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Cannabinoid Receptor-2 Ameliorates Inflammation in Murine Model of Crohn's Disease

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Abstract

Background and Aims: Cannabinoid receptor stimulation may have positive symptomatic effects on inflammatory bowel disease [IBD] patients through analgesic and anti-inflammatory effects. The cannabinoid 2 receptor [CB_2R] is expressed primarily on immune cells, including $CD4^+$ T cells, and is induced by active inflammation in both humans and mice. We therefore investigated the effect of targeting CB_2R in a preclinical IBD model.

Methods: Employing a chronic ileitis model [$TNF^{\Delta ARE/+}$ mice], we assessed expression of the CB_2R receptor in ileal tissue and on $CD4^+$ T cells and evaluated the effect of stimulation with CB_2R-selective ligand GP-1a both *in vitro* and *in vivo*. Additionally, we compared cannabinoid receptor expression in the ilea and colons of healthy human controls with that of Crohn's disease patients.

Results: Ileal expression of CB_2R and the endocannabinoid anandamide [AEA] was increased in actively inflamed $TNF^{\Delta ARE/+}$ mice compared with controls. CB_2R mRNA was preferentially induced on regulatory T cells [Tregs] compared with T effector cells, approximately 2.4-fold in wild-type [WT] and 11-fold in $TNF^{\Delta ARE/+}$ mice. Furthermore, GP-1a enhanced Treg suppressive function with a concomitant increase in IL-10 secretion. GP-1a attenuated murine ileitis, as demonstrated by improved histological scoring and decreased inflammatory cytokine expression. Lastly, CB_2R is downregulated in both chronically inflamed $TNF^{\Delta ARE/+}$ mice and in IBD patients.

Conclusions: In summary, the endocannabinoid system is induced in murine ileitis but is downregulated in chronic murine and human intestinal inflammation, and CB_2R activation attenuates murine ileitis, establishing an anti-inflammatory role of the endocannabinoid system.

Key Words: Inflammatory bowel disease; endocannabinoid; regulatory T cell

1. Introduction

Inflammatory bowel disease [IBD], including Crohn's Disease [CD] and ulcerative colitis, is one of the most costly, prevalent gastrointestinal disorders and affects approximately 1.6 million people in the USA.[1] Chronic inflammation of the gastrointestinal tract in IBD places a significant burden on affected individuals, not only due to poor quality of life and the long-term risk of malignancy, but also due to recurrent symptoms of abdominal pain, bloody diarrhoea, and weight loss, as well as the growth failure, impaired bone mineralisation and pubertal delay commonly seen in paediatric-onset IBD.[2]

The complex factors underlying IBD pathogenesis are still incompletely understood, but it is generally recognised that IBD emerges due to an aberrant immune response. $CD4^+$ T cells act as key mediators of intestinal inflammation and therefore represent an ideal target for therapeutic treatment of IBD. Of particular significance are $CD4^+FoxP3^+$ regulatory T cells [Tregs], which play a crucial role in maintaining gut homeostasis by suppressing inappropriate immune responses. Expression of the transcription factor FoxP3 is vital for the regulatory activity of Tregs, and thus mutated FoxP3 in both humans and mice results in severe autoimmunity, including immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome [IPEX] in humans.[3] Novel modulatory approaches which promote the regulatory pathway in T cells, whereby excessive effector cell responses are curtailed, hold considerable potential as future therapeutic approaches for the treatment of IBD.

The limited benefits, rapid loss of efficacy, and multiple long-term risks, including malignancy, infection, and low bone density,[4] of current immunosuppressive therapies have influenced affected individuals and physicians alike to urge the development of alternate therapeutic strategies for the treatment of IBD. The endogenous cannabinoid [endocannabinoid] system is one pathway that is rapidly gaining attention as a novel therapeutic target for its immunosuppressive effects[5] and, specifically in IBD, for its possible analgesic and anti-inflammatory effects.[6] As of March 2017, 28 states and Washington DC have legalised cannabis for medical purposes, and limited observational studies have demonstrated that medical marijuana, through interaction with the endogenous cannabinoid system [ECS], can potentially be used to alleviate a number of IBD-associated intestinal symptoms including reducing nausea, stool frequency, and abdominal pain, while improving appetite and weight gain.[7] However, it is unclear if the symptomatic relief experienced by patients using cannabis in IBD is accompanied by improvement of intestinal inflammation or any real disease-modifying effect.[8] Indeed, one such study showed that the patients with Crohn's disease who used cannabis had worse disease prognosis and higher tendency toward surgical intervention.[9] It is this uncertainty of the risks, benefits, and true impact of cannabis on IBD that has driven the intensification of basic science research investigating the biological effects of manipulation of the ECS in intestinal inflammation.

The ECS consists of endogenous ligands termed endocannabinoids, including anandamide [AEA] and 2-arachidonoylglycerol [2-AG], which mainly act via two classic cannabinoid heterotrimeric G-protein-coupled receptors: cannabinoid receptor 1 [CB_1R] and cannabinoid receptor 2 [CB_2R]. They also act through non-classical receptors: transient receptor potential vanilloid type 1 [TRPV1] and the orphan G-protein-coupled receptor GPR55.[2,10] The CB_1R modulates neurotransmitter release and is found on central and peripheral neurons, including within the enteric nervous system.[10] The CB_2R has been shown to be mostly expressed on neutrophils, macrophages, subsets of T and B cells, and epithelial cells,[11] and thus is mainly associated with immune function.[12] The ECS also includes the proteins involved in endocannabinoid biosynthesis and degradation; fatty-acid amide hydrolase [FAAH] degrades AEA to arachidonic acid and ethanolamine,[13] and monoacylglycerol lipase [MAGL] degrades 2-AG to arachidonic acid and glycerol.[14]

The ECS regulates gastrointestinal function through its effects on gastric secretions, gut-brain signalling, gut motility, and the gut microbiome, and significantly influences murine intestinal inflammation.[15] A recent review on cannabinoids in murine colitis models demonstrated that manipulation of the endocannabinoid system had immunomodulatory effects dependent on complex interactions of specific receptor pathways.[2] CB_1R activation[16–21] and CB_2R activation[19,20,22–26] both ameliorated acute colitis. Furthermore, inflamed colonic epithelium and lamina propria [LP] immune cells have increased expression of the CB_2R,[27] and activation of epithelial CB_2R inhibits tumour necrosis factor alpha [TNF-α]-induced CXCL8 release *in vitro*.[28]

Our studies made use of the $TNF^{\Delta ARE/+}$ mouse model of Crohn's-like ileitis.[29] These mice are generated by the deletion of 69 bp within the AU-rich element of the TNF gene, causing systemic overproduction of TNF and consequently chronic ileal inflammation representative of human CD. The compelling similarities with human CD in the pathogenesis and histological presentation of disease in this model reinforce its value and relevance in our investigation of the role of cannabinoids in mediating T cell homeostasis.

In summary, development of novel therapeutic pathways is imperative in the treatment of IBD. Limited data are supportive of symptomatic improvement with cannabis use in IBD, but the role of cannabinoids in immune response and immunostimulation is incompletely understood. Because CB_2R is expressed by $CD4^+$ T cells[30] and is upregulated in inflamed LP immune cells, and based on the amalgamation of the murine colitis studies described, a central anti-inflammatory role of the CB_2R is suggested. We hypothesised that stimulation of CB_2R would positively affect disease outcomes in murine ileitis. In our study, we investigated inflammation-induced changes in cannabinoid tone in both murine and human intestinal tissue, and assessed the role of cannabinoids as endogenous modulators of intestinal inflammation by defining the effect of pharmacological CB_2R targeting on inflammation, T cell function, and phenotype.

2. Materials and Methods

2.1. Mice

The B6.129S-Tnf^{tm2GKl}/Jarn strain [$TNF^{\Delta ARE/+}$; MGI: 3720980] was generated by continuous backcrosses between $TNF^{\Delta ARE}$ on a mixed background[29] to C57BL/6J mice. Homozygous wild-type [WT] C57BL/6J littermates served as non-inflamed controls. An alternate model of *Salmonella enterica* serovar Typhimurium infectious colitis was induced in adult C57BL/6J mice as previously described.[31] $CB_2R^{-/-}$ knockout mice [B6.129P2-*Cnr2*^{tm1Dgen}/J] were obtained from Jackson Laboratories. Mice were kept under specific pathogen-free conditions, and faecal samples were routinely negative for *Helicobacter* species, protozoa, and helminthes. Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver.

2.2. Analysis of endogenous cannabinoid tone

Tissue samples from WT and $TNF^{\Delta ARE/+}$ terminal ilea were assessed for presence of endogenous cannabinoids using-high performance liquid chromatography [HPLC]. RNA was isolated from ileal tissue

or T cell subsets using the RNeasy Mini Kit [QIAGEN] and converted to complementary DNA [cDNA] using a high-capacity cDNA Reverse Transcription Kit [Applied Biosystems]. Relative quantitation of mRNA expression was performed using Taqman Gene Expression Assays and the ABI PRISM 7900HT Sequence Detection System [Applied Biosystems]. Polymerase chain reaction [PCR] assays for *Cnr1* [Mm01212171_s1] and *Cnr2* [Mm02620087_s1] were carried out, with ribosomal 18s as an endogenous control.

2.3. Western blot analysis

Ilea from inflamed TNF$^{\Delta ARE/+}$ and non-inflamed WT littermates were homogenised in RIPA lysis and extraction buffer [Thermo Scientific] containing Halt protease inhibitor cocktail [Thermo Scientific]. Protein concentration was determined using the BCA Protein Assay [Pierce]. Protein extracts were separated by SDS-PAGE using Bio-Rad Mini-PROTEAN® TGX Stain-Free™ Gels. Rabbit anti-CB_2R was raised by immunising rabbits with the C-terminal fragment of recombinant CB_2R [Ac-CKYLQGLGPEGKEEGPR-amide; New England Peptide]. Blots were probed overnight at 4°C with rabbit anti-CB_2R [1:2500] or rabbit anti-Na+/K+-ATPase alpha [sc28800; 1:200; Santa Cruz], followed by a 1-h incubation at room temperature with a goat anti-rabbit peroxidase-conjugated antibody [sc2004; 1:5000; Santa Cruz]. Blots were then exposed to enhanced chemiluminescence substrate [Clarity Western ECL Substrate; Bio-Rad] and imaged using the Bio-Rad ChemiDoc™ MP system.

2.4. *In vitro* suppression assays

Treg suppression assays were performed as previously described.[32] Briefly, $CD4^+CD25^+$ Tregs and $CD4^+CD25^{Neg}$ effector T cells [Teff] were magnetically isolated from splenocytes using the EasySep Mouse CD25 Regulatory T Cell Positive Selection Kit II [Stem Cell Technologies]; 5×10^4 CellTrace Violet-labeled [Invitrogen] $CD4^+CD25^{Neg}$ Teff per well were stimulated with anti-CD3 mAb [1 µg/mL] in the presence of 7.5×10^4 irradiated syngeneic antigen-presenting cells [APC] and varying ratios of Tregs. Where indicated, cells were cultured with 10 µM GP-1a [Tocris Bioscience]. The percentage of Teff proliferation was determined by the CellTrace profile of live $CD4^+$ cells at 72 h.

2.5. Apoptosis assay

WT splenocytes were stimulated at a concentration of 2×10^6/mL with plate-bound anti-CD3 mAb [1 µg/mL] and soluble anti-CD28 [4 µg/mL] for 24 h in complete RPMI-1640 [with L-glutamine, Corning Inc., supplemented with 10% FBS, 100 IU penicillin and 100 µg/ml streptomycin; Invitrogen]. Where indicated, GP-1a was added to cultures at 10 µM. Determination of apoptosis was performed using FITC Annexin V Apoptosis Detection Kit I [BD Biosciences] according to the manufacturer's instructions.

2.6. *In vivo* GP-1a treatment

Twice per week for 2 weeks, 8-week-old actively inflamed TNF$^{\Delta ARE/+}$ mice were treated with selective CB_2R ligand GP-1a [5 mg/kg per mouse[33]] or vehicle [10% DMSO in PBS] by retro-orbital injection. At the time of sacrifice, spleens, mesenteric lymph nodes [MLN], and ilea were excised for flow cytometric analysis, histological assessment of ileitis, and cytokine analyses. Ileal sections were fixed in 10% formalin, and stained with haematoxylin and eosin. Histological assessment of ileitis was performed using a standardised semi-quantitative scoring system, as described previously.[34] Representative micrographs were obtained using an Olympus IX83 inverted microscope with cellSens Dimension v1.16 software [Olympus], made available through the Gastrointestinal Liver Innate Immunology Program at the University of Colorado.

Splenocytes, MLN, and LP mononuclear cells were isolated as previously described.[35,36] Tissues were filtered to remove any remaining undigested material before antibody labelling and flow cytometric evaluation.

2.7. Flow cytometry and intracellular cytokine staining

Cells from indicated compartments were incubated with fluorescent rat anti-mouse antibodies for T cell subset evaluation. Intracellular cytokine staining was performed by stimulating cells for 5 h with 20 ng/mL phorbol 12-myristate 13-acetate [PMA, Sigma Aldrich #P8139] and 1 µg/mL calcium ionomycin [Sigma Aldrich #I0634] in the presence of 3 µg/mL brefeldin-A [Sigma Aldrich #B7651], followed by permeabilisation and staining with antibodies against CD4 [GK1.5], IFNγ [XMG1.2], IL-10 [JES5-16E3], and FoxP3 [FJK16S]. Live cells were identified using Live/Dead Fixable Aqua dye [Invitrogen]. FoxP3 staining was performed according to manufacturer's instructions [FoxP3/Transcription Factor Staining Buffer Kit, eBiosciences]. Cells were washed and fixed with 2% paraformaldehyde and then analysed using the FACS® Canto II system [Becton-Dickinson]. Post-analyses were performed using FlowJo software [Tree Star Inc.].

2.8. Quantification of cytokine production

Terminal ileal explants [1 cm^2] were cultured for 24 h in RPMI-1640 [with L-glutamine; Corning Inc., supplemented with 10% FBS, 100 IU penicillin and 100 µg/ml streptomycin; Invitrogen]. Culture supernatants were then analysed for IL-10 concentration using enzyme-linked immunosorbent assay [ELISA], as per the manufacturer's instructions [Mouse IL-10 ELISA Ready-Set-Go! eBioscience].

2.9. Evaluation of cannabinoid receptor expression in Crohn's disease patients

The study protocol was approved by the Ethical Committee of the University Hospital, Tuebingen, Germany. All patients and controls gave their written, informed consent to participation. Biopsies from the terminal ileum and colon were sampled during routine colonoscopy at the Robert Bosch Hospital, Stuttgart, Germany, and were immediately stored in liquid nitrogen for subsequent processing and analysis. Diagnosis was performed according to standard criteria using clinical, radiological, endoscopic, and histopathological methods. Patients with CD were grouped according to standard Vienna classifications, designating L1 as CD with exclusive ileal involvement, L2 as CD with solely colonic involvement, and L3 as CD with ileocolonic involvement.

2.10. RNA preparation from human tissue and quantification of mRNA expression

RNA from human tissue was isolated using TRIzol Reagent [Life Technologies] according to manufacturer's protocol. cDNA synthesis was performed using a reverse transcription kit [Promega] and oligo[dT]15-primer. An amount of cDNA corresponding to 10 ng of RNA was applied in all assays. For quantification, we used SYBR Green I [Roche] and the Roche LightCycler 480 system. Absolute copy numbers were calculated from the crossing point [Cp] values according to respective standard plasmid curves, generated for all targets. Plasmid standards were designed using a TOPO TA Cloning Kit [Invitrogen] and confirmed by sequencing. Results were normalised to β-actin [see Table 1 for primers].

Table 1. Primer sequences.

Gene	Sense	Antisense
β-actin	5'-GCCAACCGCGAGAAGATGA-3'	5'-CATCACGATGCCAGTGGT A-3'
CXCL8	5'-ATGACTTCCAAGCTGGCCGTGGC-3'	5'-TCTCAGCCCTCTTCAAAAACTTC-3'
CNR1	5'-ATGCGAAGGGATTGCCCC-3'	5'-ATGGTGCGGAAGGTGGTATC-3'
CNR2	5'-TCCTGGGAGAGGACAGAAAAC-3'	5'-TTGTCTAGAAGGCTTGTTG-3'

2.11. Statistics

Statistical analyses were performed using one- or two-tailed Student's *t* test [as appropriate], Mann–Whitney U-test [for non-normally distributed groups], and Spearman rank analysis to test for correlation. In the absence of evidence regarding expression of CB receptors in the inflamed ileum, we chose to employ a more conservative two-tailed *t* test. In contrast, interventional studies were assessed using a one-tailed *t* test consistent with our directional research hypothesis to be tested. Graphs are presented as mean ± standard error of the mean [SEM], generated using GraphPad Prism software. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Murine ileitis induces the endocannabinoid system

The ECS is induced in acute colitis models using dextran sulphate sodium [DSS] or 2,4,6-trinitrobenzenesulphonic acid [TNBS].[2,15] To establish the validity of this finding in the context of our preclinical CD model, we isolated terminal ileal tissue from acutely inflamed 8–12-week-old male $TNF^{\Delta ARE/+}$ mice [$n = 7$] and age-matched male WT mice [$n = 5$] and assessed for the presence of endogenous cannabinoids AEA, 2-AG, oleoylethanolamide [OEA], and palmitoylethanolamide [PEA] by HPLC. We found that the endocannabinoid AEA was significantly induced in inflamed $TNF^{\Delta ARE/+}$ ilea p[26.9 ± 1.6 pmol/g] as compared with WT ilea [18.8 ± 3.1 pmol/g; $P < 0.05$] [Figure 1A]. Furthermore, endocannabinoid OEA showed an overall trend of increased expression in inflamed $TNF^{\Delta ARE/+}$ ilea [182.0 ± 32.1 pmol/g] as compared with WT [147.5 ± 26.1 pmol/g; $p = 0.45$], whereas 2-AG showed a decreased although non-significant trend in expression [49.6 ± 11.7 pmol/g versus 26.3 ± 9.5 pmol/g; $p = 0.15$] and PEA demonstrated no significant difference in expression among the two groups [Figure 1A].

As previously described, $TNF^{\Delta ARE/+}$ mice are grouped into subsets of pre-disease [age 4 weeks], peak active inflammatory state [age 8–12 weeks], and chronic inflammatory state [age ≥ 20 weeks].[29] To further evaluate ECS expression in our inflamed $TNF^{\Delta ARE/+}$ mouse model, we isolated terminal ilea from each age subset [$n \geq 4$ per age group] and measured expression of *Cnr1* and *Cnr2* [genes coding for CB_1R and CB_2R, respectively]. Neither *Cnr1* nor *Cnr2* expression was significantly altered in the ileum of pre-disease 4-week-old $TNF^{\Delta ARE/+}$ mice as compared with WT mice. In actively inflamed 8–12-week-old mice, however, *Cnr1* was found to be significantly increased approximately 1.5-fold compared with WT, and *Cnr2* increased 2.6-fold [$p < 0.05$ and $p < 0.01$]. In chronically inflamed mice [≥ 20 weeks], we saw a significant downregulation of both *Cnr1* and *Cnr2* [$p < 0.01$]. Thus, both CB_1R and CB_2R are induced by active ileitis and downregulated by chronic inflammation [Figure 1B]. We also investigated cannabinoid receptor expression in an infectious *S. Typhimurium* colitis model [$n \geq 3$ per group], and found that there was no such induction in cannabinoid receptor expression, indicating that this phenomenon was not simply a general response to intestinal insult [Supplementary Figure 1, available as Supplementary data at *ECCO-JCC* online].

Due to the expression profile of the CB_2R in the ileum, and because CB_2R is preferentially expressed on lymphocytes and other immune cells, we identified the CB_2R as our receptor of interest. We found that in line with the results of real-time [RT]-PCR, CB_2R protein was prominently increased in the actively inflamed ilea of $TNF^{\Delta ARE/+}$ mice compared with WT littermates [Figure 1C].

In summary, the compilation of data in Figure 1 highlights the induction of the ECS at the receptor level, and to a much lesser extent at the ligand level, in the setting of active murine ileitis. Furthermore, CB_2R mRNA expression is more significantly elevated than CB_1R expression in the ilea of actively inflamed $TNF^{\Delta ARE/+}$ mice and is preferentially expressed on immune cells, distinguishing CB_2R as the key receptor of interest in our investigation.

3.2. CB_2R is preferentially upregulated on regulatory T cells in murine ileitis, and targeting with GP-1a enhances suppressive function

In view of the fact that cannabinoid receptors are expressed by $CD4^+$ T cells, key regulators of intestinal inflammation, and based on their upregulation in acute murine ileitis, we focused our investigational efforts on elucidation of the role of the cannabinoid receptors on $CD4^+$ T cell phenotype and function during inflammation. First, we compared cannabinoid receptor expression on $CD4^+$ Tregs with that on $CD4^+$ non-Tregs isolated from actively inflamed 8–12-week-old male $TNF^{\Delta ARE/+}$ mice and age-matched male WT controls [$n = 3$ per group]. Tregs were magnetically enriched from splenocytes based on expression of CD25, and although CD25 can be induced on activated non-Treg cells especially *in vitro*, our experience with $TNF^{\Delta ARE/+}$ mice indicates that CD25 is not expressed on non-Tregs, even in the inflamed lamina propria.[32] It was confirmed by flow cytometry that approximately 85% of enriched $CD4^+CD25^+$ expressed FoxP3. Our study demonstrated that relative mRNA expression of *Cnr1* was preferentially upregulated 2-fold in $TNF^{\Delta ARE/+}$ Tregs as compared with Teff to a near-significant extent [$p = 0.06$], whereas there was no difference in expression of *Cnr1* between WT Tregs and Teff [Figure 2A]. Moreover, relative mRNA expression of *Cnr2* was increased 11-fold in Tregs as compared with Teff cells of actively inflamed $TNF^{\Delta ARE/+}$ mice [$p < 0.001$] and increased 2.4-fold on Tregs compared with Teff cells in WT mice, though this did not reach statistical significance [$p = 0.09$] [Figure 2A].

In consideration of the preferential upregulation of the CB_2R on Tregs in acute murine ileitis, we performed a Treg suppression assay to assess the *in vitro* suppressive capacity of Tregs upon stimulation with selective CB_2R ligand GP-1a. $CD4^+CD25^+$ Tregs were isolated from actively inflamed 8–12-week-old male $TNF^{\Delta ARE/+}$ mice and co-cultured with CellTrace Violet-labelled $CD4^+CD25^{Neg}$ Teff at varying ratios in the presence of irradiated APC and anti-CD3 stimulation. Tregs treated with GP-1a showed enhanced suppressive capacity compared with control, indicated by significantly decreased Teff proliferation [Figure 2B] [$p < 0.01$ at 1:1 ratio, and $p < 0.001$ at

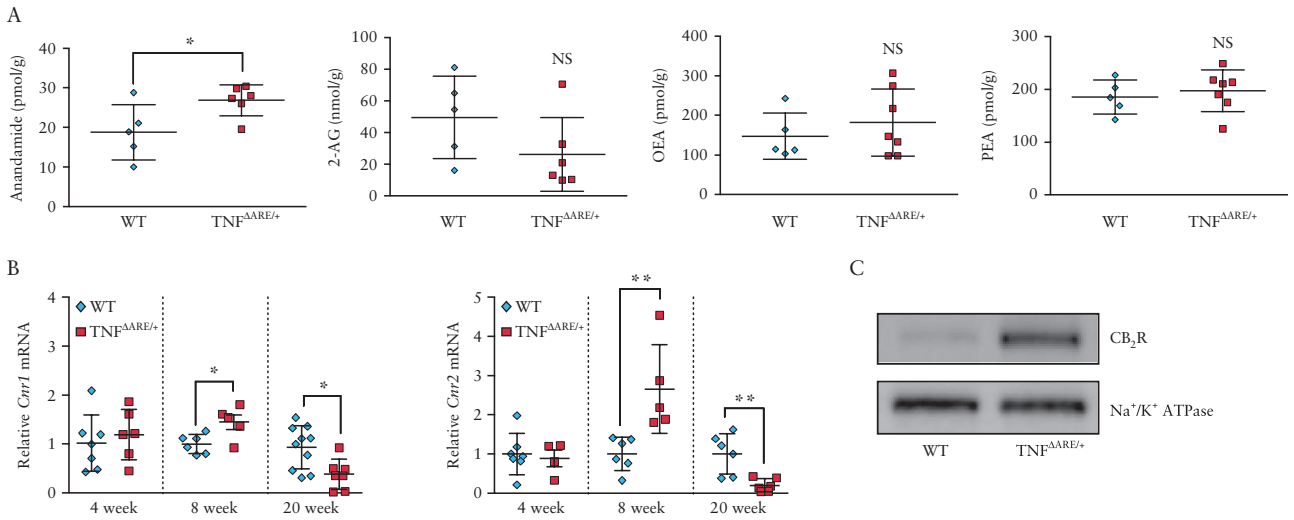

Figure 1. Murine ileitis induces the endocannabinoid system. [A] HPLC analysis of endogenous cannabinoids anandamide, 2-AG, OEA, and PEA in terminal ilea of TNF$^{\Delta ARE/+}$ mice compared with WT [mean ± SEM of $n \geq 5$ individual mice; two-tailed *t* test $^{*}p < 0.05$]. [B] mRNA expression of *Cnr1* and *Cnr2* in whole ileal tissue of 4-week [pre-disease], 8–12 week [active inflammation] and ≥ 20-week [chronic inflammation] TNF$^{\Delta ARE/+}$ mice compared with WT [mean ± SEM, $n \geq 5$ individual mice; two-tailed *t* test $^{*}p < 0.05$, $^{**}p < 0.01$]. [C] Representative Western blot of CB_2R expression in whole ileal tissue of 8–12 week TNF$^{\Delta ARE/+}$ mice and non-inflamed littermates. Na+/K+-ATPase is used as a loading control. HPLC, high-performance liquid chromatography; WT, wild type; SEM, standard error of the mean; TNF, tumour necrosis factor.

1:2, 1:4, and 1:8 ratios; assay repeated ≥ 3 times]. GP-1a did not affect proliferation of Teff in the absence of Treg. Furthermore, an ELISA was performed to assay IL-10 quantities in the culture supernatants of the suppression assay with and without GP-1a, revealing a concomitant increase in anti-inflammatory IL-10 secretion in the setting of CB_2R stimulation [vehicle = 125.4 ± 12.4 pg/ml, GP-1a = 224.7 ± 31.7 pg/ml; $p < 0.05$]. We then assessed the suppressive capacity of Tregs lacking the CB_2R, isolated from $CB_2R^{-/-}$ knockout mice. Compared with WT, we observed no significant differences in suppression by Tregs lacking CB_2R [Supplementary Figure 2, available as Supplementary data at *ECCO-JCC* online].

In order to investigate a potential direct effect of GP-1a on Teff in the absence of Treg, we evaluated cannabinoid receptor expression on TNF$^{\Delta ARE/+}$ Teff stimulated with anti-CD3 and anti-CD28 for 24 h [$n = 4$ per group]. We found that there was a significant increase in mRNA expression of *Cnr1* and *Cnr2* upon stimulation and treatment with Gp-1a. Culture with GP-1a alone in the absence of stimulation resulted in a modest but significant induction of *Cnr1* and *Cnr2* approximately 1.1-fold [$p < 0.01$], and stimulation with anti-CD3 and anti-CD28 resulted in a 1.2-fold induction of both *Cnr1* and *Cnr2* [$p < 0.01$]. When stimulated and exposed to GP-1a simultaneously, *Cnr1* mRNA increased 1.3-fold and *Cnr2* increased 1.4-fold [$p < 0.001$] [Figure 2C].

In summary, Figure 2A identified a preferential expression of CB_1R and CB_2R on Tregs, and this effect was even more pronounced for CB_2R expression in the setting of active murine ileitis, implying involvement of the CB_2R on Tregs in the active inflammatory pathway. Figure 2B indicates the ability of CB_2R stimulation with GP-1a to enhance Treg suppression, with a concomitant increase in IL-10 production suggesting an anti-inflammatory role for CB_2R stimulation in $CD4^+$ T cells. Figure 2C indicates a direct effect of GP-1a on the expression of CB_1R and CB_2R on TNF$^{\Delta ARE/+}$ Teff cells.

3.3. CB_2R stimulation attenuates murine ileitis

To investigate the potential anti-inflammatory role of CB_2R stimulation in active murine ileitis, we performed a treatment study using selective CB_2R ligand GP-1a. Eight-week-old male TNF$^{\Delta ARE/+}$ mice received pulsatile 2-week treatment with either GP-1a or vehicle [$n = 9$ mice per treatment group], and ilea were then isolated at 10 weeks of age for blinded histology review by a pathologist using the previously described Burns method.[34]

We found that CB_2R stimulation with GP-1a led to histological improvement of ileitis of TNF$^{\Delta ARE/+}$ mice [Figure 3]. Notably, both active inflammation characterised by granulocytic infiltration, and chronic inflammation characterised by lymphocytic infiltration, significantly improved [$p < 0.05$] in response to CB_2R ligand treatment [Figure 3]. There was a trend of decreased villus architecture distortion in mice treated with GP-1a, although this improvement did not reach statistical significance [$p = 0.07$]. These data support the findings in Figure 2, suggesting an anti-inflammatory role of CB_2R stimulation in murine ileitis.

3.4. CB_2R receptor stimulation reduces inflammatory infiltrate

To further elucidate the anti-inflammatory effect of CB_2R stimulation in murine ileitis, we assessed cells from spleen, MLN, and ileal LP from 10-week-old TNF$^{\Delta ARE/+}$mice following GP-1a treatment by flow cytometry. We found a significant decrease [2.2 ± 0.3% to 1.4 ± 0.2; $p < 0.05$] in IFNγ-producing Th1 cells in the TNF$^{\Delta ARE/+}$ LP when treated with GP-1a as compared with vehicle control, as well as a decrease, albeit non-significant, in the expression of IFNγ in both spleen and MLN [p = 0.1 and 0.3, respectively] [Figure 4A, B]. Furthermore, we found that whereas the frequency of $CD4^+FoxP3^+$ Tregs was unchanged in the LP of GP-1a treated mice, the proportion of Tregs producing IL-10 was significantly increased [3.9 ± 1% to 7.1 ± 0.9 %; $p < 0.05$]. Consistent with this finding, an ELISA revealed a significant increase in IL-10 in the supernatants of ileal explant culture of GP-1a treated mice [252.8 ± 43.7 pg/mg; $p < 0.05$] as compared with vehicle control [123.1 ± 26.3 pg/mg; $p < 0.05$] [Figure 4C].

Collectively, the data presented in Figures 3 and 4 indicate that CB_2R stimulation ameliorates inflammation and reduces inflammatory infiltrate in murine ileitis.

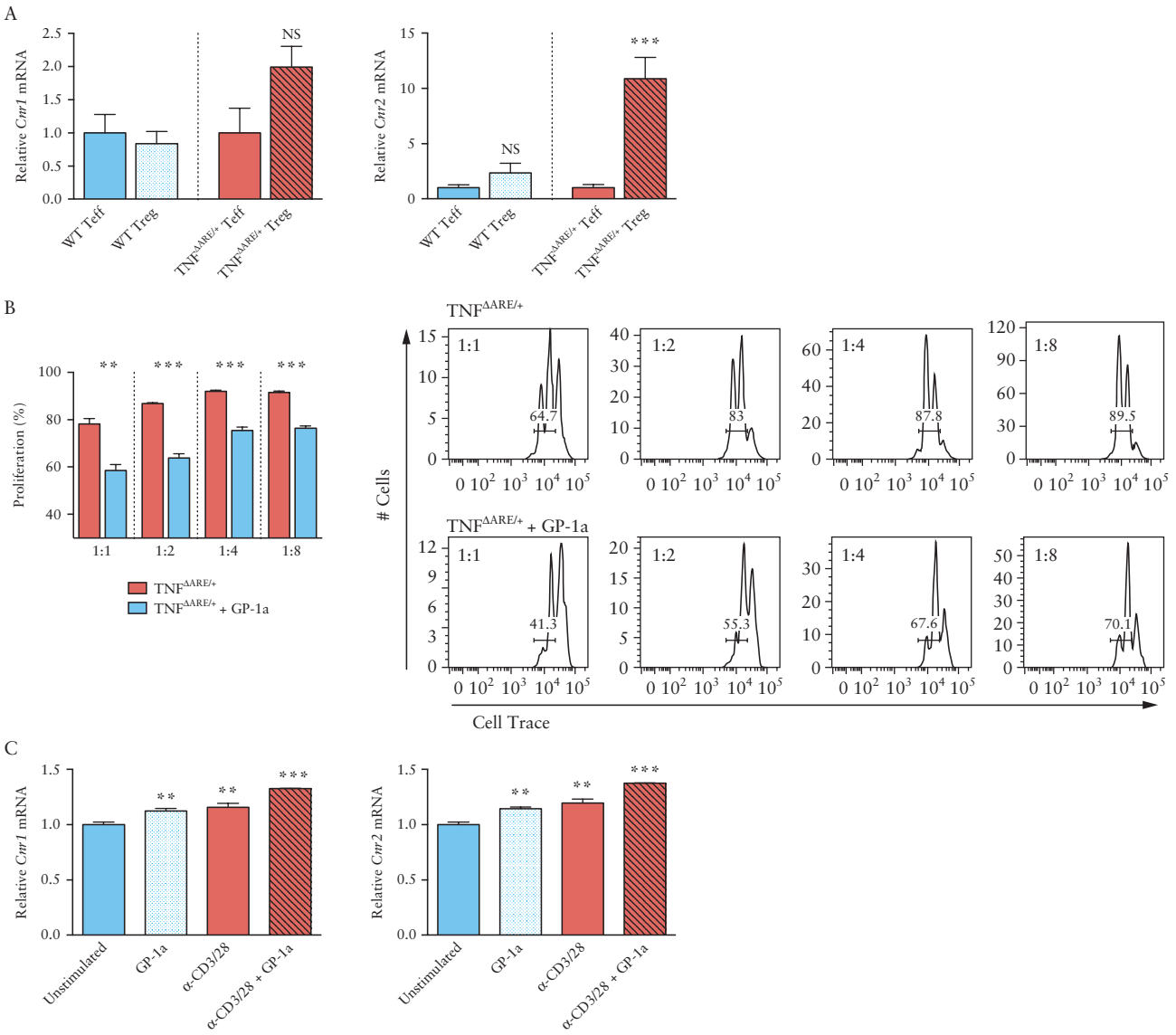

Figure 2. CB_2R is preferentially upregulated on regulatory T cells in murine ileitis and targeting with GP-1a enhances suppressive function. [A] Preferential upregulation of CB_2R on regulatory T cells in murine ileitis. *Cnr1* and *Cnr2* mRNA expression in isolated $CD4^+CD25^+$ Tregs and $CD4^+CD25^{Neg}$ Teff from 8–12 week WT and $TNF^{\Delta ARE/+}$ mice [mean ± SEM of pooled cells from $n \geq 3$ per group; two-tailed *t* test $^{***}p < 0.001$] [B] Treatment of co-culture with GP-1a restores capacity of $TNF^{\Delta ARE/+}$ Treg to suppress Teff. Data shown are percentages of proliferating cells at various ratios of Treg:Teff; representative of three experiments [mean ± SEM; two-tailed *t* test $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$]. [C] Induction of *Cnr1* and *Cnr2* mRNA in $TNF^{\Delta ARE/+}$ $CD4^+CD25^{Neg}$ Teff with anti-CD3 and anti-CD28 stimulation and GP-1a treatment [mean ± SEM of $n \geq 4$ per group; two-tailed *t* test $^{**}p < 0.01$, $^{***}p < 0.001$]. WT, wild type; SEM, standard error of the mean; TNF, tumour necrosis factor.

3.5. Cannabinoid receptor expression in Crohn's disease patients

Classification of CD patient tissue as currently inflamed or non-inflamed was based on expert diagnosis [clinical, radiological, endoscopic, and histopathological methods]. Patients with CD were grouped according to standard Vienna classifications, designating L1 as CD with exclusive ileal involvement, L2 as CD with solely colonic involvement, and L3 as CD with ileocolonic involvement.

We also assessed mucosal CXCL8 expression to confirm the inflammatory status of the included samples. In the ileum, controls displayed a mean level of 546.3 copies/10 ng total RNA [417.4 SEM], non-inflamed ileal CD patients showed 380.2 ± 120.8, and inflamed samples 7873 ± 3036 copies of CXCL8/10ng total RNA. In the colon, controls displayed an average of 115.1 CXCL8 copies/10 ng total RNA, non-inflamed colonic CD patients showed on average 339.9 ± 138, and inflamed samples 4599 ± 2097 copies of CXCL8/10 ng total RNA. Patient characteristics are listed in Table 2. The age distribution of the studied patients was typical for this disease, as was the balanced gender ratio. For our ileal studies, we compared ilea of CD patients, who all belonged to Vienna classification L1 or L3 [$n = 58$], with those of healthy controls [$n = 24$], and for colonic tissue, we combined L2 and L3 subgroups as colonic CD [$n = 49$], which we again compared with healthy controls [$n = 25$].

Gene expression on the mRNA level for both *CNR1* and *CNR2* was relatively low in all studied groups. However, expression was consistently detected in all analysed samples. Interestingly, we found

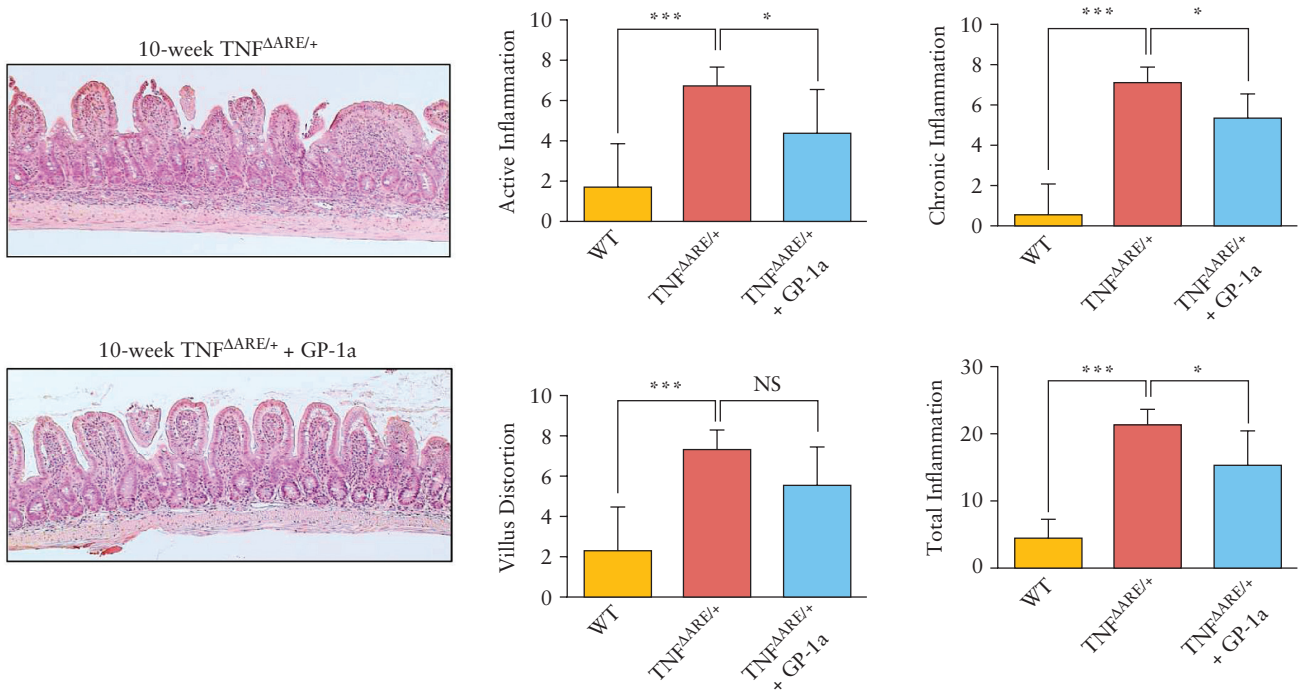

Figure 3. Significant attenuation of ileitis by GP-1a treatment. Representative H&E ilea 10x micrographs of vehicle and GP-1a treated 10-week-old TNF$^{\Delta ARE/+}$ mice. Histological assessment of inflammatory indices from ilea of 10-week-old TNF$^{\Delta ARE/+}$ mice treated with GP-1a or vehicle for 2 weeks [mean ± SEM of $n = 7$ per group; one-tailed t test $^*p < 0.05$]. H&E, haematoxylin and eosin; SEM, standard error of the mean; TNF, tumour necrosis factor.

a profound decrease in both *CNR1* [$p < 0.001$] [Figure 5A] and *CNR2* [$p < 0.05$] [Figure 5C] in the ilea of non-inflamed CD patients. In active disease, the reduction was less pronounced but still present. In the colon, however, both receptors were markedly higher in non-inflamed colonic CD patients [$p < 0.01$ for *CNR1* and $p < 0.05$ for *CNR2*] [Figure 5B, D]. Since the pattern of expression of the two receptors showed notable similarity, we checked for correlation. In both tissues, we found a moderate but statistically highly significant degree of correlation [$p < 0.001$] [Figure 5E, F].

In summary, Figure 5 reveals an overall marked reduction in cannabinoid receptor expression in the ilea of CD patients with chronic inflammation compared with healthy controls, though expression of both CB_1R and CB_2R was increased in active disease compared with non-inflamed patients nonetheless. In the colon, we saw an increase in cannabinoid receptor expression with disease, to a significant extent in non-inflamed CD patients.

4. Discussion

Our experiments demonstrate that the ECS is induced by active intestinal inflammation at receptor mRNA and protein expression levels, and to a much lesser extent at the ligand level. Similar results of receptor expression have been reported in chemical models of intestinal inflammation.[17,19,21,37–39] These findings are also supported from a broader perspective, where CB_2R expression is reported to increase in early inflammatory events in both the central nervous system [CNS] and peripheral tissue.[40,41] Thus, the effects of the ECS in models of gut inflammation appear to be primarily due to increased receptor expression and not ligand expression. In addition, these effects may be specific to the TNF$^{\Delta ARE/+}$ model of CD, as there was no induction of CB_1R and CB_2R seen in an acute infectious *Salmonella* colitis model. The compilation of past evidence and our current results validate the ECS as a promising therapeutic target in inflammatory conditions such as IBD.

Intriguingly, we found that both cannabinoid receptors were preferentially upregulated on Tregs when compared with CD4$^+$CD25^{Neg} Teff cells. In light of the importance of Treg in maintaining normal gut homeostasis, we proceeded to examine the role of ECS activity in governing Treg function. We observed that treatment *in vitro* with CB_2R ligand GP-1a led to improved suppression of Teff proliferation in the setting of inflammation. Increased IL-10 in the supernatants of treated co-cultures suggests that the effect of GP-1a is a result of improved Treg suppressive function, although increased susceptibility of Teff to suppression is also a possibility. Our efforts in investigating the mechanism of decreased Teff proliferation with Gp-1a revealed that Gp-1a appears to have some direct effect on Teff; however, this effect was lost in the absence of Tregs, suggesting a possible effect on Teff resistance to suppression. In addition, GP-1a does not directly induce apoptosis [Supplementary Figure 3, available as Supplementary data at *ECCO-JCC* online].

We then demonstrated that CB_2R stimulation with GP-1a *in vivo* significantly attenuated active murine ileitis, as determined histologically and by assessment of inflammatory infiltrate. We found that histological features of ileitis, such as leukocyte infiltration, goblet cell hyperplasia, and muscularis hypertrophy, were markedly decreased with GP-1a treatment. Furthermore, we found a decrease in the frequency of infiltrating IFNγ-producing Th1 cells and an increase in the proportion of IL-10-producing Tregs, consistent with an overall increased secretion of IL-10 from the ileal tissue of GP-1a-treated mice. These *in vivo* results are supported by previous studies which showed GP-1a-induced inhibition of Th1 and Th17 differentiation *in vitro*, under both polarising and non-polarising conditions.[33] Overall, manipulation of the CB_2R has both pro-regulatory and anti-inflammatory effects, as evidenced by enhanced

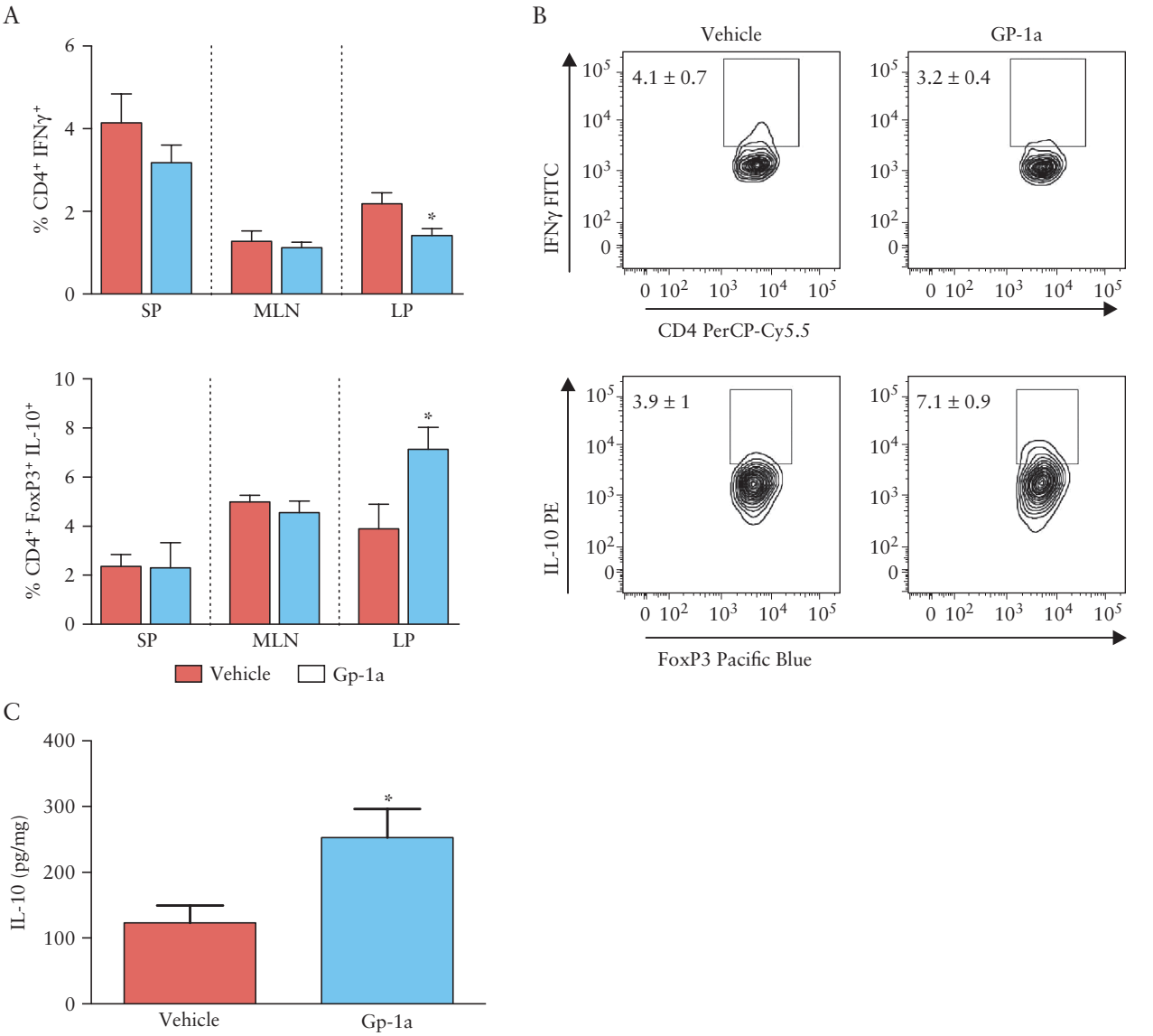

Figure 4. Reduction of inflammatory infiltrate with CB_2R stimulation. [A] Frequency of pro-inflammatory $CD4^+$ $IFN\gamma^+$ Th1 cells and $CD4^+FoxP3^+IL\text{-}10^+$ Tregs in indicated organs isolated from vehicle or GP-1a treated 10-week-old $TNF^{\Delta ARE/+}$ mice [SP, spleen; MLN, mesenteric lymph node; LP, lamina propria] [mean ± SEM, $n = 5$ per group; one-tailed t test $^*p < 0.001$]. [B] Representative dot plots display the $CD4^+$ $IFN\gamma^+$ and $CD4^+FoxP3^+IL\text{-}10^+$ expression from vehicle and GP-1a treated mice. [C] IL-10 quantities from 24-h terminal ilea explant cultures measured by ELISA [mean ± SEM, $n \geq 3$ per group; one-tailed t test $^*p < 0.05$, $^{**}p < 0.01$]. SEM, standard error of the mean; TNF, tumour necrosis factor.

Table 2. Patient characteristics.

Ileum	*N* [total]	Gender		Age in years [mean ± SD]	Localisation		Inflammation	
		F	M		L1	L3	Yes	No
Patients	57	52%	48%	51.4 ± 14.8	47%	53%	74%	25%
Controls	24	54%	46%	63.6 ± 18.6				
Colon	*N* [total]	Gender		Age in years [mean ± SEM]	Localisation		Inflammation	
		F	M		L2	L3	Yes	No
Patients	49	54%	46%	46.9 ± 11.16	41%	59%	67%	33%
Controls	25	60%	40%	59.8 ± 20.6				

F, female; M, male; SD, standard deviation; SEM, standard error of the mean.

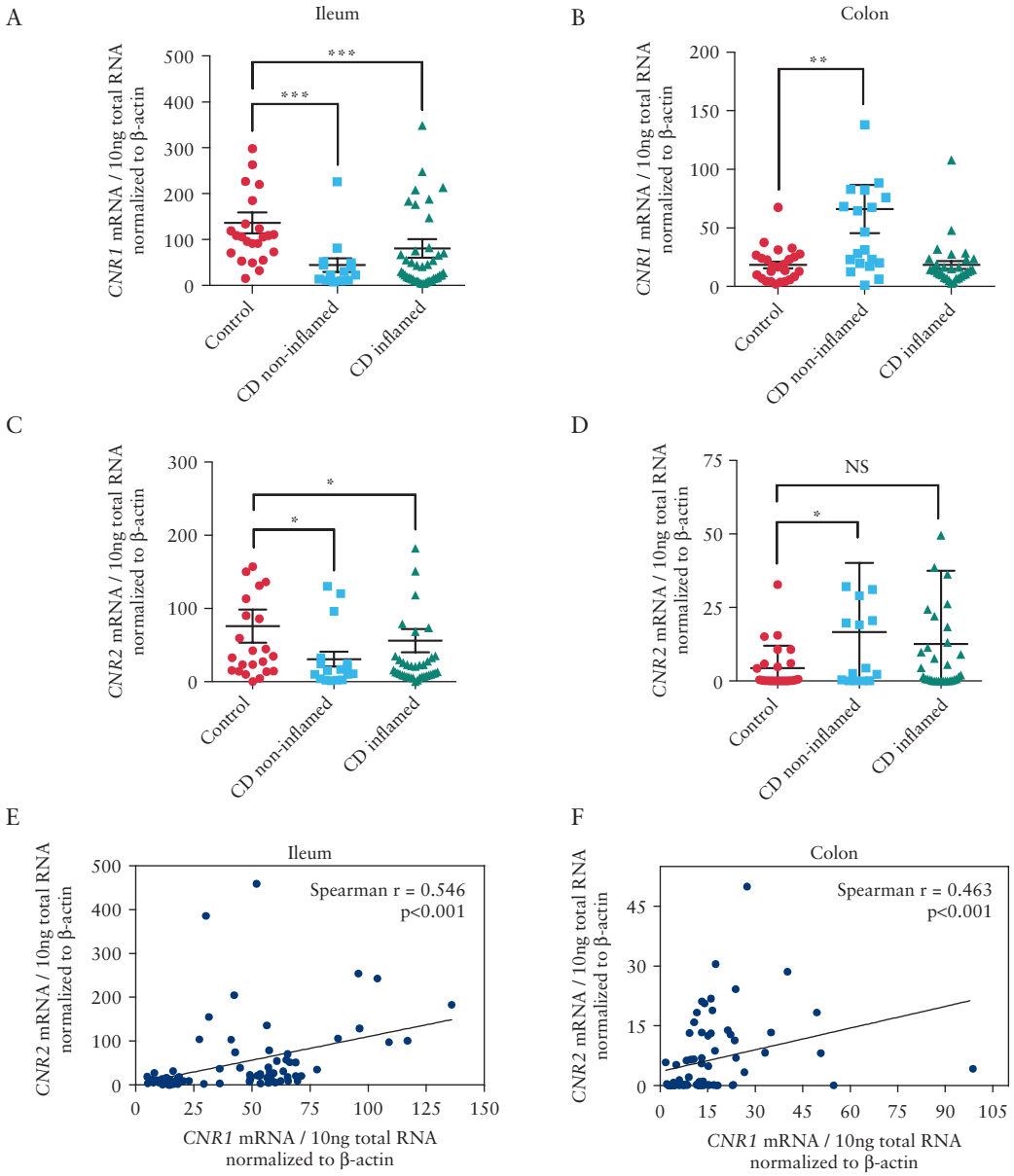

Figure 5. Cannabinoid receptor expression in Crohn's disease patients. [A, C] mRNA expression of *CNR1* and *CNR2*, respectively, in the ilea of inflamed and non-inflamed CD patients compared with healthy controls. [B, D] mRNA expression of *CNR1* and *CNR2*, respectively, in the colon of inflamed and non-inflamed CD patients compared with healthy controls [mean ± SEM; two-tailed Mann-Whitney test $*p < 0.05$, $**p < 0.01$, $***p < 0.001$]. [E] Correlation of *CNR1* and *CNR2* expression in the ilea of CD patients. [F] Correlation of *CNR1* and *CNR2* expression in the colon of CD patients [Spearman rank analysis $p < 0.001$]. CD, Crohn's disease; SEM, standard error of the mean.

suppression of Teff, increased IL-10 production, and decreased Th1 cell infiltration.

Interestingly, the previously accepted classification of GP-1a as a selective CB_2R agonist[42,43] has more recently been debated. Soethoudt *et al.* profiled various CB_2R ligands and confirmed that GP-1a is one of the most selective compounds for the CB_2R and is an active AEA reuptake inhibitor but, in contrast to previous reports, it acts *in vitro* as an inverse agonist at the CB_2R while it is inactive at the CB_1R.[44] However, cannabinoid signalling is complex, and evidence shows that cannabinoid receptor ligands may have varying pharmacological roles in native versus recombinant systems.[45] Marini *et al.* characterised several cannabinoid receptor ligands in tissue that natively express CB_2R, and found that certain cannabinoid receptor ligands can act as an agonist in human spleen but act *in vitro* as an inverse agonist in cells over-expressing the human CB_2R.[45] Although GP-1a was not specifically investigated, this work demonstrated that some cannabinoid ligands that are classified as CB_2R inverse agonists can have anti-nociceptive actions *in vivo*, and therefore demonstrate CB_2R agonist properties.[45] As a G-protein-coupled receptor, CB_2R-specific properties such as tethering of G-protein subunits, G-protein signalling regulators, receptor phosphorylation, and compartmentalisation of signalling elements within the membrane influence the efficacy of CB_2R ligands,[46] and these factors may explain the differences in the pharmacological profile of ligands between species and between the native and recombinant cell systems.[45] Our experiments did not define the exact mechanism of GP-1a-attenuated

inflammation, but we hypothesise that there is a critical importance in selecting a CB_2R inverse agonist in the therapeutic targeting of inflammation, as well as in the function of G-protein-coupled receptors such as the CB_2R.

It is well established that G-protein-coupled receptors such as the CB_2R are constitutively active, and will recruit known elements of cellular desensitisation leading to constitutive desensitisation.[47] Likewise, G-protein-coupled receptors are phosphorylated by G-protein-coupled receptor kinase and lead to desensitisation at a similar extent and rate in both the absence and presence of an agonist.[47] A study by Bouaboula *et al.* demonstrated that inverse agonism with SR144528 blocked the constitutive activation of CB_2R and did not allow desensitisation of the receptor to occur.[48] Therefore, G-protein-coupled receptors such as the CB_2R require treatment with an inverse agonist in order to reverse desensitisation, and thus allow activation of the receptor and its downstream pathways. We hypothesise that, in a similar mechanism, GP-1a acts upon the CB_2R with inverse agonism in order to reverse desensitisation and restore the innate anti-inflammatory signalling system of the ECS. One critical aspect of this assumption is that our experiments were performed using fetal bovine sera containing endocannabinoids,[49] and in contrast, our preliminary *in vitro* experiments using serum-free media have demonstrated that the effect of GP-1a treatment is abrogated in the absence of these endogenous cannabinoids [data not shown]. This incidental but significant observation reinforces our hypothesis that resensitisation of the CB_2R allows restoration of endogenous cannabinoid signalling and thereby resolution of inflammation.

Whereas we propose that GP-1a acts upon the CB_2R with inverse agonism in order to allow receptor resensitisation, promoting the endocannabinoid system to act at the CB_2R to ameliorate murine ileitis, our data reveal a novel and overarching role of Tregs in this process. It is well established that $CD4^+$ T cells are key mediators of intestinal inflammation, and more specifically $CD4^+FoxP3^+$ cells [Tregs] suppress inappropriate immune responses and maintain gut homeostasis. Our experiments parallel this concept, and we have demonstrated that CB_1R and CB_2R are upregulated on Tregs in the setting of active murine ileitis. More importantly, usng a Treg suppression assay to assess the suppressive function of Tregs, we found that treatment *in vitro* with CB_2R ligand GP-1a led to improved suppressive function of Tregs in the setting of inflammation. When we investigated the suppressive capacity of Tregs lacking the CB_2R, our data showed no significant difference in their suppressive function. It should be noted, however, that this assay was conducted in the absence of inflammation-induced upregulation of the CB_2R. This experimental finding is therefore unsurprising, as CB_2R shows low expression overall in the absence of inflammation, and thus the CB_2R would not be expected to play a role in Treg suppressive function in a WT system. Efforts to cross CB_2R-deficient mice with the $TNF^{\Delta ARE/+}$ ileitis model are currently under way and may shed additional light on these findings.

One very important aspect of this study is the striking similarities in the decreased expression of the CB_1R and CB_2R in both murine and human chronic inflammation as compared with healthy controls, and in the increased expression of CB_1R and CB_2R in active murine ileitis and active, non-remission CD patients. This similarity lends credence to the translational applicability of our study in the search for novel therapeutic targets for IBD. However, we recognise that we see a decrease in CB_2R expression in 20-week $TNF^{\Delta ARE/+}$ mice as well as in human IBD, despite previous evidence that there is continued increase in Tregs in intestinal inflammation in 20-week $TNF^{\Delta ARE/+}$ mice[32] and in IBD.[50,51] The difference in expression of Tregs and CB_2R is present because the CB_2R is not exclusively expressed on Tregs, but rather is found on many immune cell types.[2,11] In addition, whereas the differential expression of CB receptors in the colon has previously been classified,[27] the data collected in Figures 5A and C are novel in that, to the best of our knowledge, this is the first time the expression of the CB_2R has been classified in the ileum. Understanding the role of the ECS in human IBD is paramount; genetic studies have associated a common CB_2R functional variant, CB2-R63, with increased risk, worsening disease severity, and increased incidence of clinical relapse in paediatric IBD.[52] Our human data demonstrate a marked reduction in expression of the CB_2R in diseased patients as compared with healthy controls, and this finding further justifies the importance of targeting the CB_2R for resensitisation to promote restoration of gut homeostasis.

In summary, our study has demonstrated the significance of the endocannabinoid system in IBD. We propose that GP-1a acts upon the CB_2R with inverse agonism in order sto allow receptor resensitization, promoting the endocannabinoid system to act at the CB_2R on Tregs, thus attenuating inflammation in a mouse model of Crohn's disease. Given that CB_2R stimulation attenuates murine ileitis, the anti-inflammatory influences of the endocannabinoid system hold promise as a new potential treatment modality for IBD. Our work elucidates a possible mechanism in the anti-inflammatory pathways of the ECS, which will contribute to the interpretation of data and greatly benefit the ongoing clinical trials in cannabinoids and IBD.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

Author Contributions

KL: acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis. AAJ: acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis. RHH: administrative and technical support; critical revision of the manuscript for important intellectual content. PJ: blinded assessment of histological inflammatory indices; analysis and interpretation of data; critical revision of the manuscript for important intellectual content. DJK: acquisition of data; analysis and interpretation of data; critical revision of the manuscript for important intellectual content. EdeZ: acquisition of data; analysis and interpretation of data; critical revision of the manuscript for important intellectual content. SG: acquisition of data; analysis and interpretation of data; critical revision of the manuscript for important intellectual content. RM: acquisition of data; analysis and interpretation of data; critical revision of the manuscript for important intellectual content. JW: acquisition of data; analysis and interpretation of data; critical revision of the manuscript for important intellectual content; material support. MJO: acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis. JB: acquisition of data; analysis and interpretation of data; critical revision of the manuscript for important intellectual content; material support. CMAe: material support; critical revision of the manuscript for important intellectual content. CBC: study concept and design; analysis and interpretation of data; drafting of the manuscript; critical revision of the

manuscript for important intellectual content; statistical analysis; obtained funding; administrative, technical, and material support; study supervision. All authors gave final approval of the submitted version.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

References

1. Kappelman MD, Rifas-Shiman SL, Kleinman K, *et al*. The prevalence and geographic distribution of Crohn's disease and ulcerative colitis in the United States. *Clin Gastroenterol Hepatol* 2007;**5**:1424–9.
2. Leinwand KL, Gerich ME, Hoffenberg EJ, Collins CB. Manipulation of the endocannabinoid system in colitis: a comprehensive review. *Inflamm Bowel Dis* 2017;**23**:192–9.
3. Bacchetta R, Passerini L, Gambineri E, *et al*. Defective regulatory and effector T cell functions in patients with FOXP3 mutations. *J Clin Invest* 2006;**116**:1713–22.
4. Hanauer SB, Feagan BG, Lichtenstein GR, *et al*.; ACCENT I Study Group. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 2002;**359**:1541–9.
5. Carrier EJ, Auchampach JA, Hillard CJ. Inhibition of an equilibrative nucleoside transporter by cannabidiol: a mechanism of cannabinoid immunosuppression. *Proc Natl Acad Sci U S A* 2006;**103**:7895–900.
6. Gerich ME, Isfort RW, Brimhall B, Siegel CA. Medical marijuana for digestive disorders: high time to prescribe? *Am J Gastroenterol* 2015;**110**:208–14.
7. Hoffenberg EJ, Newman H, Collins C, Tarbell S, Leinwand K. Cannabis and pediatric inflammatory bowel disease: change blossoms a mile high. *J Pediatr Gastroenterol Nutr* 2017;**64**:265–71.
8. Naftali T, Bar-Lev Schleider L, Dotan I, Lansky EP, Sklerovsky Benjaminov F, Konikoff FM. Cannabis induces a clinical response in patients with Crohn's disease: a prospective placebo-controlled study. *Clin Gastroenterol Hepatol* 2013;**11**:1276–80.e1.
9. Storr M, Devlin S, Kaplan GG, Panaccione R, Andrews CN. Cannabis use provides symptom relief in patients with inflammatory bowel disease but is associated with worse disease prognosis in patients with Crohn's disease. *Inflamm Bowel Dis* 2014;**20**:472–80.
10. Massa F, Storr M, Lutz B. The endocannabinoid system in the physiology and pathophysiology of the gastrointestinal tract. *J Mol Med* 2005;**83**:944–54.
11. Galiègue S, Mary S, Marchand J, *et al*. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem* 1995;**232**:54–61.
12. Izzo AA, Camilleri M. Emerging role of cannabinoids in gastrointestinal and liver diseases: basic and clinical aspects. *Gut* 2008;**57**:1140–55.
13. Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB. Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* 1996;**384**:83–7.
14. Bisogno T, Sepe N, Melck D, Maurelli S, De Petrocellis L, Di Marzo V. Biosynthesis, release and degradation of the novel endogenous cannabimimetic metabolite 2-arachidonoylglycerol in mouse neuroblastoma cells. *Biochem J* 1997;**322**[Pt 2]:671–7.
15. Di Patrizio Nicholas V. Endocannabinoids in the Gut. *Cannabis Cannabinoid Res* 2016;**1**:67–77.
16. Li YY, Yuece B, Cao HM, *et al*. Inhibition of p38/Mk2 signaling pathway improves the anti-inflammatory effect of WIN55 on mouse experimental colitis. *Lab Invest* 2013;**93**:322–33.
17. Matos I, Bento AF, Marcon R, Claudino RF, Calixto JB. Preventive and therapeutic oral administration of the pentacyclic triterpene α,β-amyrin ameliorates dextran sulfate sodium-induced colitis in mice: the relevance of cannabinoid system. *Mol Immunol* 2013;**54**:482–92.
18. Cluny NL, Keenan CM, Duncan M, Fox A, Lutz B, Sharkey KA. Naphthalen-1-yl-[4-pentyloxynaphthalen-1-yl]methanone [SAB378], a peripherally restricted cannabinoid CB1/CB2 receptor agonist, inhibits gastrointestinal motility but has no effect on experimental colitis in mice. *J Pharmacol Exp Ther* 2010;**334**:973–80.
19. Kimball ES, Schneider CR, Wallace NH, Hornby PJ. Agonists of cannabinoid receptor 1 and 2 inhibit experimental colitis induced by oil of mustard and by dextran sulfate sodium. *Am J Physiol Gastrointest Liver Physiol* 2006;**291**:G364–71.
20. Fichna J, Bawa M, Thakur GA, *et al*. Cannabinoids alleviate experimentally induced intestinal inflammation by acting at central and peripheral receptors. *PLoS One* 2014;**9**:e109115.
21. Massa F, Marsicano G, Hermann H, *et al*. The endogenous cannabinoid system protects against colonic inflammation. *J Clin Invest* 2004;**113**:1202–9.
22. Singh UP, Singh NP, Singh B, Price RL, Nagarkatti M, Nagarkatti PS. Cannabinoid receptor-2 [CB2] agonist ameliorates colitis in IL-10[-/-] mice by attenuating the activation of T cells and promoting their apoptosis. *Toxicol Appl Pharmacol* 2012;**258**:256–67.
23. Engel MA, Kellermann CA, Burnat G, Hahn EG, Rau T, Konturek PC. Mice lacking cannabinoid CB1-, CB2-receptors or both receptors show increased susceptibility to trinitrobenzene sulfonic acid [TNBS]-induced colitis. *J Physiol Pharmacol* 2010;**61**:89–97.
24. Storr MA, Keenan CM, Zhang H, Patel KD, Makriyannis A, Sharkey KA. Activation of the cannabinoid 2 receptor [CB2] protects against experimental colitis. *Inflamm Bowel Dis* 2009;**15**:1678–85.
25. El Bakali J, Muccioli GG, Body-Malapel M, *et al*. Conformational restriction leading to a selective CB2 cannabinoid receptor agonist orally active against colitis. *ACS Med Chem Lett* 2015;**6**:198–203.
26. Tourteau A, Andrzejak V, Body-Malapel M, *et al*. 3-Carboxamido-5-aryl-isoxazoles as new CB2 agonists for the treatment of colitis. *Bioorg Med Chem* 2013;**21**:5383–94.
27. Wright K, Rooney N, Feeney M, *et al*. Differential expression of cannabinoid receptors in the human colon: cannabinoids promote epithelial wound healing. *Gastroenterology* 2005;**129**:437–53.
28. Ihenetu K, Molleman A, Parsons ME, Whelan CJ. Inhibition of interleukin-8 release in the human colonic epithelial cell line HT-29 by cannabinoids. *Eur J Pharmacol* 2003;**458**:207–15.
29. Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F, Kollias G. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity* 1999;**10**:387–98.
30. Graham ES, Angel CE, Schwarcz LE, Dunbar PR, Glass M. Detailed characterisation of CB2 receptor protein expression in peripheral blood immune cells from healthy human volunteers using flow cytometry. *Int J Immunopathol Pharmacol* 2010;**23**:25–34.
31. Barthel M, Hapfelmeier S, Quintanilla-Martínez L, *et al*. Pretreatment of mice with streptomycin provides a *Salmonella enterica* serovar Typhimurium colitis model that allows analysis of both pathogen and host. *Infect Immun* 2003;**71**:2839–58.
32. Collins CB, Aherne CM, Kominsky D, *et al*. Retinoic acid attenuates ileitis by restoring the balance between T-helper 17 and T regulatory cells. *Gastroenterology* 2011;**141**:1821–31.
33. Kong W, Li H, Tuma RF, Ganea D. Selective CB2 receptor activation ameliorates EAE by reducing Th17 differentiation and immune cell accumulation in the CNS. *Cell Immunol* 2014;**287**:1–17.
34. Burns RC, Rivera-Nieves J, Moskaluk CA, Matsumoto S, Cominelli F, Ley K. Antibody blockade of ICAM-1 and VCAM-1 ameliorates inflammation in the SAMP-1/Yit adoptive transfer model of Crohn's disease in mice. *Gastroenterology* 2001;**121**:1428–36.
35. Collins CB, Strassheim D, Aherne CM, Yeckes AR, Jedlicka P, de Zoeten EF. Targeted inhibition of heat shock protein 90 suppresses tumor necrosis factor-α and ameliorates murine intestinal inflammation. *Inflamm Bowel Dis* 2014;**20**:685–94.
36. Collins CB, Aherne CM, McNamee EN, *et al*. Flt3 ligand expands CD103$^+$ dendritic cells and FoxP3$^+$ T regulatory cells, and attenuates Crohn's-like murine ileitis. *Gut* 2012;**61**:1154–62.
37. Izzo AA, Fezza F, Capasso R, *et al*. Cannabinoid CB1-receptor mediated regulation of gastrointestinal motility in mice in a model of intestinal inflammation. *Br J Pharmacol* 2001;**134**:563–70.
38. Romano B, Borrelli F, Fasolino I, *et al*. The cannabinoid TRPA1 agonist cannabichromene inhibits nitric oxide production in macrophages and ameliorates murine colitis. *Br J Pharmacol* 2013;**169**:213–29.

39. Borrelli F, Romano B, Petrosino S, *et al*. Palmitoylethanolamide, a naturally occurring lipid, is an orally effective intestinal anti-inflammatory agent. *Br J Pharmacol* 2015;**172**:142–58.
40. Guindon J, Hohmann AG. Cannabinoid CB2 receptors: a therapeutic target for the treatment of inflammatory and neuropathic pain. *Br J Pharmacol* 2008;**153**:319–34.
41. Cabral GA, Griffin-Thomas L. Emerging role of the cannabinoid receptor CB2 in immune regulation: therapeutic prospects for neuroinflammation. *Expert Rev Mol Med* 2009;**11**:e3.
42. Mussinu JM, Ruiu S, Mulè AC, *et al*. Tricyclic pyrazoles. Part 1: synthesis and biological evaluation of novel 1,4-dihydroindeno[1,2-c]pyrazol-based ligands for CB1and CB2 cannabinoid receptors. *Bioorg Med Chem* 2003;**11**:251–63.
43. Murineddu G, Ruiu S, Mussinu JM, *et al*. Tricyclic pyrazoles. Part 2: synthesis and biological evaluation of novel 4,5-dihydro-1H-benzo[g]indazole-based ligands for cannabinoid receptors. *Bioorg Med Chem* 2005;**13**:3309–20.
44. 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.
45. Marini P, Cascio MG, 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.
46. Sutor S, Heilmann J, Seifert R. Impact of fusion to Gα[i2] and coexpression with RGS proteins on pharmacological properties of human cannabinoid receptors CB_1R and CB_2R. *J Pharm Pharmacol* 2011;**63**:1043–55.
47. Pei G, Samama P, Lohse M, Wang M, Codina J, Lefkowitz RJ. A constitutively active mutant beta 2-adrenergic receptor is constitutively desensitized and phosphorylated. *Proc Natl Acad Sci U S A* 1994;**91**:2699–702.
48. 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.
49. Marazzi J, Kleyer J, Paredes JM, Gertsch J. Endocannabinoid content in fetal bovine sera - unexpected effects on mononuclear cells and osteoclastogenesis. *J Immunol Methods* 2011;**373**:219–28.
50. Eastaff-Leung N, Mabarrack N, Barbour A, Cummins A, Barry S. Foxp3+ regulatory T cells, Th17 effector cells, and cytokine environment in inflammatory bowel disease. *J Clin Immunol* 2010;**30**:80–9.
51. Ban H, Andoh A, Shioya M, Nishida A, Tsujikawa T, Fujiyama Y. Increased number of FoxP3+CD4+ regulatory T cells in inflammatory bowel disease. *Mol Med Rep* 2008;**1**:647–50.
52. Strisciuglio C, Bellini G, Miele E, *et al*. Cannabinoid receptor 2 functional variant contributes to the risk for pediatric inflammatory bowel disease. *J Clin Gastroenterol* 2016, Novmber 21. doi: 10.1097/MCG.0000000000000755. [Epub ahead of print.]