Cannabidiol and Palmitoylethanolamide are anti-inflammatory in the acutely inflamed human colon

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No conflicts of interest disclosed.

No funding sources disclosed.

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Conception and design of the study; Couch DG, Lund J, O'Sullivan SE conceived and designed the study

DG Couch, C Tasker and E Theophilidou collected tissue samples, conducted experimentation and collected data.

DG Couch, C Tasker, J Lund and S O'Sullivan analysed data.

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Drafting or revision of the manuscript; all

Approval of the final version of the manuscript: all

Clinical Perspectives

i) Inflammatory bowel disease remains a major clinical problem, as current therapies available to treat these collection of disease carry significant side effects. Cannabis-based medicines have been used informally to treat colonic inflammation for many years, however investigation into the therapeutic utility of these drugs has only recently been undertaken. We therefore sought to quantify the anti-inflammatory properties of two cannabinoid drugs, cannabidiol (CBD) and palmitoylethanolamide (PEA) in the inflamed human colon.

ii) We found that CBD and PEA agents demonstrate significant anti-inflammatory actions in experimentally and clinically inflamed colon reducing the production of multiple key pro-inflammatory cytokines, and down regulating pro-inflammatory intracellular pathways.

iii) As these two drugs are well tolerated in humans with few side effects, their clinical use in treating inflammatory bowel disease is expected. Appropriately seized clinical trials should now assess their clinical efficacy.

ABSTRACT

Objective: We sought to quantify the anti-inflammatory effects of two cannabinoid drugs: cannabidiol (CBD) and palmitoylethanolamide (PEA), in cultured cell lines and compared this effect with experimentally inflamed explant human colonic tissue. These effects were explored in acutely and chronically inflamed colon, using inflammatory bowel disease and appendicitis explants.

Design: Caco-2 cells and human colonic explants collected from elective bowel cancer, inflammatory bowel disease (IBD) or acute appendicitis resections, and were treated with the following drug treatments: vehicle, an inflammatory protocol of IFNγ and TNFα (10 ng/ml), inflammation and PEA (10μM), inflammation and CBD (10μM), & PEA or CBD alone. PEA, CBD or vehicle were added simultaneously with IFNγ. Nine intracellular signalling phosphoproteins were determined by multiplex. Inflammatory cytokine secretion was determined using ELISA. Receptor mechanisms were investigated using antagonists for CB_1, CB_2, PPARα, PPARγ, TRPV1 and GPR55.

Results: IFNγ and TNFα treatment increased phosphoprotein and cytokine levels in Caco-2 cultures and colonic explants. Phosphoprotein levels were significantly reduced by PEA or CBD in Caco-2 cultures and colonic explants. CBD and PEA prevented increases in cytokine production in explant colon, but not in Caco-2 cells. CBD effects were blocked by the CB_2antagonist AM630 and TRPV1 antagonist SB366791. PEA effects were blocked by the PPARα antagonist GW6471. PEA and CBD were anti-inflammatory in IBD and appendicitis explants.

Conclusion: PEA and CBD are anti-inflammatory in the human colon. This effect is not seen in cultured epithelial cells. Appropriately sized clinical trials should assess their efficacy.

INTRODUCTION

In health, the gut absorbs nutrients from the lumenal environment into the sterile submucosa without absorbing noxious material, such as bacteria and lipopolysaccharide. The barrier between lumen and submucosa is formed by epithelial cells, which allow selective absorption of particles into the enteric circulation via paracellular and transcellular pathways, whilst preventing bacterial translocation (1–5). Inflammation causes this barrier to become compromised (6). Inflammation is caused by conditions such as diverticulitis, infective colitis and appendicitis, and also inflammatory bowel disease (IBD), such as ulcerative colitis and Crohn's disease (7). When inflammation occurs, lumenal bacteria and lipopolysaccharide are able to translocate into the submucosal space and beyond, resulting in secondary complications such as endotoxaemia, sepsis and death (there are 30,000 deaths from sepsis per year in the UK alone) (8,9). Currently, there are no clinical treatments to counter permeability changes seen in systemic inflammation and sepsis. Development of an agent to prevent bacterial and lipopolysaccharide translocation across the gut wall with the intention of reducing or preventing the triggering of sepsis is therefore of high clinical importance.

We have previously demonstrated, in Caco-2 culture models, that inflammation stimulated by tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ) increases epithelial permeability, shown by falls in trans-epithelial electrical resistance (TEER) (10). The non-pyschotrophic constituent of cannabis sativa, cannabidiol (CBD), and the endogenous fatty acid amide palmitoylethanolamide (PEA) prevented these changes when used prophylactically, and restored membrane resistance when given therapeutically, acting via the CB_1 and PPARα receptors respectively (10,11). Others have also observed a protective effect of PEA and CBD on the gut barrier during inflammation (12,13).

What is not clear is the mechanism by which PEA and CBD modify permeability during inflammation. Recently we showed that PEA may act through modification of the actin

cytoskeleton by inducing FAK production and down regulating aquaporins 3 and 4, but it was not clear if this was secondary to an anti-inflammatory effect of PEA or due to direct action of PEA (14). PEA and CBD exert an anti-inflammatory effect on enteric glial cells, which can modify the immune response *in vivo* (15–18), therefore any *in vivo* permeability effects may indeed be secondary to an anti-inflammatory effect rather than direct action on cellular structures which contribute to regulation of permeability.

Previous studies have shown both PEA and CBD to blunt increases in permeability in experimentally inflamed explant colonic tissue (17–19). As the extent of the anti-inflammatory effect of PEA and CBD *in vitro* and *in vivo* has not yet been quantified, we examined the effect of PEA and CBD on the local inflammatory response in cultured Caco-2 cells and explant human colonic tissue. We hypothesised that CBD and PEA cause changes in intestinal permeability through suppression of the local immune response. We therefore examined the effect of CBD and PEA on the intracellular signalling phosphoproteins in response to inflammation, and on down-stream production of inflammatory cytokines. This allowed us to compare the effects of PEA and CBD on the epithelium alone, to their effect on whole tissue. In order to assess the effect of CBD and PEA on clinically inflamed colonic tissue we examined the effects of these drugs on explant colon from patients with established inflammation caused by acute appendicitis and inflammatory bowel disease.

MATERIALS AND METHODS

All experiments and procedures received prior approval of the University of Nottingham Ethics Committee and local NHS Research Ethics Committee.

Caco-2 Cell Culture

Caco-2 cells were purchased from European Collection of Cell Culture (Wiltshire, UK; passages 21-42). Cells were cultured in Eagle's minimum essential medium supplemented with L-glutamine, 10% foetal bovine serum (FBS) 1% penicillin/streptomycin and 1% non-essential amino acids mixture (all Sigma-Aldrich). Cells were kept at 37°C in 5% CO_2 and 95% humidity. Cells were seeded at $4x10^5$ cells per well in polystyrene 12 well plates (Corning Incorporated, USA), and grown for 2 weeks until fully differentiated. Cells were used for experimentation at day 14-16. The medium was changed on alternate days.

Randomly assigned wells (n=8) were treated with the following drug treatments: vehicle, an inflammatory protocol of IFNγ (10ng.ml^{-1}, Sigma-Aldridge) for 8 hours, followed by TNFα (10ng.ml^{-1}, Sigma-Aldridge) for 16 hours, inflammation and PEA (10μM, PEA was added simultaneously with IFNγ at the start of the 24 hour inflammatory period), inflammation and CBD (10μM, CBD was added simultaneously with IFNγ at the start of the 24 hour inflammatory period), PEA (10μM) alone, or CBD (10μM) alone. PEA and CBD were purchased from Tocris Bioscience (Bristol, UK). At the end of the 24 hour experimental period, media was collected and stored at -80 °C until analysis. Cells were washed twice with ice-cold phosphate-buffered saline (PBS), and treated with radioimmunoprecipitation assay (RIPA) buffer supplemented with phosphatase and protease inhibitors (Sigma-Aldridge) at 4°C for one hour on a rocking platform to cause cell lysis. Cell lysates were then collected and stored at -80 °C until analysis.

Human Colon Experimentation

Experiments on *ex vivo* human tissue were performed by obtaining colonic samples from patients having elective bowel resections for bowel cancer (n=13), planned resections for quiescent inflammatory bowel disease (n=6) or emergency appendectomies for acute appendicitis (n=6) at Derby Teaching Hospitals NHS Trust, Derbyshire, UK. Samples of normal colon at least 10cm proximal to right sided bowel tumours (in the case of bowel cancer resections), sections of inflamed colon (in inflammatory bowel disease resection), or sections of inflamed appendix (in the case of emergency appendicectomy) were obtained immediately after resection in the operating theatre. Sections of tissue 2cm x 2cm were removed from the resected specimen and transferred on ice to the laboratory within ten minutes, in pre-chilled Eagle's minimum essential medium supplemented with 1% FBS 1% penicillin/streptomycin and 1% non-essential amino acids mixture (Sigma-Aldrich). The remaining operative specimen was sent to pathology for routine analysis. Once in the laboratory samples were pinned on Stylgard plates. Mucosa with submucosa was dissected free from the underlying muscularis layer. Mucosal samples were then further dissected into 2mm x 2mm sections and placed in individual wells of 24-well polystyrene plates (Corning Incorporated, USA), each containing 1ml of media. Samples of colonic tissue were then treated with TNFα and IFNγ, in the absence or presence of PEA and CBD as described above within Caco-2 experiments. Experiments were carried out in triplicate, with final values derived from the mean result of three measurements. Samples were incubated for 24 hours at 37°C in 5% CO2 and 95% humidity. At the end of the 24 hour experimental period media was collected and stored at -80 °C until analysis. Explant tissue was washed with ice cold PBS and stored frozen at -80 °C until homogenisation and analysis. Prior to analysis colonic samples were thawed on ice and cryohomogenised using the method described by von Ziegler (20). Collected homogenates were then dissolved in 215µl of RIPA buffer, incubated on an oscillating thermomixer for 30

minutes at 60 °C, then centrifuged at 10,000G for 15 minutes. Supernatant was collected, vortexed for 20 seconds and then analysed.

Intracellular signalling pathways

To determine the effect of CBD and PEA on signalling proteins, Luminex xMAP technology (Austin, TX, USA) was used to detect changes in phosphorylated cAMP response element-binding protein (CREB; pS133), ERK (pT185/pY187), NF-kB (pS536), JNK (pT183/pY185), p38 (pT180/pY182), p70 S6K (pT412), STAT3 (pS727), STAT5A/B (pY694/699), and Akt (pS473) (Milliplex 48-680MAG; Merck Millipore, Darmstadt, Germany) from undiluted cell lysates or homogenised colonic lysates according to the manufacturer's instructions using a Magpix plate reader (MAGPX 11326002, Luminex, Texas, US). Signalling phosphoprotein concentrations were corrected for total protein content using the bicinchoninic acid (BCA) assay determination of cell lysate protein concentration against a standard curve.

Cytokine production

We measured specific proteins induced as a consequence of TNFα stimulation. To quantify the effects of PEA and CBD on the inflammatory response we measured media concentrations from cell or colonic cultures of seven cytokines at the end of the 24 hour experimental period using ELISA. Cytokines measured were interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), matrix metallopeptidase-3 (MMP-3); DUOSET ELISA kits R&D Systems Minneapolis, US. Interleukin-17 (IL-17), granulocyte-macrophage-colony stimulating factor (GM-CSF), interleukin-6 (IL-6); ready-set-go ELISA kits, Affymetrix eBioscience, San Diego, CA. Cytokine concentrations were normalised for protein content as previously using BCA assay determination of cell lysate protein concentration against a standard curve. Experimental conditions were averaged from triplicate readings, as above.

Target sites of action of PEA & CBD

To identify target sites of action of PEA and CBD we co-applied the following antagonists to healthy colonic explant tissue with PEA or CBD under inflammatory conditions: AM251 100nM (CB_1 antagonist, Tocris Bioscience, Bristol, UK), AM630 100nM (CB_2 antagonist, Tocris Bioscience, Bristol, UK), GW6471 500nm (PPARα antagonist, Tocris Bioscience, Bristol, UK), GW9662 100nM (PPARγ antagonist, Tocris Bioscience, Bristol, UK), SB366791 500nM ($TRPV_1$ antagonist, Tocris Bioscience, Bristol, UK), and CID16020046 500nM (GPR_{55} antagonist, Tocris Bioscience, Bristol, UK). Following co-incubation for 24 hours, media concentrations of IL-8 and MCP-1 were measured using ELISA. IL-6 concentrations were measured using ready-set-go ELISA kits (Affymetrix eBioscience, San Diego, CA).

Statistical Analysis

Data are presented as mean (or mean percentage change from baseline where indicated) ± SEM. Caco-2 cytokine and signalling protein concentration results were compared using one-way ANOVA. Human tissue cytokine secretion and phosphorylated protein levels are expressed as percentage change compared to vehicle per patient or [pg/ml]/[mg/ml] where indicated, and analysed using repeated measures ANOVA. For human tissue, each value represents the mean of three independent values. A p-value of <0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 7.01 for Windows (GraphPad Software, San Diego, USA).

RESULTS

Intracellular response to inflammation

Stimulation of Caco-2 cells with IFNγ and TNFα significantly increased the levels of CREB, JNK, NF-KB, p38, ERK1/2, Akt, p70S6k, STAT3, and STAT5 (figure 1). CBD, in the presence of inflammation compared to inflammation alone, prevented the production of CREB ($p=0.002$), p38 ($p=0.08$), NF-κB ($p=0.009$), JNK ($p=0.02$), p70S6K ($p=0.07$), Akt ($p=0.08$), STAT3 ($p=0.06$) and STAT5 ($p=0.03$). PEA in the presence of inflammation, compared to inflammation alone, prevented increases in CREB ($p=0.01$), JNK ($p=0.01$) and STAT5 ($p=0.08$). CBD or PEA treatment alone did not affect the level of any of the phosphorylated proteins compared to control tissue.

Stimulation of explant colonic tissue with IFNγ and TNFα resulted in an increase in all of the measured signalling phosphoproteins (figure 2). PEA in the presence of inflammation, compared to inflammation alone, prevented increases in CREB ($p=0.055$), NF-κB ($p=0.007$), Akt ($p=0.002$), p70S6K ($p=0.005$), STAT3 ($p=0.01$) and STAT5 ($p=0.007$). CBD in the presence of inflammation, compared to inflammation alone, prevented increases in STAT3, though did not reach significance ($p=0.09$). PEA or CBD alone did not affect the production of any phosphoprotein compared to vehicle.

Effects of PEA and CBD on cytokine production in response to inflammation

Stimulation of Caco-2 cells with IFNγ and TNFα caused an increase in the secretion of IL-8 and IL-6 although did not increase in the secretion of IL-17 (figure 3). In human colonic tissue IFNγ and TNFα caused and increase in the production of IL-8, IL-6 and IL-17 (figure 3). Both PEA and CBD did not affect the production of these cytokines in Caco-2 cultures, but did prevent their increased in human colonic explants.

Stimulation of Caco-2 cells with the inflammatory protocol did not increase the production of GM-CSF, but both PEA alone and CBD alone increased GM-CSF production in the absence of inflammation (figure 4). In human tissue IFNγ and TNFα stimulation increased the production of GM-CSF which was prevented by CBD but not PEA (figure 4).

IFNγ and TNFα markedly increased MCP-1 production by Caco-2 cells and human colonic tissue (figure 4). CBD and PEA had no effect on Caco-2 production of MCP-1, but did significantly reduce production in human tissue.

Stimulation of Caco-2 cells with IFNγ and TNFα increased the production of ICAM-1 (figure 5, A). CBD and PEA did not prevent this increase in production, but PEA alone caused a marked increase in ICAM-1 production compared to vehicle. In human colonic tissue, an increase in ICAM-1 caused by IFNγ and TNFα was prevented by the administration of PEA (figure 5, C). Treatment of inflammation-stimulated colonic tissue with CBD also decreased production, though did not reach significance. PEA alone, CBD in the presence of inflammation and CBD alone had no effect on ICAM-1 production in colonic tissue compared to vehicle.

Stimulation of Caco-2 cells with IFNγ and TNFα had no effect on the production of the enzyme MMP-3 (figure 5, B). In human tissue the inflammatory protocol did not significantly increase the production of MMP-3, however both PEA and CBD did significantly reduce its production in the presence of IFNγ and TNFα (figure 5, D).

Antagonist studies

When investigating for a receptor mechanism for PEA and CBD we found again that stimulation of colonic explant tissue with the inflammatory protocol caused a significant rise in IL-8, IL-6 and MCP-1 production compared to baseline, whilst treatment of inflamed colon with simultaneous PEA or CBD prevented these increases in cytokine production

(figure 6, A to F). The anti-inflammatory effects of PEA on IL-8, IL-6 and MCP-1 production were prevented by the addition of the PPARα antagonist GW6471. The anti-inflammatory effects of CBD on IL-8, IL-6 and MCP-1 production were prevented by the CB_2 antagonist AM630. The anti-inflammatory effects of CBD on IL-8 and MCP-1 production were also inhibited by the addition of the TRPV1 antagonist SB366791. SB366791 had no effect on the anti-inflammatory effect of CBD in the presence of inflammation. GW6471, AM630 and SB366791 had no effect on cytokine production in the presence of IFNγ and TNFα alone (data not shown).

Effects of PEA and CBD on cytokine production in response to inflammation in IBD colonic explants

Because we found PEA and CBD had an anti-inflammatory effect on experimentally inflamed tissue we collected samples of inflamed colon from patients with IBD and acute appendicitis.

Explants of six patients with quiescent IBD were collected. IBD colonic explants demonstrated significantly higher levels of all measured cytokines, in comparison to vehicle-treated sections of healthy tissue. Treatment of IBD explants with inflammatory cytokines further increased the secretion of IL-8, IL-6, IL-17, GM-CSF, MCP-1 and ICAM-1 (figure 7), but not MMP-3. Treatment of cytokine-stimulated tissue with PEA and CBD supressed this increased secretion of inflammatory mediators (figure 7). Treatment of unstimulated IBD tissue with either PEA or CBD alone produced a trend of decreased inflammatory cytokine secretion, however this only reached significance in the case of IL-6 in CBD-treated tissue.

Effects of PEA and CBD on cytokine production in response to inflammation in appendicitis explants

Explants of six patients with acute appendicitis were collected. Appendicitis explants secreted significantly higher baseline levels of all measured cytokines compared to healthy colonic tissue. Treatment of appendicitis explants with cytokines caused an increase in the secretion of inflammatory cytokines, though this only reached significance in GM-CSF, MCP-1 and ICAM-1 (figure 8). Treatment of cytokine-stimulated appendicitis tissue with PEA decreased the secretion of IL-8, IL-6 and MCP-1 compared to appendicitis tissue stimulated with cytokines alone. Treatment of cytokine-stimulated appendicitis tissue with CBD decreased the secretion of MCP-1 compared to appendicitis tissue stimulated with cytokines alone. Compared to vehicle-treated appendicitis tissue, PEA alone significantly supressed the secretion of IL-8, IL-6, MCP-1 and ICAM-1. Compared to vehicle-treated appendicitis tissue, CBD alone significantly supressed the secretion of GM-CSF, MCP-1 and ICAM-1.

DISCUSSION

The aim of this study was to examine the anti-inflammatory properties of PEA and CBD in Caco-2 cell lines and explant human colonic tissue. We demonstrate that under inflammatory conditions, PEA and CBD supress the phosphorylation of several intracellular proteins in Caco-2 cells, however this does not supress the secretion of pro-inflammatory cytokines. Conversely, in explant human colonic tissue stimulated with IFNγ and TNFα, PEA and CBD both supressed the phosphorylation of intracellular proteins which were up-regulated by inflammation, and also prevented the increased secretion of pro-inflammatory cytokines. Additionally we have shown that PEA and CBD have an anti-inflammatory effect in explant IBD and appendicitis tissue.

Caco-2 cell cultures

We previously demonstrated that CBD and PEA prevent changes in the permeability of Caco-2 monolayers under inflammatory conditions. Prior to this study we had hypothesized that this effect on permeability was secondary to a local anti-inflammatory action, as it had been seen in work from other centres that CBD and PEA supress the inflammatory response in the inflamed colon of mice and humans (17,18,21–23). For this hypothesis to be correct we would expect PEA and CBD to have an anti-inflammatory action in Caco-2 monolayers. IFNγ and TNFα, caused a significant increase in all measured intracellular signalling proteins, in line with similar experiments using TNFα stimulation on Caco-2 cell lines (24). We found that increases were suppressed by PEA and CBD. It is possible that both of these effects could be due to increased anandamide (AEA) tone. PEA has been shown to increase the action of local AEA either by preventing hydrolysis of AEA through substrate competition or FAAH inhibition (25), or by enhancing AEA potency at villanoid receptors (26). Secondly CBD has been shown to prevent AEA uptake and catabolism (27). AEA itself has been shown to down regulate NF-KB and exert anti-inflammatory properties

through IL-10, and may therefore been at least partly responsible for these effects on signal phosphorylation (28). We then quantified the effect of PEA and CBD on the extracellular inflammatory response, measuring seven pro-inflammatory cytokines representing five aspects of immune activation. As an indicator of pro-inflammatory cytokine production we assayed IL-8, IL-6, and IL-1 (29–31). As a marker of leucocyte recruitment and activation we assayed MCP-1 and GM-CSF (32,33). As a marker of extracellular matrix remodelling we measured MMP-3 concentrations (34), and as a marker of cell-to-cell adhesion we measured concentrations of ICAM-1. We found that stimulation of Caco-2 cultures with IFNγ and TNFα caused an increase in all measured cytokines, except MMP-3. Surprisingly, neither PEA nor CBD prevented the increased secretion of these cytokines. We may suggest therefore that the effects of PEA and CBD on signal phosphorylation and permeability are distinct from their effects on inflammation, and not due to suppression of a local extracellular immune response.

Ex vivo Colonic Tissue

In explant human tissue we found that the local inflammatory response caused by stimulation with IFNγ and TNFα was inhibited by treatment with PEA and CBD. This finding, in contrast to results in cultured Caco-2 cells, could be explained by the presence of submucosal immunocytes such as dendritic cells and macrophage activity. Explant colonic samples contain lymphoid aggregates and innate immune cells, as opposed to monolayers of Caco-2 cells (35). It has previously been described that within the gut the CB_2 receptor, AEA and 2-AG are found in highest abundance within these submucosal immune and also nervous tissues such as enteric glial cells, and that this may be the primary site of endocannabinoid activation during inflammatory episodes (12,36). Within our colonic explant tissue PEA and CBD may be acting on such macrophage and dendritic cell colonies, thus supressing the local inflammatory response (35,37). This would explain why cytokine secretion was decreased in

the presence of IFNγ and TNFα, but no decrease was found in tissue treated with PEA or CBD alone, compared to vehicle treated tissue. Further work therefore could examine the effect of CBD and PEA on Caco-2 cells in a co-culture model exploring possible receptor mechanisms in these specialised tissues.

Receptor sites of PEA and CBD are not clear. Capasso et al. (2001) first demonstrated that the action of PEA on gut hypermotility in mice caused by caustic inflammation (croton oil), was prevented by blockade of the CB_2, but not CB_1 receptor. (38) The same group also reported that in a different model of hypermotility (oil of mustard), PEA had an anti-inflammatory effect that was inhibited by blockade of the CB_1 and PPARα receptors, but not by blockade of CB_2. (39) Esposito et al. demonstrated that anti-inflammatory effects of PEA in both dextran-sulphate sodium treated mice and colonic biopsies from patients with ulcerative colitis were inhibited by blockade of the PPARα receptor (18). As mentioned above, it has been suggested that PEA may exert its effects through increasing the local concentration or potency of a second agent, such as anandamide, and therefore antagonising receptor targets of PEA or the second agent would inhibit their biologic effects (40). In view of this we examined the anti-inflammatory effect of PEA in the presence of six receptor antagonists at which cannabinoid agonists have been postulated to act; CB_1, CB_2, PPARα, PPARγ, $TRPV_1$ and GPR55. We found that across three measured cytokines the effect of PEA was prevented by antagonism of PPARα. This is the second human colonic study to demonstrate PEA action at PPARα, suggesting that this PEAs primary site of action in colonic mucosa (14).

Similarly, multiple studies have suggested various sites of action of CBD in the mammalian gut. Two murine studies demonstrating the beneficial effect of CBD on inflammation-induced effects on gut transport showed that CB_1 rather than CB_2 was the target receptor of CBD (41,42). However two independent studies examining the effect of CBD on the

immune response in human and murine colonic tissue suggested that PPARα was the dominant receptor target of CBD, with a possible role for CB_1, but again suggesting that CB_2 was not a receptor target (17,43). However our data show a role for CB_2 and TRPV1. It is possible than these data differ from pre-existing literature because of site of colonic sampling and mode of inflammation used for simulating colitis. It has been demonstrated that the distribution of endocannabinoid receptors and differs across the colon, and that these receptors are activated by inflammation. Within our study we collected colonic samples from right sided colonic resections, whereas mouse-colonic studies previously cited have used whole colonic homogenates, and the pre-existing human colon studies have used left-sided (sigmoid) colonic biopsies. It is possible therefore the sites of CBD activation differ throughout the gut, hence in the proximal gastrointestinal tract PPARα is the pre-dominant site of activation, whereas distally CB_2, TRPV1 and then CB_1 become more important. This may be supported by evidence showing that in established inflammation of the gut and other organ systems CBD has epithelial protective effects medicated by CB_2 and TRPV1 (44,45). One study in particular found that during a murine model of colitis-induced sepsis, CBD prevented peripheral organ oxidation, further demonstrating the effect of CBD on epithelial barriers during sepsis (46). Further work could compare the receptor targets of CBD across the gut, using a similar model of inflammation.

We then compared the anti-inflammatory effect of PEA and CBD in experimentally inflamed normal colonic tissue, explant IBD tissue, and explant appendicitis. We found that although appendicitis tissue had higher baseline levels of cytokine production, similar increases in cytokine secretion was caused by IFNγ and TNFα treatment in all three tissue types. These differences in baseline cytokine production are likely to represent the acute inflammatory nature of acute appendicitis, versus the chronic low-levels of inflammation in long-standing IBD, compared with healthy tissue. CBD and PEA were strongly anti-inflammatory in acute

appendicitis tissue, but not paralleled in IBD tissue. We also did not find that PEA and CBD were effective in preventing increased cytokine production in cytokine-treated IBD and appendicitis tissue. This may suggest that the receptor profile in acute inflammation differs from that in long-standing chronic inflammation, and therefore any benefit seen in IBD may not be secondary to an anti-inflammatory effect, and may be secondary to effects on mucosal permeability. This is supported by a clinical study from Naftali et al (2013) who showed an improvement in disease activity scores and quality of life in IBD patients receiving *cannabis sativa* cigarettes, though did not find any change in biochemical markers of inflammation using serum CRP levels as a marker (13). Furthermore a study from Di Sabatino (2011) conducted in a similar explant manner found differences in the expression of endocannabinoid ligands between control and inflamed IBD explants, the inflammatory response of which was down regulated with the addition of methanandamide (47). A recent study from the same centre used low dose CBD in inflammatory bowel disease, though did not find any benefit in improving quality of life scores (48). Before drawing conclusions however regarding the efficacy of CBD in IBD it is important to highlight that this study may have been hampered by small group sizes and the ultra-low doses of CBD employed.

This study provides further evidence that PEA and CBD may play a role in the treatment of acute inflammation of the gut, including Crohn's and ulcerative colitis. Our data are limited by using explant tissue, rather than any clinical *in vivo* data, and the generic nature of the explant models used. Furthermore we did not carry out tissue viability assays on explant tissue to ensure the mucosa had not been damaged by dissection, or had become necrotic. Further clinical work examining the use of these two drugs in the treating inflammatory disease of the gut should now be considered. Additionally we have hypothesized that the effects of CBD and PEA on signal phosphorylation and the inflammatory response may be due to increased efficacy or presence of AEA. Further work should now be conducted within

both Caco-2 models and healthy colon, appendicitis and inflammatory bowel disease explants to quantify the effect of CBD and PEA on endocannabinoid production. Lastly within this study we did not examine for any additive effects of CBD together with PEA on the immune response, which may have been positive.

In summary we have demonstrated that CBD and PEA prevent cytokine production in human colonic explant tissue via PPARα, CB_2 and TRPV1, but not in cultured epithelial cells. These effects extend into chronic inflammatory processes such as IBD, but also acute inflammatory conditions such as appendicitis. Further clinical work must examine the effects of these two drugs at higher doses, and clarify their clinical role.

REFERENCE LIST

1. van der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol. 2009 Jan;71:241–60.

2. Kim YS, Ho SB. Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr Gastroenterol Rep. 2010 Oct;12(5):319–30.

3. Coskun M. Intestinal Epithelium in Inflammatory Bowel Disease. Front Med. 2014;1(August):1–5.

4. Peterson LW, Artis D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nat Rev Immunol. 2014 Mar;14(3):141–53.

5. Lee SH. Intestinal Permeability Regulation by Tight Junction : Implication on Inflammatory Bowel Diseases. 2015;9100(1):11–8.

6. de Punder K, Pruimboom L. Stress induces endotoxemia and low-grade inflammation by increasing barrier permeability. Front Immunol. 2015;6:223.

7. Okamoto R, Watanabe M. Role of epithelial cells in the pathogenesis and treatment of inflammatory bowel disease. J Gastroenterol. 2016 Jan 3;51(1):11–21.

8. Harrison DA, Welch CA, Eddleston JM. The epidemiology of severe sepsis in England, Wales and Northern Ireland, 1996 to 2004: secondary analysis of a high quality clinical database, the ICNARC Case Mix Programme Database. Crit Care. 2006;10(2):R42.

9. Vaishnavi C. Translocation of gut flora and its role in sepsis. Indian J Med Microbiol. 2013 Jan 1;31(4):334–42.

10. Alhamoruni A, Wright KL, Larvin M, O'Sullivan SE. Cannabinoids mediate opposing

effects on inflammation-induced intestinal permeability. Br J Pharmacol. 2012;165:2598–610.

11. Alhamoruni A, Lee C, Wright KL, Larvin M, O'Sullivan SE. Pharmacological effects of cannabinoids on the Caco-2 cell culture model of intestinal permeability. J Pharmacol Exp Ther. 2010;335(1):92–102.

12. Borrelli F, Romano B, Petrosino S, Pagano E, Capasso R, Coppola D, et al. Palmitoylethanolamide, a naturally occurring lipid, is an orally effective intestinal anti-inflammatory agent. Br J Pharmacol. 2015;172:142–58.

13. 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 Oct;11(10):1276–1280.e1.

14. Karwad MA, Macpherson T, Wang B, Theophilidou E, Sarmad S, Barrett DA, et al. Oleoylethanolamine and palmitoylethanolamine modulate intestinal permeability in vitro via TRPV1 and PPARα. FASEB J. 2016 Sep 13;fj.201500132.

15. Parrella E, Porrini V, Iorio R, Benarese M, Lanzillotta A, Mota M, et al. PEA and luteolin synergistically reduce mast cell-mediated toxicity and elicit neuroprotection in cell-based models of brain ischemia. Brain Res. 2016 Jul 13;

16. Skaper SD, Facci L, Giusti P. Glia and mast cells as targets for palmitoylethanolamide, an anti-inflammatory and neuroprotective lipid mediator. Mol Neurobiol. 2013 Oct;48(2):340–52.

17. de Filippis D, Esposito G, Cirillo C, Cipriano M, de Winter BY, Scuderi C, et al. Cannabidiol reduces intestinal inflammation through the control of neuroimmune axis.

PLoS One. 2011;6(12):1–8.

18. Esposito G, Capoccia E, Turco F, Palumbo I, Lu J, Steardo A, et al. Palmitoylethanolamide improves colon inflammation through an enteric glia/toll like receptor 4-dependent PPAR-α activation. Gut. 2014;1300–12.

19. Harvey BS, Sia TC, Wattchow D a., Smid SD. Interleukin 17A evoked mucosal damage is attenuated by cannabidiol and anandamide in a human colonic explant model. Cytokine. 2014;65(2):236–44.

20. von Ziegler LM, Saab BJ, Mansuy IM. A simple and fast method for tissue cryohomogenization enabling multifarious molecular extraction. J Neurosci Methods. 2013 Jun 15;216(2):137–41.

21. Borrelli F, Izzo AA. Role of acylethanolamides in the gastrointestinal tract with special reference to food intake and energy balance. Best Pract Res Clin Endocrinol Metab . 2009 Feb;23(1):33–49.

22. Jamontt JM, Molleman A, Pertwee RG, Parsons ME. The effects of delta 9-tetrahydrocannabinol and cannabidiol alone and in combination on damage, inflammation and in vitro motility disturbances in rat colitis. Br J Pharmacol. 2010;160(November 2009):712–23.

23. Cuzzocrea S, Campolo M, Impellizzeri D, Paterniti I, Bruschetta G, Cordaro M, et al. Palmitoylethanolamide ameliorates development of colitis caused by injection of dinitrobenzene sulfonic acid in mice (840.3). FASEB J. 2014 Apr 1;28(1_Supplement):840.3-.

24. Erlejman AG, Jaggers G, Fraga CG, Oteiza PI. TNFα-induced NF-κB activation and cell oxidant production are modulated by hexameric procyanidins in Caco-2 cells.

Arch Biochem Biophys. 2008;476(2):186–95.

25. Di Marzo V, Melck D, Orlando P, Bisogno T, Zagoory O, Bifulco M, et al. Palmitoylethanolamide inhibits the expression of fatty acid amide hydrolase and enhances the anti-proliferative effect of anandamide in human breast cancer cells. Biochem J. 2001 Aug 15 ;358(Pt 1):249–55.

26. De Petrocellis L, Davis JB, Di Marzo V. Palmitoylethanolamide enhances anandamide stimulation of human vanilloid VR1 receptors. FEBS Lett. 2001 Oct 12;506(3):253–6.

27. Bisogno T, Hanus L, De Petrocellis L, Tchilibon S, Ponde DE, Brandi I, et al. Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. Br J Pharmacol. 2001;134:845–52.

28. Correa F, Hernangómez M, Mestre L, Loría F, Spagnolo A, Docagne F, et al. Anandamide enhances IL-10 production in activated microglia by targeting CB_2 receptors: Roles of ERK1/2, JNK, and NF-κB. Glia [Internet]. 2010 Jan 15 [cited 2017 Jul 31];58(2):135–47. Available from: http://doi.wiley.com/10.1002/glia.20907

29. Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N, Matsushima K. Essential involvement of interleukin-8 (IL-8) in acute inflammation. J Leukoc Biol. 1994 Nov;56(5):559–64.

30. Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF, et al. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. J Clin Invest 1998 Jan 15;101(2):311–20.

31. Zhang Z, Zheng M, Bindas J, Schwarzenberger P, Kolls JK. Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. Inflamm Bowel Dis. 2006

May;12(5):382–8.

32. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res. 2009 Jun29(6):313–26.

33. Wicks IP, Roberts AW. Targeting GM-CSF in inflammatory diseases. Nat Rev Rheumatol. 2015 Dec 3;12(1):37–48.

34. Nagase H, Woessner JF. Matrix metalloproteinases. J Biol Chem. 1999 Jul 30;274(31):21491–4.

35. Jarry A, Crémet L, Caroff N, Bou-Hanna C, Mussini JM, Reynaud A, et al. Subversion of human intestinal mucosa innate immunity by a Crohn's disease-associated E. coli. Mucosal Immunol. 2015 May];8(3):572–81.

36. Nagarkatti P, Pandey R, Rieder SA, Hegde VL, Nagarkatti M. Cannabinoids as novel anti-inflammatory drugs. Future Med Chem. 2009 Oct;1(7):1333–49.

37. Kozela E, Pietr M, Juknat A, Rimmerman N, Levy R, Vogel Z. Cannabinoids Delta(9)-tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-kappaB and interferon-beta/STAT proinflammatory pathways in BV-2 microglial cells. J Biol Chem 2010 Jan 15;285(3):1616–26.

38. Izzo a a, Capasso R, Pinto L, Di Carlo G, Mascolo N, Capasso F. Effect of vanilloid drugs on gastrointestinal transit in mice. Br J Pharmacol. 2001;132:1411–6.

39. Capasso R, Orlando P, Pagano E, Aveta T, Buono L, Borrelli F, et al. Palmitoylethanolamide normalizes intestinal motility in a model of post-inflammatory accelerated transit: involvement of CB_1 receptors and TRPV1 channels. Br J Pharmacol. 2014 Sep;171(17):4026–37.

40. Ho W-S V, Barrett DA, Randall MD. "Entourage" effects of N-palmitoylethanolamide

and N-oleoylethanolamide on vasorelaxation to anandamide occur through TRPV1 receptors. Br J Pharmacol. 2008 Nov;155(6):837–46.

41. Capasso R, Borrelli F, Aviello G, Romano B, Scalisi C, Capasso F, et al. Cannabidiol, extracted from Cannabis sativa, selectively inhibits inflammatory hypermotility in mice. Br J Pharmacol. 2008;154:1001–8.

42. de Filippis D, Iuvone T, D'amico A, Esposito G, Steardo L, Herman AG, et al. Effect of cannabidiol on sepsis-induced motility disturbances in mice: involvement of CB receptors and fatty acid amide hydrolase. Neurogastroenterol Motil. 2008 Aug;20(8):919–27.

43. Krohn RM, Parsons SA, Fichna J, Patel KD, Yates RM, Sharkey KA, et al. Abnormal cannabidiol attenuates experimental colitis in mice, promotes wound healing and inhibits neutrophil recruitment. J Inflamm (Lond). 2016 Dec 14;13(1):21.

44. De Petrocellis L, Orlando P, Moriello a. S, Aviello G, Stott C, Izzo a. a., et al. Cannabinoid actions at TRPV channels: Effects on TRPV3 and TRPV4 and their potential relevance to gastrointestinal inflammation. Acta Physiol. 2012;204:255–66.

45. Pazos MR, Mohammed N, Lafuente H, Santos M, Martínez-Pinilla E, Moreno E, et al. Mechanisms of cannabidiol neuroprotection in hypoxic–ischemic newborn pigs: Role of 5HT1A and CB2 receptors. Neuropharmacology. 2013 Aug;71:282–91.

46. Cassol-Jr OJ, Comim CM, Silva BR, Hermani F V., Constantino LS, Felisberto F, et al. Treatment with cannabidiol reverses oxidative stress parameters, cognitive impairment and mortality in rats submitted to sepsis by cecal ligation and puncture. Brain Res. 2010 Aug 12;1348:128–38.

47. Di Sabatino A, Battista N, Biancheri P, Rapino C, Rovedatti L, Astarita G, et al. The

endogenous cannabinoid system in the gut of patients with inflammatory bowel disease. Mucosal Immunol. 2011;4(5):574–83.

48. Naftali T, Mechulam R, Marii A, Gabay G, Stein A, Bronshtain M, et al. Low-Dose Cannabidiol Is Safe but Not Effective in the Treatment for Crohn's Disease, a Randomized Controlled Trial. Dig Dis Sci. 2017 Mar 27

FIGURE LEGENDS

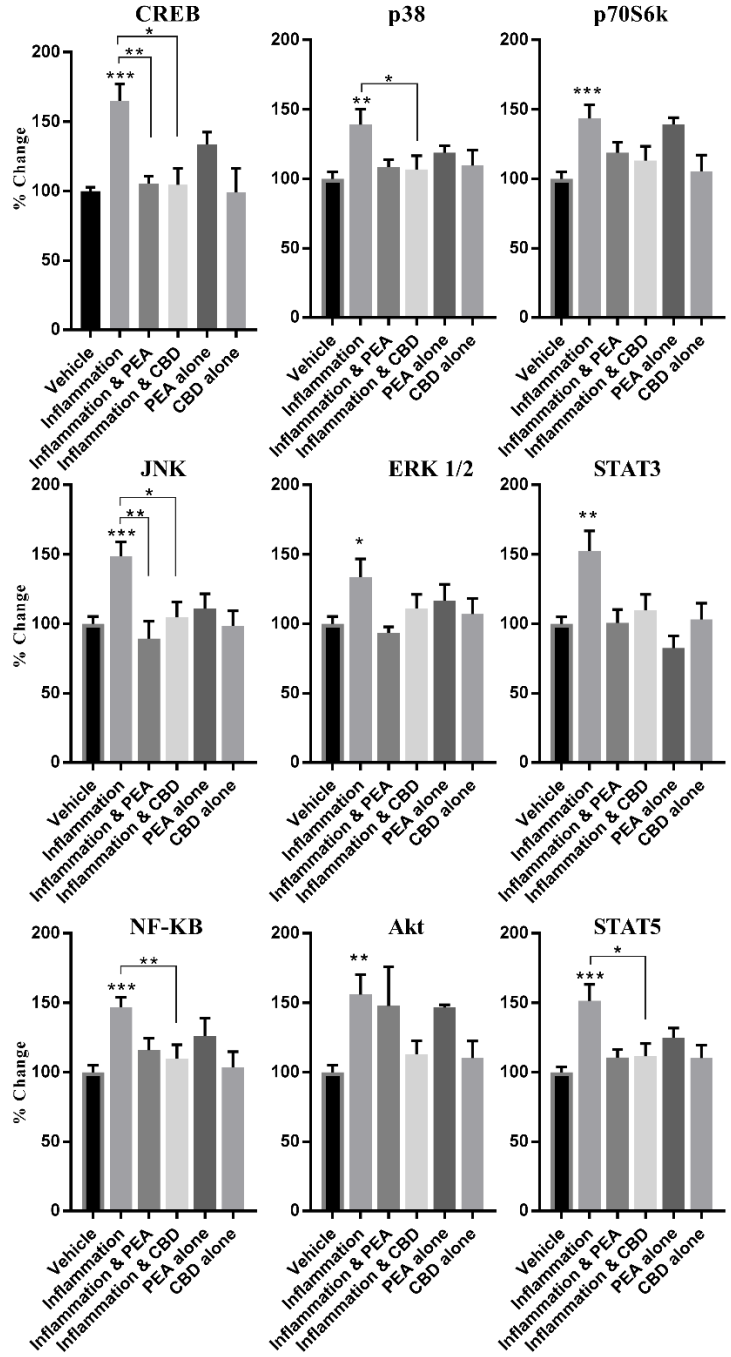

Figure 1

The effects of PEA and CBD on the intracellular levels of phosphorylated nuclear signalling proteins in response to an inflammatory protocol in cultured Caco-2 monolayer, measured by multiplex. Data is presented as percentage change from vehicle per plate +/- SEM, n=8 per condition. Data was analysed by one-way ANOVA comparing against the vehicle control or inflammation (*<0.05, **<0.01 and ***<0.001).

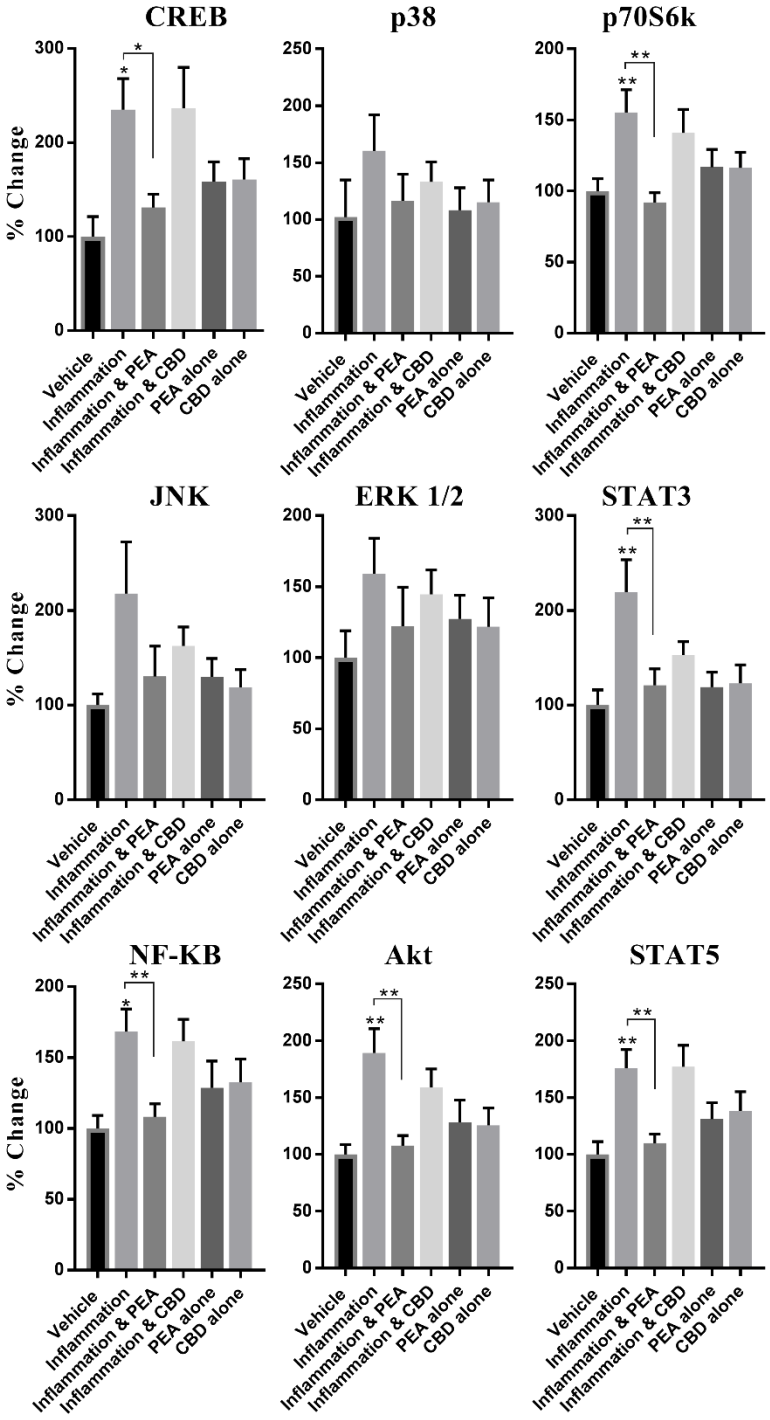

Figure 2

The effects of PEA and CBD on the intracellular levels of phosphorylated signalling proteins in response to 24hr exposure to TNFα and IFNγ in cultured human colonic explants, measured by multiplex. Data is presented as percentage change from vehicle +/- SEM, n=13 per condition. Data was analysed by repeated measures ANOVA comparing against the vehicle control (*), *<0.05, **<0.01.

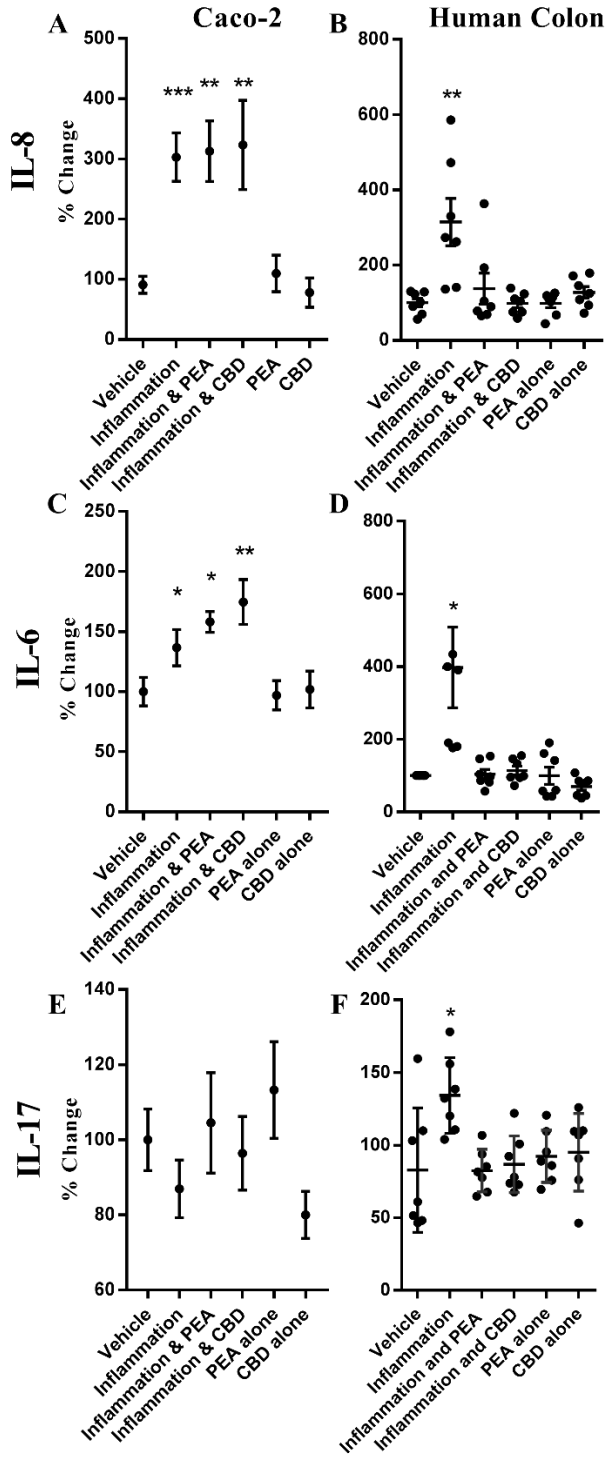

Figure 3

The effects of PEA and CBD on the secreted cytokine response to an inflammatory protocol in cultured Caco-2 monolayers (A, C and E, column 1, n=8, percentage change from vehicle, one way ANOVA) and human colonic tissue (B, D and F, column 2, n=7, percentage change compared to vehicle, repeated measures ANOVA), measured by ELISA. Error bars represent +/- SEM per condition. Asterixes (*) represent significant difference from vehicle, *<0.05, **<0.01, ***<0.001.

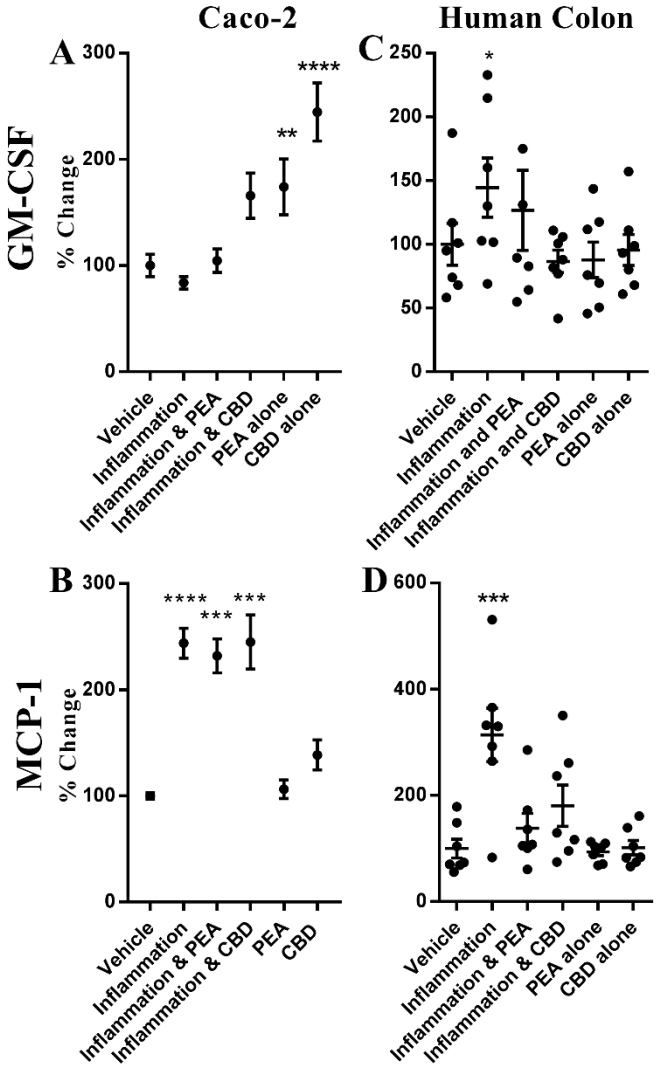

Figure 4

The effects of PEA and CBD on granulocyte macrophage colony stimulation (A, C) and monocyte chemotaxis (B, D) response to an inflammatory protocol in cultured Caco-2 monolayers (column 1, n=8, percent change from vehicle per plate compared by one-way ANOVA) and human colonic tissue (column 2, percentage change from vehicle, N=7, compared by repeated measures ANOVA), measured by ELISA. Error bars represent +/- SEM per condition. Asterixes (*) represent significant difference from vehicle, *<0.05, **<0.01, ***<0.001, ****<0.0001.

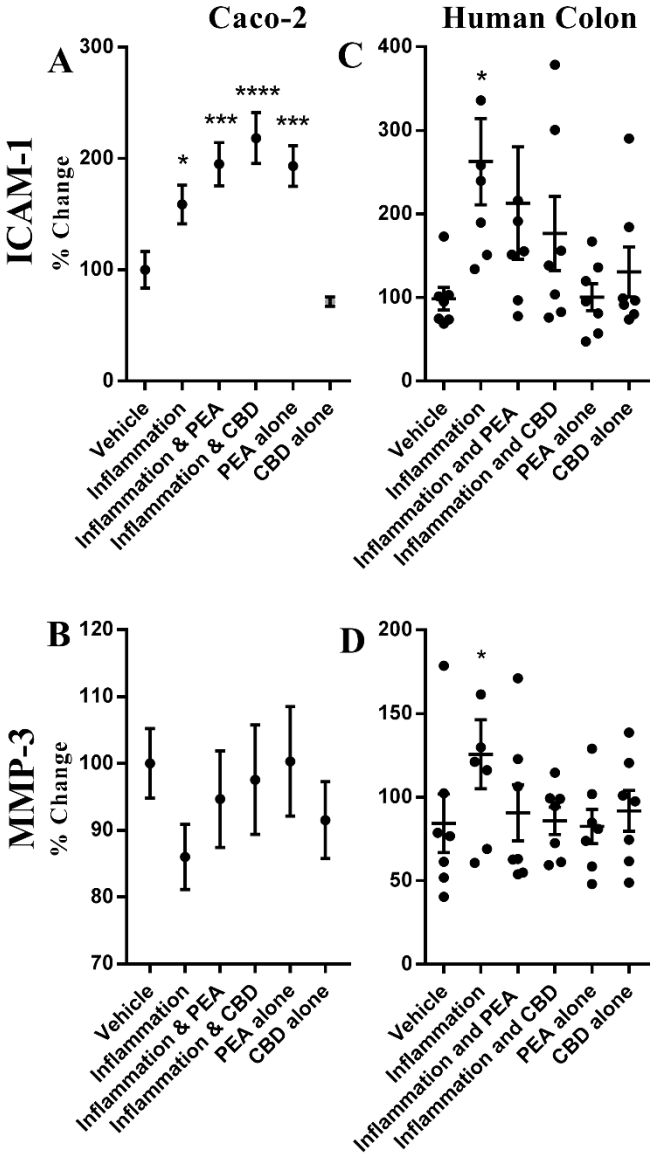

Figure 5

The effects of PEA and CBD on intracellular adhesion (A,D) and matrix metalloproteinase secretion (B,D) response to an inflammatory protocol in cultured Caco-2 monolayers (column 1, n=7, percent change compared by one-way ANOVA) and human colonic tissue (column 2, percentage change compared by repeated measures ANOVA), measured by ELISA. Error bars represent +/- SEM per condition. Asterixes (*) represent significant difference from vehicle, *<0.05, **<0.01, ***<0.001, ****<0.0001.

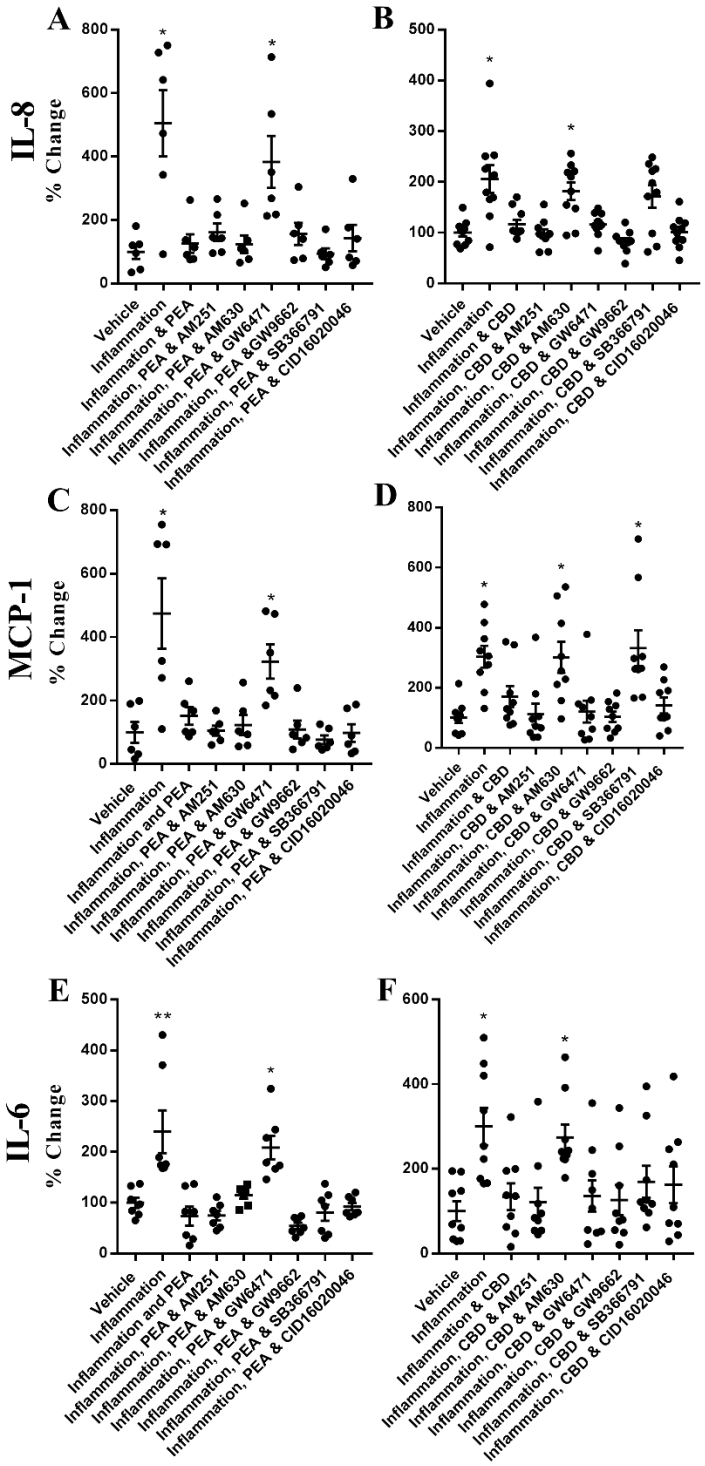

Figure 6

The effects of PEA (A,C,E) and CBD (B,D,F) on the secretion of IL-8, MCP-1 and IL-6 in response to an inflammatory protocol in explant human colonic tissue in the presence of receptor antagonists, measured by ELISA (compared by repeated measures ANOVA, n=7). Data presented as mean +/- SEM per condition. Asterixes (*) represent significant difference from vehicle, *<0.05, **<0.01.

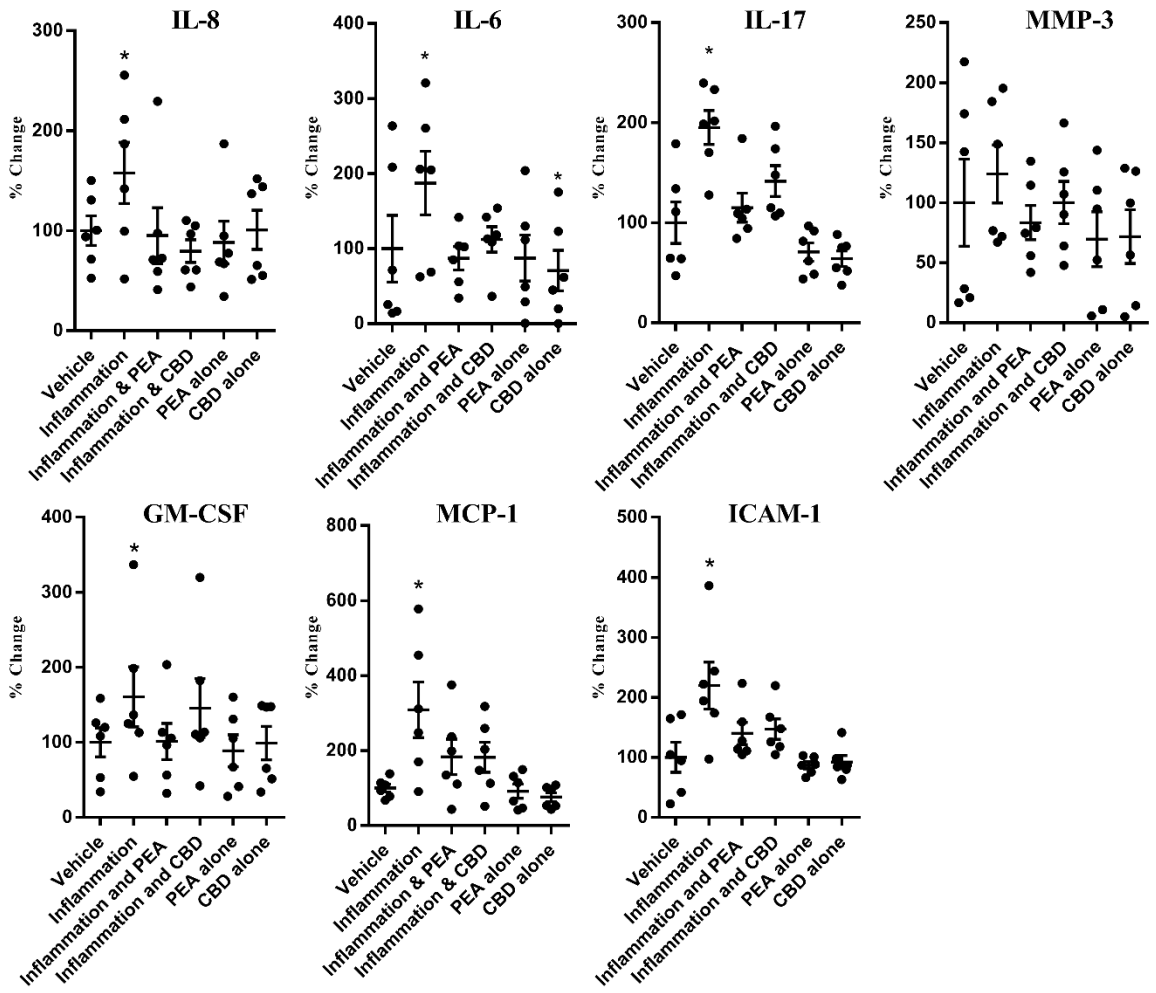

Figure 7

The effects of PEA and CBD on the secreted inflammatory response in IBD colonic explants treated with an inflammatory protocol (n=6, percent change from vehicle, compared by repeated measures ANOVA), measured by ELISA. Error bars represent +/- SEM per condition. Asterixes (*) represent significant difference from vehicle, *<0.05, **<0.01, ***<0.001, ****<0.0001.

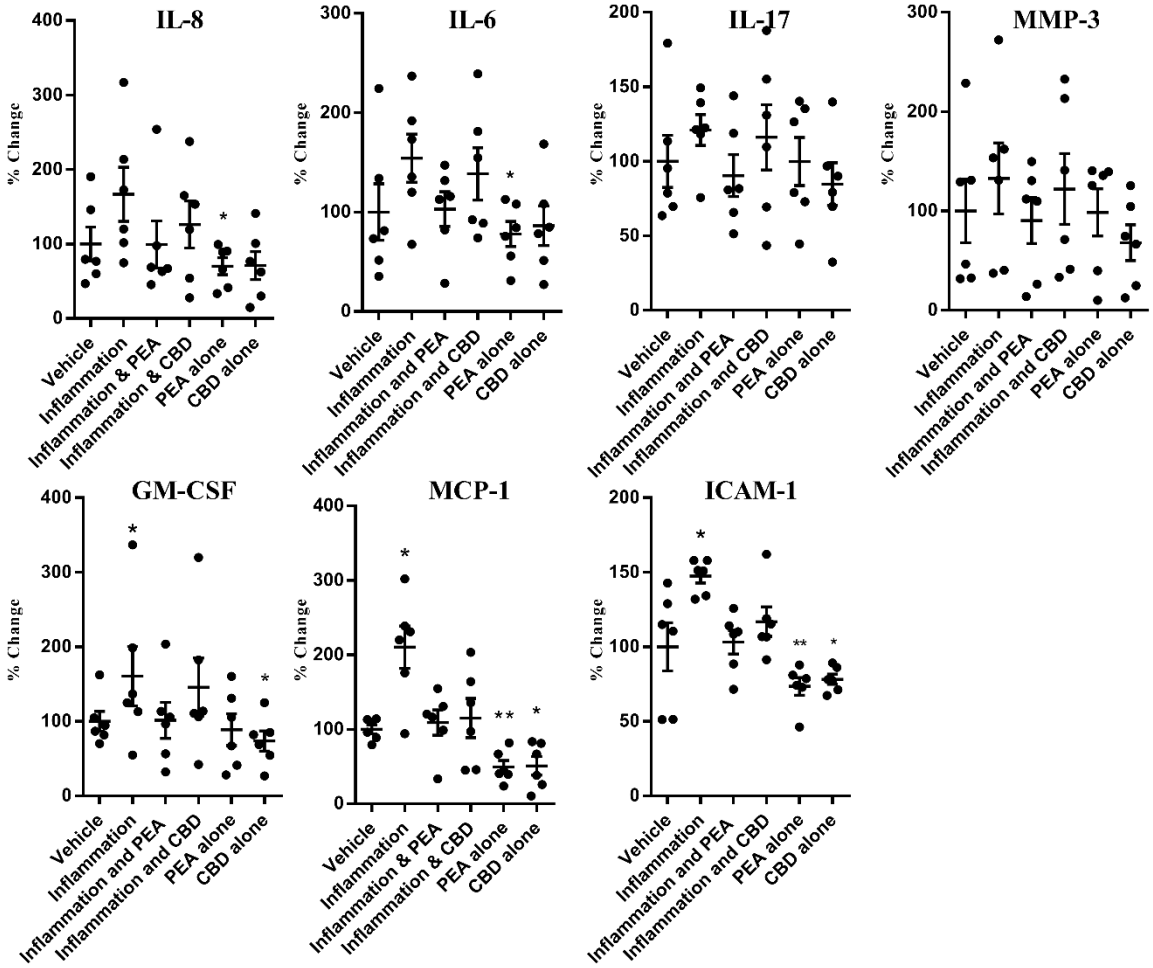

Figure 8

The effects of PEA and CBD on the secreted inflammatory response in appendicitis explants treated with an inflammatory protocol (n=6, percent change from vehicle, compared by repeated measures ANOVA), measured by ELISA. Error bars represent +/- SEM per condition. Asterixes (*) represent significant difference from vehicle, *<0.05, **<0.01, ***<0.001, ****<0.0001.