus particles were collected and used to transfect Sf9 cells. Sixty-six hours after the third transfection, cells were lysed to collect whole cell extract and TAK1-TAB2_V5 fusion protein was purified for use in further experiments.

Immunoprecipitation (IP)
IP method was adapted from the method previously described (19). Briefly, RASFs were grown to 80% confluency in 150 mm plates and treated with JWH-133 (20 μM) for 10 minutes prior to the addition of IL-1β for 30 minutes. Whole cell lysates were collected with RIPA buffer as previously described. One hundred and twenty μl of V5 affinity gel volume equivalent (Sigma) was incubated with TAK1-TAB2 fusion protein overnight at 4ºC on a rotator, then added evenly to the samples with equivalent protein (1 mg). Mixture was incubated for 4 hours to capture associated proteins. The protein complex was eluted out with 2X sample buffer for 15 minutes at 70ºC.

Small-interfering RNA (siRNA)
siRNA sequences for CB2 were purchased from Sigma MISSION predesigned siRNA [Catalogue SASI_Hs01_00041077, SASI_Hs01_00041084, Sigma] and RASFs were transfected as previously described (20). RASFs were transfected with 120 pmoles of negative (SIC001) or CB2 siRNA with Lipofectamine 2000 (Thermo Fisher Scientific) in Opti-MEM media for 8 hours. Media was replaced with RPMI media supplemented with the next day. Forty-eight hours after transfection, RASFs were serum starved overnight prior to IL-1β simulation for 24 hours.

Overexpression
CB2 (Cat: U446D5DA300) and control plasmids were purchased from GenScript (Piscataway, NJ). Plasmids were inserted into TOP10 cells and amplified in lysogeny broth (LB) supplemented with ampicillin. Plasmid was purified with MidiPrep Kit (Qiagen) and cells were transfected with 100 ng of plasmid with Lipofectamine for 24 hours prior to serum starvation and IL-1β stimulation.

Gelatin zymography
MMP-2 and MMP-9 activity was determined as previously described (18, 21). Briefly, conditioned media from siRNA and overexpression experiments was resolved under non-reducing conditions on SDS-polyacrylamide gels polymerised with 1 mg/ml gelatin (type B from bovine skin). Gels were washed in 2.5%

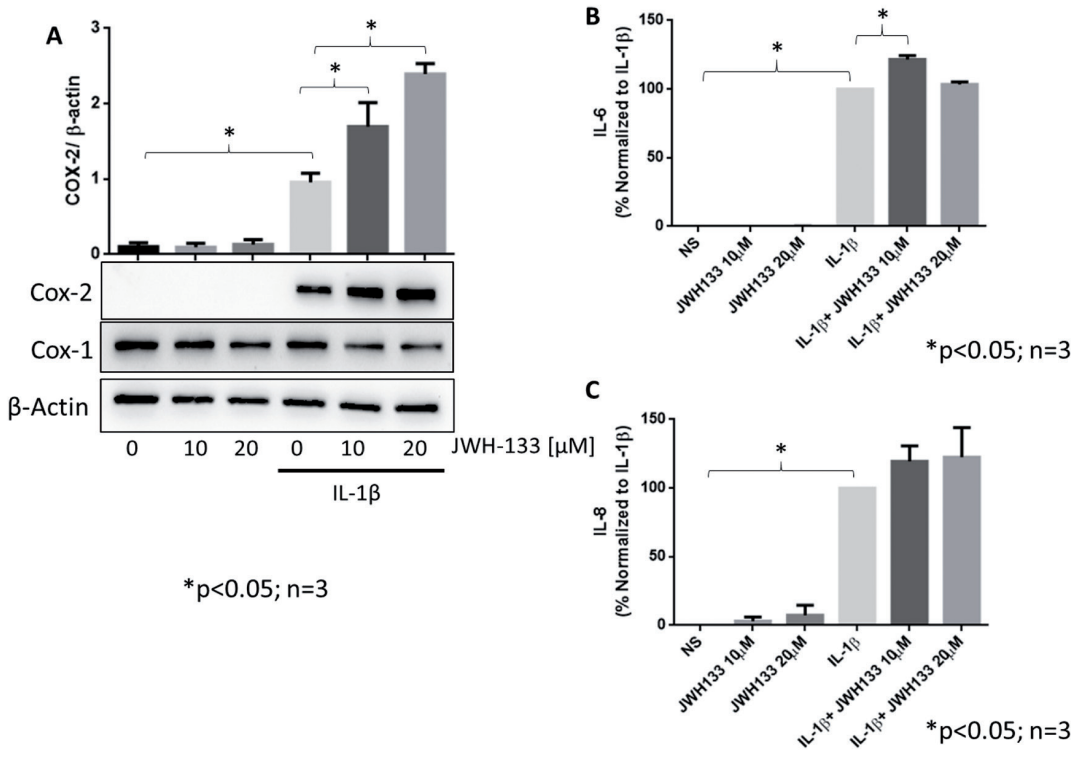

Fig. 2. JWH-133 treatment up regulates or maintains IL-1β induced IL-6, IL-8, and COX-2 production.
(A) RASFs were grown to confluency and serum starved overnight. Next morning JWH-133 was added to RASFs at 10 and 20μM concentrations 10 minutes prior to IL-1β (10 ng/ml) stimulation for 24 hours. Whole cell lysate was assayed for COX-1 and COX-2 (**A**) and the conditioned media was used to determine IL-6 (**B**) and IL-8 (**C**) production. Densitometric analysis was performed on COX-2 which was normalised to β-actin loading control.
*p<0.05, one-way ANOVA n=3 where 3 different cell lines derived from 3 different RA patients was used. NS= non-stimulated.

Triton X-100 for 30 minutes with gentle shaking, then washed again in 1X developing buffer (50 mM Tris HCl [pH 8.0], 5 mM $CaCl_2$, and 0.02% NaN_3) for 30 minutes. Afterwards, gels were incubated overnight in fresh developing buffer at 37°C, stained in Coomassie brilliant blue R250 for two hours then destained using a solution of 7% glacial acetic acid and 5% methanol.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software. Student's *t*-test was used between RA and NL tissue (Fig. 1). One-way ANOVA was used to evaluate differences of means between NS, IL-1β, TNF-α (Fig. 1) and NS, IL-1β + JWH-133, JWH-133 (Figs. 2, 3 and 5). Two-way ANOVA was used between siRNA and overexpression groups (Fig. 4). All ANOVA tests were followed with Tukey's multiple-comparison test. Due to large variability in protein expression, data was either normalised to positive control (IL-1β) or negative control (NS). All tests assumed normal distribution where $\alpha = 0.05$ was considered significant. All data are presented as the mean ± SEM where error bars in the figures represent the SEM. Mean was taken from data obtained from RASFs derived from 3 or more RA patients unless otherwise noted.

Results

CB2 is upregulated in RASFs

Previous studies suggest higher expression of CB2 in RA compared to OA at the tissue and synovial fibroblast levels (9, 22), however, the changes compared to normal/non-diseased (NL) samples were not previously published. Western blot analysis showed RA synovial tissues (RASTs) had 1.5-fold higher expression of CB2 compared to NLSTs (Fig. 1A; p<0.05; n=3). As reported in previous studies, CB2 was seen as 60, 55, and 40 kDa bands in RASTs due to various glycosylation states and CB1 has been observed at 65 kDa (22). Furthermore, the evaluation of CB1 and CB2 expression in NLSFs, OASFs, and RASFs showed no change in CB1 expression at the protein level (Fig. 1B), but there was an increase in CB1 expression at the mRNA level (Fig. 1C). Nevertheless, there was a consistent increase of CB2 expression in RASFs compared to NLSFs or OASFs at the mRNA and protein levels (Figs. 1D and E, p<0.05, n=5). RASFs stimulated with IL-1β for 8 hours showed an upregulation of CB2 mRNA (Fig. 1G, p<0.05, n=5). RASFs stimulated for 24 hours with IL-1β resulted in a modest upregulation in CB2 expression by ~20% in RASFs compared to the non-stimulated (NS) control. (Fig. 1F; p<0.05, n=5). Because TNFα did not have a pronounced effect, we conducted further studies using IL-1β only.

JWH-133 upregulates IL-1β-induced COX-2, IL-6 and IL-8 expression in RASFs

Next, we studied the effect of CB2 agonist JWH-133 on IL-1β-induced inflammatory mediators in human RASFs. Our Western immunoblotting results and densitometric analysis showed that IL-1β-induced COX-2 expression was further increased by ~70% and ~150% with the pretreatment of JWH-133 at 10 and 20 μM, respectively (Fig. 2A; p<0.05). COX-1 expression remained unchanged, suggesting that JWH-133 selectively enhances IL-1β-induced COX-2 expression in human RASFs (Fig. 2A). Furthermore, we analysed IL-6 and IL-8 production in the conditioned media collected after 24 hours of IL-1β stimulation alone or in the presence of JWH-133 at 10 and 20 μM. Results of the ELISA assay showed that JWH-133 significantly enhanced

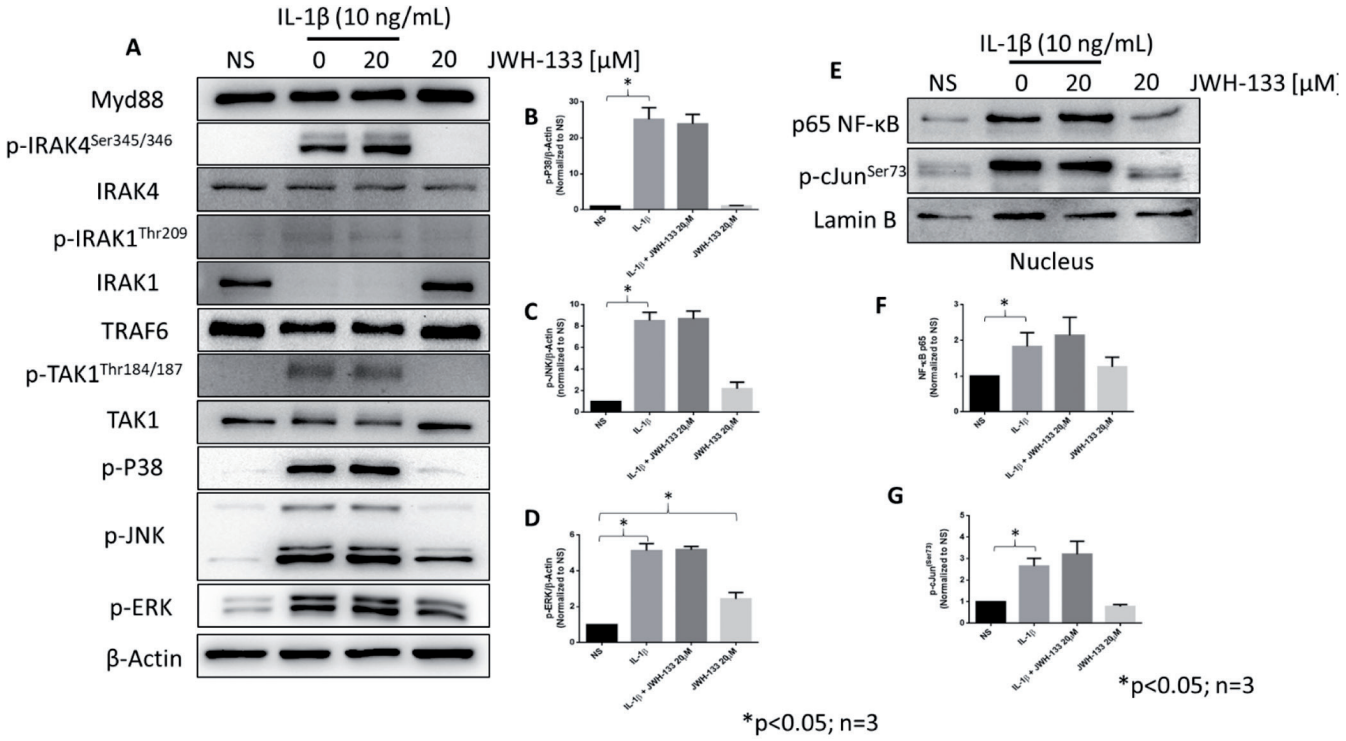

Fig. 3. JWH-133 treatment enhances IL-1β signalling pathway in human RASFs.
(A) RASFs were grown to confluency and serum starved overnight. Next morning JWH-133 was added to RASFs at 10 and 20 μM concentrations 10 minutes prior to IL-1β (10 ng/ml) stimulation for 30 minutes. Signalling proteins that are part of the IL-1 signalling cascade were assayed using Western immunoblotting. **(B)** Nuclear and Cytosolic fractions from similarly treated samples were analysed for nuclear translocation of p-cJun and NF-κBp65. Densitometric analysis was performed on COX-2 which was normalised to NS.
*p<0.05 one-way ANOVA, n=3 where 3 different cell lines derived from 3 different RA patients was used. NS= non-stimulated.

IL-1β-induced IL-6 production by ~21% and IL-8 production by ~12% in RASFs (Figs. 2B and C). Surprisingly, the highest tested dose of JWH-133 (20 M) either maintained or deceased when treated IL-6 and IL-8 levels with the highest dose whereas the lower dose (10 μM) seemed to upregulate them.

JWH-133 does not affect IL-1β signalling pathways in human RASFs

Since we observed upregulation in inflammatory mediators, we sought to examine the level of expression or activation of signalling proteins that play a pivotal role in the IL-1β signalling pathway in human RASFs (19). RASFs were stimulated with IL-1β alone or with JWH-133 (20 μM) for 30 minutes to study the expression pattern of IL-1β signalling proteins. Our results showed that the expression levels of many of the IL-1 signalling phosphorylated proteins did not change with JWH-133 in combination with IL-1β without changing the levels of total protein (Fig. 3A). However, JWH-133 by markedly affected the basal phosphorylation of MAPKs, including p-JNK/p46 and p-ERK, and exhibited slightly activation of P38, suggesting JWH-133 activation of CB2 may have the capability to co-operate with IL-1β signalling pathways (Figs 3B-D). This correlates with a modest increase in basal IL-8 production observed in Fig. 2C.

In addition to whole cell lysates, nuclear and cytosolic fractions were analysed to determine the nuclear translocation of transcription factors NF-κBp65 and p-c-Jun (AP-1). The presence of JWH-133 further enhanced IL-1β-induced nuclear levels of NF-Bp65 by ~17% and p-cJun levels by ~21%, respectively (Figs. 3E-G), which ultimately results in the observed upregulation of COX-2 and increase in IL-6 and IL-8 production.

The CB2 receptor enhances inflammatory signalling in human RASFs

To decipher the impact of CB2 receptor in IL-1β-induced signalling in RASFs, we knocked down CB2 expression using siRNA prior to stimulation with IL-1β for 24 hours. Data was normalised to IL-1β of control (CTR siRNA) for clarity of the effect of CB2 knockdown. Interestingly, evaluation of the conditioned media showed that IL-6 and IL-8 production was significantly reduced to ~50% in CB2 siRNA treated samples as compared to the control (CTR) siRNA group (Figs. 4A and 4B). Similarly, IL-1β-induced RANTES and ENA-78 production were also significantly reduced in CB2 siRNA treated RASFs when compared to CTR siRNA treated RASFs (Figs. 4C and D). Evaluation of the conditioned media using gelatin zymography showed that CB2 knockdown significantly inhibited IL-1β-induced MMP-2 and MMP-9 activity in RASFs by ~40% and ~50%, respectively (Figs. 4J and K). In addition, IL-1β-induced COX-2 expression was significantly reduced by ~85% by CB2 knockdown compared to the CTR siRNA (Fig. 4L), with no effect on COX-1 expression.

To complement our siRNA experiments, we overexpressed CB2 by trans-

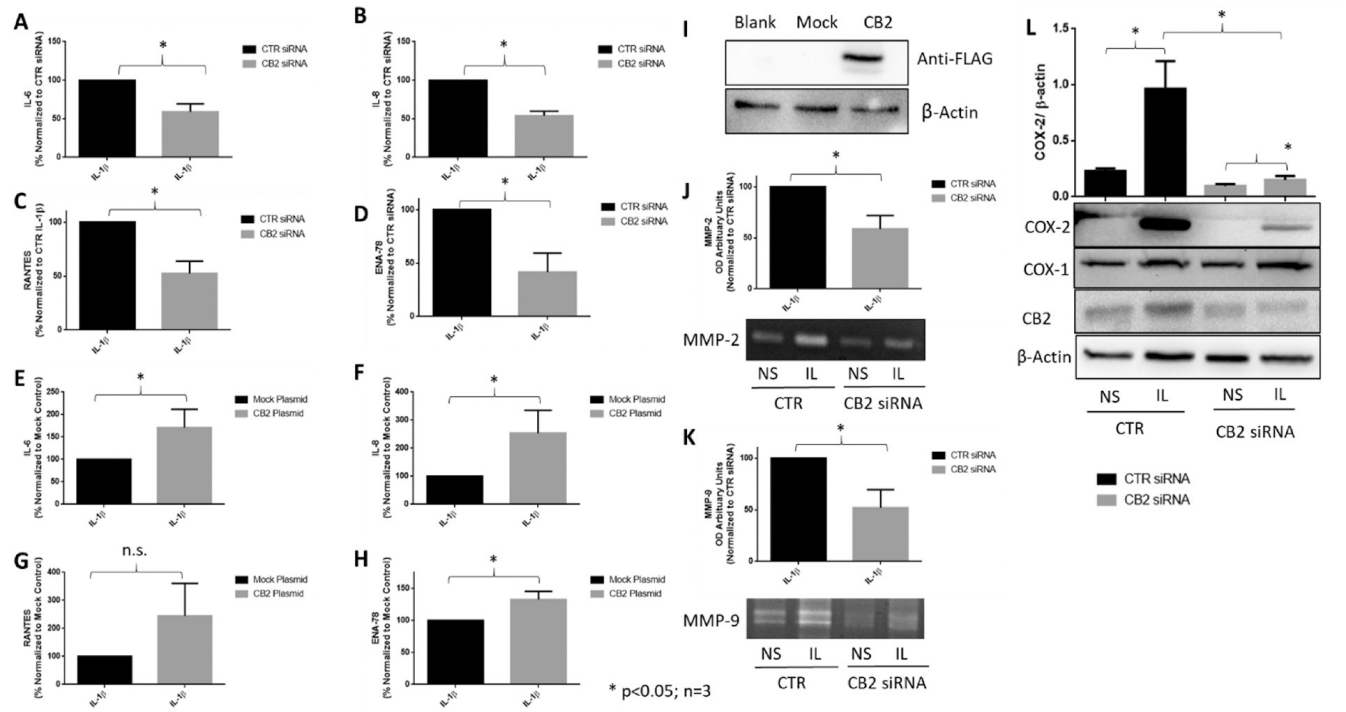

Fig. 4. Effect of CB2 knockdown and overexpression on IL-1β-induced inflammation in human RASFs.
RASFs were grown to ~80% confluency before being transfected with 120pM CB2-targeted siRNA using Lipofectamine 2000 for 8 hours. Media was replaced 24 hours after transfection then serum starved overnight 48 hours later. Next morning cells were stimulated with IL-1β (10 ng/ml) for 24 hours. Conditioned media was assayed for IL-6 (**A**), IL-8 (**B**), ENA-78 (**D**), and RANTES (**C**) production and whole cell lysates were assayed for COX-1, COX-2, and CB2 expression (**L**). Densitometric analysis was performed on COX-2 which was normalised to β-actin loading control. Finally, the effect of CB2 knockdown on MMP-2 (**J**) and MMP-9 (**K**) activity was evaluated using gelatin zymography. In a similar experiment, RASFs were transfected with CB2_FLAG overexpression plasmid with Lipofectamine 2000 for 24 hours. Cells were then serum starved and stimulated with IL-1β for 24 hours. Conditioned media was assayed for IL-6 (**E**) and IL-8 (**F**) ENA-78 (**H**), and RANTES (**G**) production. Data was normalised to IL-1β of control (CTR or Mock plasmid) for clarity of the effect of CB2 absence or presence. (**I**) FLAG tag western was used to confirm the expression of the plasmid.
**p*<0.05 two-way ANOVA n=3 where 3 different cell lines derived from 3 different RA patients was used.

fecting RASFs with a CB2_FLAG plasmid followed by IL-1β stimulation for 24 hours. Again, data was normalised to IL-1β of control (Mock plasmid) for clarity of the effect of CB2 overexpression. Transfection of CB2 plasmid was validated using Western blotting (Fig. 4I). Once transfection was confirmed, we evaluated the conditioned media which showed that CB2 overexpression resulted in a ~2-fold increase in IL-6, a ~3-fold increase in IL-8, and ~1.5 fold increase in ENA-78 compared to IL-1β stimulated group transfected with mock plasmid (Fig. 4E, F and H). We also observed a similar trend with RANTES, however this increase was not statistically significant (Fig 4G).

The CB2 receptor interacts with IL-1β signalling proteins to enhance inflammation

We performed protein association studies in order to decipher the potential mechanism of CB2 association with TAK1 to increase the IL-1β inflammatory milieu (Fig. 5). TAK1 was selected for further analysis because TAK1 is a central upstream MAPK for inflammatory signalling in RASFs (23). For association experiments, recombinant TAK1-TAB2_V5 fusion protein was purified from Sf9 cells. Upon terminating transposition experiments, Western immunoblotting was used to confirm the correct molecular weight and tag (Fig 5A). An Equal amount of fusion protein was incubated with whole cell lysate stimulated with IL-1β or JWH-133 for 30 minutes. V5 affinity gel was used to pull down the TAK-TAB fusion protein and any associated proteins. Pulldown lysate was assayed for CB1 and CB2 expression using Western immunoblotting. Interestingly, we observed that CB2 associates with active TAK-TAB fusion protein upon treatment with IL-1β (Fig. 5B). Importantly, JWH-133 treatment also triggered association of CB2 with TAK-TAB fusion protein, suggesting that it has capability to engage TAK1 mediated signalling in RASFs, similar to IL-1β. In contrast, we found only a modest association of CB1 with TAK-TAB fusion protein with IL-1β stimulation, whereas no association was observed in the presence of JWH-133.

Discussion

Medical use of marijuana is on the rise, yet our current understanding on the endocannabinoid system and its effects in the management of pain and inflammation is limited. The current study provides novel insight of CB2 in mediating IL-1β-induced signalling pathways in human RASFs to propagate pain and inflammation. Using CB2 siRNA and overexpression approaches, we provided preliminary evidence that CB2 can interact with mediators of

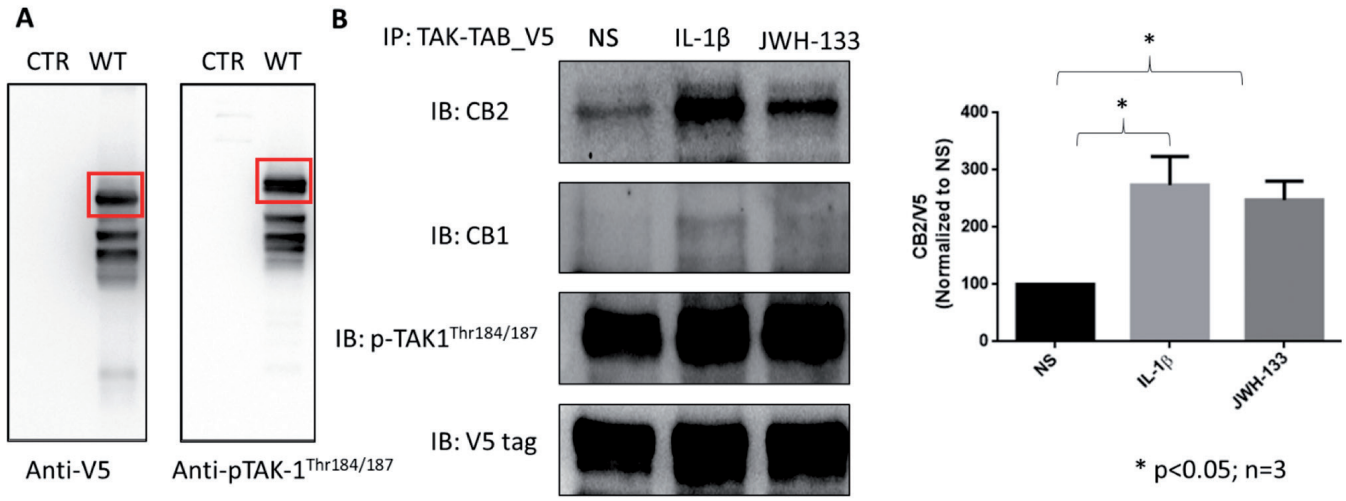

Fig. 5. CB2 may directly interact with TAK1 to propagate IL-1β signalling in human RASFs. **(A)** Recombinant TAK1-TAB2_V5 fusion protein was purified from Sf9 cells to synthetise an active TAK1 kinase to confirm the correct molecular weight and tag. **(B)** Equal amount of fusion protein (1 mg) was incubated with 1 mg of whole cell lysate obtained from IL-1β or JWH-133 stimulated samples. V5 affinity gel was used to pull down the TAK-TAB fusion protein and any associated proteins. Pulldown lysate was assayed for CB1 and CB2 using Western and further densitometric analysis was performed. Densitometric analysis was performed on CB2 which was first normalised to V5 loading control before being normalised to NS.
*p<0.05 one-way ANOVA, n=3 where different cell lines derived from 3 RA patients was used. NS = non-stimulated CTR = control vector WT = wild type TAK1-TAB1 plasmid.

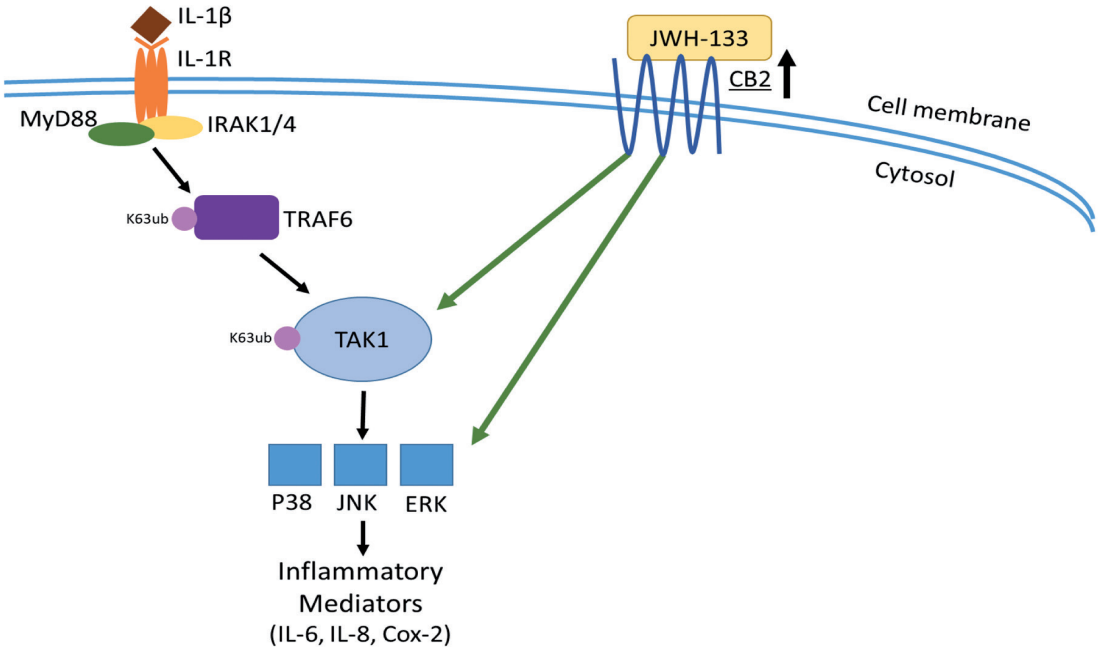

Fig. 6. Schematic diagram of key findings. Upon activation with JWH-133, IL-1β induced signalling is enhanced as evident by increased IL-6 and IL-8 production, and COX-2 expression. This may result from CB2 interactions with the active form of TAK-1. The reduction of IL-1β-induced pain and inflammation mediators with CB2 knockdown and their up-regulation in response to CB2 overexpression suggest CB2 actively participates in IL-1β signalling to cause RASF-mediated inflammation.

IL-1β signalling, especially TAK1, and affect NF-κBp65 and JNK pathways. Furthermore, our results show that the CB2-specific agonist JWH-133 has a pro-inflammatory effect in RASFs as summarised in Figure 6. These findings provide a rationale for targeting CB2 as an adjunct therapeutic approach in the management of pain and inflammation in RA.

Previous studies in RA have used other CB2 agonists to characterise the effects of the endocannabinoid system activation. However, not all CB2 agonists show the same affinity. A study done by Gui *et al*. used the CB2 agonist HU-308 which has a K_i value of 20 nM to CB2 (9). A more recent study used WIN 55,212-2 which is more specific to CB2 (K_i=3.3 nM) however, this compound has been classified as an aminoalkyl indole which structurally differs from classical cannabinoids (THC and CBD) (24). A recent study by Soethoudt *et al*. validated several CB2 agonists and concluded that HU-910, HU-308, and JWH-133 should be used to study CB2 specific effects (11). Therefore, we selected JWH-133 for further studies because it is highly specific to CB2 and is structurally a part of classical cannabinoid family (K_i=3.4 nM) (25).

Since our primary interest is pain and inflammation in RA, we focused on studying IL-1β signalling pathways. In RASFs, IL-1β signalling results in

production of IL-6, IL-8 and increased expression of COX-2 (15, 26). Interestingly, JWH-133 treatment caused an upregulation COX-2 without affecting IL-6 or IL-8 production. However, previous reports with other CB2 agonists shows significantly reduced IL-6 and IL-8 production (9, 24). Another study done by Fukuda *et al*. also used JWH-133 on RASFs and reported anti-inflammatory effects (13). However, these effects were seen with TNF-α stimulation, which suggests that CB2 activation may produce a unique response with different pro-inflammatory cytokines. In our studies, we hypothesised that direct activation of CB2 may coordinate IL-1β-mediated pain and inflammation.

To confirm our hypothesis, we performed siRNA knockdown and overexpression studies in RASFs. Looking specifically at IL-1β signalling, we observed that CB2 siRNA reduced the expression of inflammatory mediators and chemokines whereas its overexpression enhanced them. We further extended our studies to chemokines (ENA-78 and RANTES) and MMP -2 and MMP-9 which are important in the RA pathogenesis and strongly induced by IL-1β and observed a similar result (18, 27). Altogether, this suggests the CB2 receptor itself may play a role in inflammation, which has not been described until now in RA. A previous study done with $CB1^{-/-}$ and $CB2^{-/-}$ mouse embryonic fibroblasts also showed a reduction in p-JNK and p-ERK expression (28). In another study, $CB2^{-/-}$ mice were resistant to LPS driven preterm birth with lower levels of IL-6 (29). Altogether, these findings support our conclusion that CB2 is an important mediator in pain and inflammation in RA.

While our results are open to interpretation, we postulate that one-way CB2 may work to enhance inflammation is through association. When CB2 is activated, the receptor gets internalised (30, 31). Indeed, Atwood *et al*. showed that JWH-133 does cause internalisation of CB2 and activates MAPK signalling (30) with an unknown mechanism. Here we show for the first time that CB2 associates with the active form of TAK1, which is an essential MAPKKK for proinflammatory signalling (19, 23). Because TAK1 is upstream of the MAPKs, it is apparent that CB2 activation may modulate the MAPK signalling and inflammation by maintaining or stabilising TAK1 activation. This is further confirmed by our analysis of IL-1β proteins where we observed JWH-133 alone could markedly activate basal expression of MAPKs. Since JWH-133 alone was also able to activate CB2 binding to TAK-TAB fusion protein, we suggest that JWH-133 may potentially coordinate CB2 signalling by exploiting mediators of IL-1β inflammatory signalling. However, this work further warrants in-depth understanding of its nature of interaction.

Despite our findings, there are reports that suggest CB2 activation is anti-inflammatory and is therapeutic target for RA (10, 32, 33). Based on our results and previously shown by Lowin *et al*., there is a possibility that CB2 agonists may exert their anti-inflammatory effects through other receptor(s) besides CB2 (24). In our study, the highest tested dose of JWH-133 (20 μM) seems to lower or maintain IL-6 and IL-8 levels whereas the lower dose (10 μM) seemed to upregulate them. Since IL-1β is a potent inducer is of IL-6 and IL-8, JWH-133 treatment does not result in further significant upregulation of IL-6 and IL-8. Soethoudt *et al*. reported that JWH-133 does activate Trp channel receptor TRPA1 and perhaps at higher doses this effect is further amplified (11). Besides off-target effects, JWH-133 may perform differently *in vivo*. The data presented here suggests that JWH-133 administration would not reduce pain and inflammation in animal model of RA that relies heavily on IL-1β. One *in vivo* study with JWH-133 showed an increased hyperemia in healthy rat joints thus promoting inflammation (34). Yet in the monosodium iodate-induced OA rat model, JWH-133 administration reduced pain related behavior in rats (12). Therefore, further research is needed using preclinical models of RA to validate the CB2 specific therapy for RA.

In conclusion, we describe a novel role of CB2 receptor in mediating IL-1β-induced inflammation in human RASFs. To our knowledge, this study is the first to show that CB2 receptor and its signals are proinflammatory in RASFs, contrary to the current notion that endocannabinoids are only anti-inflammatory. It is worth noting that an increasing number of RA patients are looking into the uses of medical marijuana as an alternative therapy (35). Our study suggests that therapies specific to CB2 may not be beneficial in RA, assuming that the drug does get to the target (the joint) before being metabolised. However, further *in vivo* research is needed to confirm these findings before ruling out CB2-targeted therapies for RA altogether. Perhaps therapies with some level of non-specificity such as selective agonists like the endocannabinoids or molecules that differentially engage CB1 or CB2 receptors, may offer a therapeutic advantage in this context by triggering different signalling pathways that may be beneficial.

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References

1. LEE YC, CUI J, LU B *et al*.: Pain persists in DAS28 rheumatoid arthritis remission but not in acr/eular remission: A longitudinal observational study. *Arthritis Res Ther* 2011; 13: R83.
2. MARTIN GIMENEZ VM, NORIEGA SE, KASSUHA DE, FUENTES LB, MANUCHA W: Anandamide5 and endocannabinoid system: An attractive therapeutic approach for cardiovascular disease. *Ther Adv Cardiovasc Dis* 2018; 12: 177-90.
3. FRAGUAS-SANCHEZ AI, MARTIN-SABROSO C, TORRES-SUAREZ AI: Insight into the effects of the endocannabinoid system in cancer: a review. *Br J Pharmacol* 2018; 175: 2566-80.
4. WAKLEY AA, CRAFT RM: Antinociception and sedation following intracerebroventricular administration of delta(9)-tetrahydrocan-

nabinol in female vs. Male rats. *Behav Brain Res* 2011, 216: 200-6.
5. MALFITANO AM, BASU S, MARESZ K, BIFULCO M, DITTEL BN: What we know and don't know about the cannabinoid receptor 2 (cb2). *Semin Immunol* 2014; 26: 369-79.
6. BASU S, DITTEL BN: Unraveling the complexities of cannabinoid receptor 2 (cb2) immune regulation in health and disease. *Immunol Res* 2011; 51: 26-38.
7. DEJESUS. E, RODWICK BM, BOWERS D, COHEN CJ, PEARCE D: Use of dronabinol improves appetite and reverses weight loss in hiv/aids-infected patients. *J Int Assoc Physicians AIDS Care* (Chic) 2007; 6: 95-100.
8. PRYCE. G, BAKER D: Emerging properties of cannabinoid medicines in management of multiple sclerosis. *Trends Neurosci* 2005; 28: 272-6.
9. GUI H, LIU X, WANG ZW, HE DY, SU DF, DAI SM: Expression of cannabinoid receptor 2 and its inhibitory effects on synovial fibroblasts in rheumatoid arthritis. *Rheumatology* (Oxford) 2014; 53: 802-9.
10. GUI H, TONG Q, QU W, MAO C-M, DAI S-M: The endocannabinoid system and its therapeutic implications in rheumatoid arthritis. *Int Immunopharmacol* 2015; 26: 86-91.
11. SOETHOUDT M, GRETHER U, FINGERLE J *et al*.: Cannabinoid cb2 receptor ligand profiling reveals biased signalling and off-target activity. *Nat Commun* 2017; 8: 13958.
12. BURSTON JJ, SAGAR DR, SHAO P *et al*.: Cannabinoid cb2 receptors regulate central sensitization and pain responses associated with osteoarthritis of the knee joint. *PLoS One*. 2013; 8: e80440.
13. FUKUDA S, KOHSAKA H, TAKAYASU A *et al*.: Cannabinoid receptor 2 as a potential therapeutic target in rheumatoid arthritis. *BMC Musculoskelet Disord* 2014; 15: 275.
14. HYTTI M, ANDJELIC S, JOSIFOVSKA N *et al*.: Cb2 receptor activation causes an erk1/2-dependent inflammatory response in human rpe cells. *Sci Rep* 2017; 7: 16169.
15. AHMED S, MAROTTE H, KWAN K *et al*.: Epigallocatechin-3-gallate inhibits il-6 synthesis and suppresses transsignaling by enhancing soluble gp130 production. *PNAS* 2008; 105: 14692-7.
16. AHMED S, PAKOZDI A, KOCH AE: Regulation of interleukin-1beta-induced chemokine production and matrix metalloproteinase 2 activation by epigallocatechin-3-gallate in rheumatoid arthritis synovial fibroblasts. *Arthritis Rheum* 2006; 54: 2393-401.
17. BARTOK B, FIRESTEIN GS: Fibroblast-like synoviocytes: Key effector cells in rheumatoid arthritis. *Immunol Rev* 2010; 233: 233-55.
18. FECHTNER S, SINGH A, CHOURASIA M, AHMED S: Molecular insights into the differences in anti-inflammatory activities of green tea catechins on il-1beta signaling in rheumatoid arthritis synovial fibroblasts. *Toxicol Appl Pharmacol* 2017; 329: 112-20.
19. SINGH AK, UMAR S, RIEGSECKER S, CHOURASIA M, AHMED S: Regulation of tak1 activation by epigallocatechin-3-gallate in ra synovial fibroblasts: suppression of k63-linked autoubiquitination of traf6. *Arthritis Rheumatol* 2016; 68: 347-58.
20. KIM EY, SUDINI K, SINGH AK *et al*.: Ursolic acid facilitates apoptosis in rheumatoid arthritis synovial fibroblasts by inducing sp1-mediated noxa expression and proteasomal degradation of mcl-1. *FASEB J* 2018 [Epub ahead of print].
21. AHMED S, PAKOZDI A, KOCH AE: Regulation of interleukin-1beta-induced chemokine production and matrix metalloproteinase 2 activation by epigallocatechin-3-gallate in rheumatoid arthritis synovial fibroblasts. *Arthritis Rheum* 2006; 54: 2393-401.
22. RICHARDSON D, PEARSON RG, KURIAN N, LATIF ML, GARLE MJ, BARRETT DA: Characterisation of the cannabinoid receptor system in synovial tissue and fluid in patients with osteoarthritis and rheumatoid arthritis. *Arthritis Res Ther* 2008; 10: R43.
23. FECHTNER S, FOX DA, AHMED S: Transforming growth factor beta activated kinase 1: A potential therapeutic target for rheumatic diseases. *Rheumatology* (Oxford) 2017; 56: 1060-68.
24. LOWIN T, PONGRATZ G, STRAUB RH: The synthetic cannabinoid win55,212-2 mesylate decreases the production of inflammatory mediators in rheumatoid arthritis synovial fibroblasts by activating cb2, trpv1, trpa1 and yet unidentified receptor targets. *J Inflamm* (Lond) 2016; 13: 15.
25. MARINI P, CASCIO M-G, KING A, PERTWEE RG, ROSS RA: Characterization of cannabinoid receptor ligands in tissues natively expressing cannabinoid cb2 receptors. *Br J Pharmacol* 2013; 169: 887-99.
26. AHMED S, RAHMAN A, HASNAIN A, LALONDE M, GOLDBERG VM, HAQQI TM: Green tea polyphenol epigallocatechin-3-gallate inhibits the il-1 beta-induced activity and expression of cyclooxygenase-2 and nitric oxide synthase-2 in human chondrocytes. *Free Radic Biol Med* 2002; 33: 1097-105.
27. AGERE SA, AKHTAR N, WATSON JM, AHMED S: Rantes/ccl5 induces collagen degradation by activating mmp-1 and mmp-13 expression in human rheumatoid arthritis synovial fibroblasts. *Front Immunol* 2017; 8: 1341.
28. ZHENG D, BODE AM, ZHAO Q *et al*.: The cannabinoid receptors are required for ultraviolet-induced inflammation and skin cancer development. *Cancer Res* 2008; 68: 3992-8.
29. SUN X, CAPPELLETTI M, LI Y, KARP CL, DIVANOVIC S, DEY SK: Cnr2 deficiency confers resistance to inflammation-induced preterm birth in mice. *Endocrinology* 2014; 155: 4006-14.
30. ATWOOD BK, WAGER-MILLER J, HASKINS C, STRAIKER A, MACKIE K: Functional selectivity in cb(2) cannabinoid receptor signaling and regulation: implications for the therapeutic potential of cb(2) ligands. *Mol Pharmacol* 2012; 81: 250-63.
31. BOUABOULA M, DUSSOSSOY D, CASELLAS P: Regulation of peripheral cannabinoid receptor cb2 phosphorylation by the inverse agonist sr 144528. Implications for receptor biological responses. *J Biol Chem* 1999; 274: 20397-405.
32. RUSSO EB: Cannabinoids in the management of difficult to treat pain. *Ther Clin Risk Manag* 2008; 4: 245-59.
33. LU D, VEMURI VK, DUCLOS RI JR, MAKRIYANNIS A: The cannabinergic system as a target for anti-inflammatory therapies. *Curr Top Med Chem* 2006; 6: 1401-26.
34. MCDOUGALL JJ, YU V, THOMSON J: *In vivo* effects of cb(2) receptor-selective cannabinoids on the vasculature of normal and arthritic rat knee joints. *Br J Pharmacol* 2008; 153: 358-66.
35. BRUCE D, BRADY JP, FOSTER E, SHATTELL M: Preferences for medical marijuana over prescription medications among persons living with chronic conditions: alternative, complementary, and tapering uses. *J Altern Complement Med* 2018; 24: 146-53.