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Δ^9-Tetrahydrocannabinol Reverses TNFα-induced Increase in Airway Epithelial Cell Permeability through CB_2 Receptors

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Abstract

Despite pharmacological treatment, bronchial hyperresponsiveness continues to deteriorate as airway remodelling persists in airway inflammation. Previous studies have demonstrated that the phytocannabinoid Δ^9-tetrahydrocannabinol (THC) reverses bronchoconstriction with an anti-inflammatory action. The aim of this study was to investigate the effects of THC on bronchial epithelial cell permeability after exposure to the pro-inflammatory cytokine, TNFα.

Calu-3 bronchial epithelial cells were cultured at air-liquid interface. Changes in epithelial permeability were measured using transepithelial electrical resistance (TEER), then confirmed with a paracellular permeability assay and expression of tight junction proteins by Western blotting.

Treatment with THC prevented the TNFα-induced decrease in TEER and increase in paracellular permeability. Cannabinoid CB_1 and CB_2 receptor-like immunoreactivity was found in Calu-3 cells. Subsequent experiments revealed that pharmacological blockade of CB_2, but not CB_1 receptor inhibited the THC effect. Selective stimulation of CB_2 receptors displayed a similar effect to that of THC. TNFα decreased expression of the tight junction proteins occludin and ZO-1, which was prevented by pre-incubation with THC.

These data indicate that THC prevents cytokine-induced increase in airway epithelial permeability through CB_2 receptor activation. This highlights that THC, or other cannabinoid receptor ligands, could be beneficial in the prevention of inflammation-induced changes in airway epithelial cell permeability, an important feature of airways diseases.

Keywords: Airway, epithelium, cannabinoid receptors, THC, tight junctions.

Chemical compounds studied in this article: Δ^9-tetrahydrocannabinol (PubChem CID: 16078); AM251 PubChem CID: 2125; SR144528 (PubChem CID: 3081355); HU-210 (PubChem CID: 9821569); ACEA (PubChem CID: 5311006); JWH133 (PubChem CID: 6918505)

Abbreviations: Δ^9-Tetrahydrocannabinol, THC; ALI, air-liquid interface; CB_1 receptor, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; DMSO, dimethylsulfoxide; EtOH, ethanol; EVOM2, epithelial volt-ohm-meter version 2; interleukin-1β, IL-1β; LLI, liquid-liquid interface; TEER, Transepithelial Electrical Resistance; TNFα, tumour necrosis factor-α, ZO, zonula occludens.

1 Introduction

The airway epithelium provides a physical barrier, which prevents harmful agents from penetrating into the smooth muscle compartment and activating inflammatory responses [1]. This barrier function is regulated by tight junctions between cells, comprising of a complex of proteins, including occludin, claudin, junctional adhesion molecules, and zonula occludens (ZO-1, ZO-2, and ZO-3) [2]. Reduced expression of these proteins results in altered tight junction function, increased epithelial permeability and, consequently, increased transit of pro-inflammatory mediators and cytokines, leading to stimulation of the afferent sensory nerves and airway hyperreactivity [3]. Cytokines, such as TNFα have been shown to lead to loss of occludin staining, which is associated with increased epithelial permeability [4]. Therefore, regulation of tight junction protein expression and hence airway epithelial permeability is a target for preventing aggravation or progression of inflammatory airway diseases such as asthma [5]. Interestingly, tight junction disruption is present in biopsies from patients with asthma irrespective of treatment suggesting that current treatments for asthma may not prevent epithelial dysfunction [6]. Δ^9-tetrahydrocannabinol (THC), the main phytocannabinoid derived from the Cannabis plant, binds readily to both CB_1 and CB_2 receptors as a partial agonist [7]. Cannabinoid receptors have been shown to have anti-inflammatory effects in the airways. For example, THC prevents the enhanced nerve-evoked airway contractions in guinea pig trachea exposed to TNFα [8] through stimulation of both CB_1 and CB_2 cannabinoid receptors. The cannabinoid receptor agonist CP55,940 prevents inflammation-induced bronchoconstriction and mast cell degranulation in ovalbumin-sensitised guinea-pigs [9] and the endocannabinoid anandamide reverses leukotriene D4-induced airway constriction [10]. Although the effects of THC on

inflammation-induced changes in airway epithelial permeability are unknown, it has recently been shown that THC reverses the increase in colonic epithelial permeability caused by cytokines through activation of CB_1 receptors [11]. It is not known whether the effects of THC on colonic epithelial cells can be replicated in airway epithelial cells and, hence, whether cannabinoid receptor agonists might be exploited therapeutically to reverse the increase in airway epithelial permeability as seen in airway inflammation. Therefore, this present study determined the effect of THC on TNFα-induced increase in permeability and reduced tight junction protein expression in airway epithelial cells.

2 Materials and Methods

2.1 Cell culture

Calu-3 cells obtained from ATCC (Rockville, MD, USA) were cultured (from passages 5 to 20) on polyester membrane of Transwells® (pore size 0.4 µm, inserts surface area 1.12 cm^2) (Corning CoStar, Arlington, UK). Cell culture medium, Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 Ham contained 10% of fetal bovine serum, 1% L–glutamine 1% non-essential amino acids, and 1% penicillin/streptomycin. Cells were seeded at a density of 1 x 10^5 cells per well until day 5 until confluent. Medium from the apical and basolateral side were removed and washed with 0.5 ml of phosphate-buffered saline (PBS) every 2 to 3 days. Air-liquid interface (ALI) was established and maintained for three weeks, at which only the basolateral compartment was replaced with medium.

2.2 Transepithelial Electrical Resistance (TEER) Measurements

Changes in Calu-3 epithelial permeability were assessed by measuring TEERs using STX2 electrodes (World Precision Instruments, Stevenage, UK). Prior to TEER measurement, the apical face of cells were washed with 0.5 ml warmed PBS. Basal (i.e. time zero) TEER was recorded after replacing the basolateral and apical compartments of Transwells® with 1.5 ml and 0.5 ml respectively. The resistance expressed by Calu-3 cells alone was obtained by subtracting resistance of the filter membrane. TEER values were presented as epithelial resistance per cm^2 of Transwells® membrane.

The effect of THC on cytokine-induced bronchial epithelial permeability was determined by pre-treating the cells with THC (3, 10 or 30 µM) or vehicle control

(0.3% v/v EtOH) basolaterally, prior to addition of TNFα (10 ng/ml) or IL-1β (1 ng/ml). TEERs were measured at various time points up to 48 hours post-drug application. In some experiments, AM251 (100 nM) and SR144528 (100 nM) were included to determine the role of CB_1 and CB_2 receptors respectively in the THC response. The involvement of cannabinoid receptor was further investigated using the potent cannabinoid receptor agonist, HU-210 (100 nM) and selective CB_1 or CB_2 receptor agonists ACEA (100 nM) and JWH133 (3 µM) respectively.

2.3 Paracellular Permeability Assay using Fluorescein Isothiocyanate (FITC) Dextran

Changes in epithelial permeability were confirmed by measuring transfer of FITC-labelled dextran (4 kDa) across the epithelial layer. In these experiments, media on the apical side was removed and replaced with 0.3% (w/v) N-acetyl cysteine (NAC), dissolved in warm (37^0C) medium, to remove the apical mucus layer. After 30 minutes, NAC was aspirated and 500 µg/mL of FITC-dextran in warm medium added and cells incubated at 37^oC. Permeability of the epithelial layer was estimated by sampling 100 µl of basolateral medium at basal, then every 30 minutes, for up to 3 hours. The amount of FITC-dextran present in the basolateral solution was determined by measuring the fluorescence intensity using a FluoStarGalaxy® fluorometer, set at wavelengths 485 nm (excitation) and 520 nm (emission). The apparent permeability coefficient (P_{app}) is calculated according to the following equation:-

$$P_{app} = \frac{(\frac{\Delta Q}{\Delta t})}{A.C_0}$$

Where Papp is the apparent permeability coefficient (cm/sec)

ΔQ is the change in FD4 concentration over time (Δt)

A is the surface area of the Transwell filter (1.12cm^2)

C_0 is the initial concentration of FD4 applied to the apical side of the cells (500µg/ml)

2.4 Western blot analysis

Treated cells were lysed with 200 µl lysis buffer (20 mM Tris, 1 mM sodium fluoride, 1 mM ethyleneglycoltetraacetic acid (EGTA), 0.1% (v/v) Triton X100 and 10 mM β-glycerophosphate, pH 7.6) with protease inhibitor cocktail (Sigma Aldrich, Dorset, UK). Lysates were centrifuging at 6000 x *g* at 4^{0}C for 5 minutes. Supernatants were removed and diluted with 6 x Laemmli buffer and then heated at 95^{0}C for 5 minutes. Samples (10 µl) were separated using a 4%-20% precast SDS-PAGE gel and then transferred onto nitrocellulose membrane by Western blotting. After transfer, membranes were blocked in 5% w/v fat-free milk dissolved in Tris-buffered saline solution containing 0.1% v/v Tween-20 (TBS-T) for 1 hour. The membrane then was probed overnight at 4^{o}C with one of the following primary antibodies in blocking buffer: anti-occludin rabbit antibody (ab31721; Abcam, Cambridge, UK), anti-ZO-1 rabbit antibody (40-2200) (Zymed, San Francisco, USA), anti-CB_1 receptor antibody (1006590, Cayman Chemical, Michigan, USA) and anti-CB_2 receptor antibody (ADI-905-749-100, Enzo Life Sciences, New York, USA). Membranes were also probed with anti-GAPDH mouse antibody at 1:20,000 dilution (Sigma Aldrich, Dorset, UK) as a loading control. The following day, primary antibodies were removed and membranes washed three times with TBS-T buffer. Membranes were then incubated with secondary antibodies (both at 1:10,000 dilution); goat anti-rabbit IgG (IRDye®800CW Conjugate, Licor Biosciences, Cambridge, UK) and goat anti-mouse

IgG (IRDye®680CW Conjugate, Licor Biosciences, Cambridge, UK), as appropriate. After washing with TBS-T buffer, bands were detected and quantified using LI-COR Image Studio infrared imaging system (Lincoln, NE).

2.5 Statistical Analysis

Time-dependent changes in TEER were analysed using a 2-way ANOVA, followed by a Bonferroni post-hoc test, using GraphPad Prism. Western blotting data were analysed by 1-way ANOVA followed by a Bonferroni post-hoc test. Results of $p<0.05$ were considered significant.

2.6 Materials

THC, AM251, SR144528 were obtained from Tocris Bioscience (Bristol, UK). All other reagents were obtained from Sigma Aldrich (Dorset, UK).

3 Results

3.1 Effect of THC on Cytokine-Induced Reductions in TEER

Basolateral application of THC alone at 3 and 10 μM had no effect on the Transepithelial electrical resistance (TEER) reading in Calu-3 airway epithelial cells (fig. 1). At 30 μM THC, there was a small increase in TEER (fig. 1). Resistance readings for vehicle control 0.3% (v/v) EtOH were maintained throughout the whole duration of experiment. In other experiments, THC was added 24 hours after addition of TNFα (10 ng/ml; fig. 2). In these experiments, TNFα caused a reduction in TEER over the first 24 hours, which was reversed by the subsequent addition of THC (30 μM). In cells treated with TNFα alone, the reduction in TEER was maintained for the duration of the experiment (up to 48 hours post TNFα addition; fig. 2 & 3A). Pre-treatment with THC reduced the effect of TNFα on TEER responses in a concentration-dependent manner (Fig. 3A). Similarly, pre-treatment with THC reduced the subsequent reduction in TEER as a result of IL-1β treatment, although the effect of IL-1β was not completely prevented (fig. 3B).

3.2 Effect of THC on FITC-Dextran Permeability

Treatment with TNFα alone produced an increase in FD4 dextran paracellular permeability (fig. 4). The basolateral application of THC alone did not alter FD4 dextran permeability through the Calu-3 cell layers. However, the increase in FD4 dextran permeability caused by TNFα was prevented in the presence of THC. These data thus confirmed the correlation that a decrease in TEER is mirrored by an increase in P_{app}.

3.3 Role of Cannabinoid Receptors in THC-Mediated Effect

In order to determine whether the effect of THC was mediated through cannabinoid receptor activation, the effects of either the CB_1 receptor antagonist AM251 (100 nM; [12]), or the CB_2 receptor antagonist SR144528 (100 nM; [13]) were determined. As in previous experiments, TEER responses in the presence of TNFα (10 ng/ml) with THC (30 μM) were maintained around vehicle control levels over the duration of the experiment. The presence of SR144528 (fig. 5B), but not AM251 (fig. 5A) prevented the inhibitory effect of THC on the TNFα-induced reduction in TEER (fig. 5).

In order to determine whether CB receptor-selective agonists are able to replicate the effect of THC, the $CB_{1/2}$ receptor agonist HU-210 (100 nM), the selective CB_1 receptor agonist ACEA (100 nM), or the selective CB_2 receptor agonist JWH 133 (3 μM) were used. None of these agonists had any effect on TEER responses on their own over the 48 hour duration of the experiment (fig. 6A). However, pre-incubation with either HU-120 or JWH 133 prevented the reduction in TEER caused by TNFα (10 ng/ml; fig. 6B). Pre-incubation with ACEA did not prevent the immediate effects of TNFα. However, in cells treated with ACEA, the TEERs returned to baseline within 24 hours, whereas in TNFα-treated cells TEERs remained low (fig. 6B).

3.4 CB Receptors Expression in Calu-3 Cells

Western immunoblotting detected bands for both CB_1 and CB_2 receptors at the appropriate molecular weights (fig. 7 A&B). Rat cerebellum, used as a positive control for CB_1 receptor expression, exhibited two major bands of slightly higher molecular weight than that seen in Calu-3 cells. No bands were seen in BV-2 cells, used as a negative control for CB_1 expression. The band obtained with the anti-CB_2

receptor antibody corresponded to the band obtained with rat spleen, used as a positive control. No band was seen in SH-SY5Y cells, used as a negative control. Expression of both receptors appeared to be increased in cells grown at air-liquid interface compared to cells grown at liquid-liquid interface (fig. 7 A & B).

3.5 Effect of THC on TNFα-Induced Alteration of Occludin and ZO-1 Expression

Western blotting detected a band for occludin at around 64 kDa, whereas ZO-1 was expressed at approximately 225 kDa (fig. 8A & 9A). Other bands of lower molecular were also obtained, as observed by other groups using Calu-3 cells [14] (Wan et al., 2000). TNFα (10 ng/ml) reduced the expression of occludin and ZO-1 by half, compared to untreated (basal) Calu-3 cells (fig. 8B & 9B). Treatment with THC alone had no effect on the expression of either occludin or ZO-1, but prevented the decrease in expression caused by TNFα (fig. 8 & 9).

4 Discussion

The phytocannabinoid THC has been previously shown to improve airway function in asthmatic patients [15] and activation of cannabinoid receptors prevents inflammation-induced changes in the airways [9]. Airway inflammation leads to increased permeability of the epithelial layer, resulting in a loss of barrier function, which is thought to be involved in development of airway hyperreactivity. This present study has demonstrated that pre-treatment with THC prevents the increase in permeability across a confluent monolayer of Calu-3 airway epithelial cells caused by the cytokines TNFα and IL-1β. THC appeared to have more of a protective effect against TNFα compared to IL-1β, which may be related to differences in the signalling pathways activated by these two cytokines. Interestingly, THC added 24 hours after the addition of TNFα reverses the increase in epithelial permeability. The effect of THC was inhibited by a CB_2 receptor antagonist and mimicked by a selective CB_2 receptor agonist. Both TNFα and IL-1β are important inflammatory cytokines involved in airway inflammation in both asthma and COPD [16, 17, 18]. Therefore, these data suggest that cannabinoid receptor ligands, particularly CB_2 agonists, could play a role in preventing or reversing inflammation-induced increases in airway epithelial permeability, and, hence, preventing airway hyperreactivity.

Previous investigations have demonstrated an inverse relationship between paracellular permeability of Calu-3 cells cultured at ALI and TEER measurements, whereby an increase in paracellular transport of solutes through the bronchial epithelial cell layer is mirrored by a decrease in transepithelial resistance reading [19, 20, 21]. Results obtained from the present study showed a marked increase in paracellular permeability (i.e. high P_{app} value) in Calu-3 cells treated with TNFα,

which was prevented by pre-treatment with THC (fig. 4). These data thus confirm that the changes in TEER reflect an increase in paracellular permeability of the epithelial cells, and *vice versa*.

The involvement of CB_1 and CB_2 receptors as the potential site of action of THC was assessed using the selective cannabinoid receptor antagonists, AM251 [22] and SR144528 [13]. SR144528, but not AM251, inhibited the THC-induced response in Calu-3 bronchial epithelial cells, suggesting that the action of THC in preventing the decrease in epithelial permeability by TNFα was mediated through CB_2 receptors. Immunoreactivity with antibodies against CB_1 and CB_2 receptors was also detected in samples from Calu-3 cells, indicating the presence of both receptor subtypes. Interestingly, expression of both receptors was apparently higher in cells grown at ALI compared to cells grown at liquid-liquid interface (LLI). An ultrastructure study reported by a separate group of investigators revealed that ALI enhanced the differentiation of Calu-3 cells into a mucociliary phenotype, which was not seen in LLI [23]. Therefore, growing cells at ALI may have promoted the CB receptor expression demonstrated in the present study.

In order to investigate the role of CB receptor subtypes further, TEER measurements were conducted using CB receptor-selective agonists. HU-210, a highly selective cannabinoid receptor agonist that acts on both CB_1 and CB_2 receptors in the nanomolar range [24], prevented the TNFα-induced decrease in TEER, in a similar manner to THC. JWH133, a highly-selective CB_2 receptor agonist [25] also prevented the effect of TNFα. On the other hand, ACEA, a selective CB_1 receptor agonist [26], did not prevent the immediate TEER reduction as seen with TNFα, but

caused a reversal of the TNFα-effect after 24 hours. It is possible, therefore, that CB_2 receptors mediate acute reversal of the TNFα-induced reduction in TEER, whereas CB_1 receptor activation requires chronic activation. However, the CB_2 agonist does not completely prevent the reduction in TEER with TNFα and still requires 3-4 hours to reverse the response suggesting it reverses rather than prevents the TNFα response. A previous study has indicated that CB_2 receptors prevent TNFα-induced release of IL-8 from airway epithelial cells, potentially through a cAMP-mediated alteration in gene expression [27]. Therefore, it is possible that the delayed effect of the CB_2 agonist in this present study is due to the time required to alter gene expression.

The expression of epithelial tight junction proteins such as occludin and ZO-1 are directly linked to the transepithelial resistance of the Calu-3 bronchial epithelial cell line [28]. Previous studies in the same cell model have demonstrated a decrease in Calu-3 cell transepithelial resistance following the exposure to TNFα, accompanied by reduced immunoreactivity against occludin and ZO-1 proteins [29]. In the present study, Western blotting demonstrated that the reduction in the level of both occludin (fig. 8) and ZO-1 (fig. 9) expression when cells were treated with TNFα could be prevented by pre-treatment with THC. THC alone had no effect on the expression of the tight junction proteins. This is consistent with the changes in TEERs and the permeability assay. The effect of THC could be to inhibit the TNFα signalling pathway leading to a reduction in occludin and ZO-1 expression, or it could be acting through a separate pathway which counteracts the changes in tight junction proteins. The fact that THC reverses the TNFα-induced reduction in TEER 24 hours after

addition of TNFα, suggests that it may be reversing rather than preventing the changes in protein expression.

In summary, these data indicate that THC, through activation of cannabinoid receptors and subsequent prevention of decreases in tight junction protein expression, has the ability to inhibit cytokine-induced airway epithelial barrier function disruption. Reduced barrier function is associated with hyperreactivity of the airways in inflammation. Therefore, epithelial cannabinoid receptors may be a therapeutic target for the prevention of airway epithelial dysfunction, as seen in asthma and COPD.

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Conflict of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Figure 1 THC has little direct effect on TEER.

Effects of basolateral application of at various THC concentrations (3, 10 and 30 µM) or vehicle control, 0.3% v/v EtOH onto 21-day old Calu-3 cells. Average basal TEER value was 695±2.8 $\Omega.cm^2$. Data are expressed as percentage TEER by calculating the relative change in resistance at various time points from basal reading, and are presented as mean ± SD; n=9, **P<0.01, 2-way ANOVA followed by a Bonferroni post-hoc test, compared to vehicle, 0.3% v/v EtOH, except THC + cytokine which is compared to TNF(10 ng/mL).

Figure 2 THC reverses the decrease in TEER caused by TNFα.

Effect of basolateral application of THC (30 µM) alone or vehicle control, 0.3% v/v EtOH onto 21-day old Calu-3 cells 24 hours after application of 10ng/ml TNFα. Average basal TEER value was 721±6.1 $\Omega.cm^2$. Data are expressed as percentage TEER by calculating the relative change in resistance at various time points from basal reading, and are presented as mean ± SD; n=9, ***P<0.001, 2-way ANOVA followed by a Bonferroni post-hoc test, compared to vehicle, 0.3% v/v EtOH, except THC + cytokine which is compared to TNF(10 ng/mL).

Figure 3 THC prevents decrease in TEER caused by TNFα and IL-1β.

Effects of basolateral application of THC (at 3, 10 or 30 μM) in the presence of a cytokine, **A.** TNFα (10 ng/ml) or, **B.** the effect of basolateral application of THC (30 μM) in the presence of IL-1β (1 ng/ml) onto 21-day old Calu-3 cells cultured in ALI. Average basal TEER values were **A.** 700±2.3 $\Omega.cm^2$ and **B.** 690±3.4 $\Omega.cm^2$. Data are expressed as percentage TEER by calculating the relative change in resistance at various time points from basal reading, and are presented as mean ± SD; n=9-15, *P<0.05, **P<0.01, ***P<0.001, 2way ANOVA followed by a Bonferroni post-hoc test, compared to vehicle, 0.3% v/v EtOH, except THC + cytokine which is compared to their respective cytokine.

Figure 4 THC prevents increase in epithelial permeability caused by TNFα.

Effect of the corresponding treatments on FD4 dextran paracellular permeability in 21-day old Calu-3 cells. Epithelial permeability is represented as apparent permeability coefficient (P_{app}) calculated according to the equation in section 2.3. Data are presented as mean ± SD; n=9; ***P<0.001 1way ANOVA followed by a Bonferroni post-hoc test, compared to vehicle control for THC, (0.3%v/v) EtOH. THC + TNFα treatment is also compared against treatment with TNFα alone.

Figure 5 CB_2 receptor antagonism prevents effect of THC.

Effect of basolateral application of either **A.** the selective CB_1 antagonist, AM 251 (100 nM) or **B.** the selective CB_2 antagonist, SR144528 (100 nM) in the presence of THC (30 μM) and TNFα (10 ng/mL). Average basal TEER values were **A.** 669±5.5 $\Omega.cm^2$ and **B.** 687±5.3 $\Omega.cm^2$. Data are expressed as percentage TEER by calculating the relative change in resistance at various time points from basal reading, and are presented as mean ± SD; n=15, *P<0.05, **P<0.01, ***P<0.001, 2way ANOVA followed by a Bonferroni post-hoc test, compared to THC + TNFα; except THC +TNFα, which is compared to vehicle control (0.01% v/v) EtOH.

Figure 6 CB receptor agonists prevent TNFα-induced reductions in TEER.

Effects of basolateral application of **A.** Non-selective cannabinoid receptor agonist HU-210 (100 nM), selective CB_1R agonist ACEA (100 nM) or selective CB_2R agonist JWH 133 (3 μM) alone and; **B.** Cannabinoid receptor agonists in the presence of TNFα (10 ng/mL). Average basal TEER values were **A.** 721±4.5 $\Omega.cm^2$ and **B.** 687±7.2 $\Omega.cm^2$. Data are expressed as percentage TEER by calculating the relative change in resistance at various time points from basal reading, and are presented as mean ± SD; n=9-18, *P<0.05, **P<0.01, ***P<0.001, 2way ANOVA followed by a Bonferroni post-hoc test. TEER data were compared to **A.** vehicle control (0.01% v/v) EtOH. **B.** TNFα (10 ng/mL); except TNFα, which is compared to vehicle control (0.01% v/v) EtOH.

Figure 7 Calu-3 cells express CB_1 and CB_2 receptors.

Typical immunoblot showing samples that were treated with polyclonal **A.** anti-CB_1 or **B.** anti-CB_2 receptor rabbit antibody (green bands). Samples of Calu-3 cells grown at ALI were harvested at day 21 of culture; whereas cells of the LLI were lysed at day 5 of culture, when cells were fully confluent in Transwell® inserts. GAPDH (red band) was used as a loading control. Blot is representative of 3 separate experiments.

Figure 8 THC prevents the TNFα-induced reduction in occludin expression.

A. Typical immunoblot showing expression of occludin in Calu-3 cells treated following treatment of TNFα (10 ng/mL), vehicle control (0.3% v/v) EtOH, THC (30 μM) alone or in the presence of TNFα in 21-day old Calu-3 cells for 48 hours. Basal represents untreated cells cultured at air-liquid interface; i.e. no drug. **B.** Data presented as mean of fold change to protein expression over vehicle, (0.3% v/v) EtOH ± SD; n=3-8, **P<0.01, 1way ANOVA followed by a Bonferroni post-hoc test, compared to vehicle control, (0.3% v/v) EtOH, except combined treatment of THC + TNFα which is compared to TNFα (10 ng/mL).

Figure 9 THC prevents the TNFα-induced reduction in ZO-1 expression.

A. Typical immunoblot showing expression of ZO-1 in Calu-3 cells treated following treatment of TNFα (10 ng/mL), vehicle control (0.3% v/v) EtOH, THC (30 μM) alone or in the presence of TNFα in 21-day old Calu-3 cells for 48 hours. Basal represents untreated cells cultured at air-liquid interface; i.e. no drug. **B.** Data presented as mean of fold change to protein expression over vehicle, (0.3% v/v) EtOH ± SD; n=3-8, *P<0.05, 1way ANOVA followed by a Bonferroni post-hoc test, compared to vehicle control, (0.3% v/v) EtOH, except combined treatment of THC + TNFα which is compared to TNFα (10 ng/mL).