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Cannabidiol attenuates inflammatory impairment of intestinal cells expanding biomaterial-based therapeutic approaches

Elisa Boehm, Linda Droessler, Salah Amasheh *

Institute of Veterinary Physiology, School of Veterinary Medicine, Freie Universität Berlin, Oertzenweg 19b, 14163, Berlin, Germany

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ABSTRACT

Cannabis-based biomaterials have the potential to deliver anti-inflammatory therapeutics specifically to desired cells, tissues, and organs, enhancing drug delivery and the effectiveness of anti-inflammatory treatment while minimizing toxicity. As a major component of Cannabis, Cannabidiol (CBD) has gained major attention in recent years because of its potential therapeutic properties, e.g., for restoring a disturbed barrier resulting from inflammatory conditions. The aim of this study was to test the hypothesis that CBD has beneficial effects under normal and inflammatory conditions in the established non-transformed intestinal epithelial cell model IPEC-J2. CBD induced a significant increase in transepithelial electrical resistance (TER) values and a decrease in the paracellular permeability of [^{3}H]-D-Mannitol, indicating a strengthening effect on the barrier. Under inflammatory conditions induced by tumor necrosis factor alpha (TNFα), CBD stabilized the TER and mitigated the increase in paracellular permeability. Additionally, CBD prevented the barrier-disrupting effects of TNFα on the distribution and localization of sealing TJ proteins. CBD also affected the expression of TNF receptors. These findings demonstrate the potential of CBD as a component of Cannabis-based biomaterials used in the development of novel therapeutic approaches against inflammatory pathogenesis.

1. Introduction

In recent years, cannabidiol (CBD), one of the main secondary plant compounds of *Cannabis sativa* L., has been established as a novel compound for intestinal drug targeting and development [1–3]. Moreover, a combination of CBD with other biomaterials such as chitosan and alginate-based hydrogel has gained major attention [4,5]. The historical use of *Cannabis sativa* for various ailments, such as nausea, vomiting, diarrhea, and pain, dates back to antiquity [6]. Cannabinoids exert their effects by binding to specific cannabinoid receptors, namely cannabinoid receptor 1 (CBR-1) and cannabinoid receptor 2 (CBR-2), which are part of the endocannabinoid system [7]. Additionally, cannabinoids can interact with non-cannabinoid receptors, such as the transient receptor potential vanilloid subtype 1 (TRPV1; [8], the orphan receptor GPR55 [9,10], the peroxisome proliferator-activated receptors (PPARs; [11], and the serotonin receptor 5-HT$_{1A}$ [12]. The endocannabinoid system, comprising receptors and endogenous ligands, plays a crucial role in controlling numerous physiological processes, such as food intake, cell metabolism, memory, gastrointestinal motility, and barrier function [7, 13] making it a potential therapeutic target for pathophysiological processes, including inflammation, pain, and depression.

The intestinal barrier, as the first line of defense against antigens, toxins, and pathogens in the environment, plays a pivotal role in immune defense due to its large surface area [14]. However, disruptions in the integrity of the intestinal barrier have been linked to various diseases, including inflammatory bowel disease (IBD) and celiac disease. Therefore, the targeting of intestinal barrier defects represents a viable therapeutic approach in managing these diseases [15]. The integrity of the intestinal barrier is maintained by transmembrane tight junction (TJ) proteins, which form a belt-like connection between the apicolateral membranes of neighboring cells [16]. Members of the protein family of claudins and occludin primarily form the TJ between two neighboring cells to provide a selective paracellular barrier [17–19]. These TJ proteins are crucial for the paracellular permeability of the epithelium, thereby preventing the entry of harmful substances into the bloodstream [20]. Numerous endogenous and exogenous factors (e.g.,

Abbreviations: ANOVA, Analysis of Variance; CBD, cannabidiol; CBR, cannabinoid-receptor; MLCK1, myosin light chain kinase 1; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; TER, Transepithelial resistance; TJ, Tight junction; TNFα, Tumor necrosis factor alpha; TNFR, Tumor necrosis factor receptor.

* Corresponding author.
E-mail address: salah.amasheh@fu-berlin.de (S. Amasheh).

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TNFα [21] and natural bioactive compounds, e.g., chitosan [22], caprate [23], laurate [24], quercetin [25], and berberine [26,27]) have been found to impact epithelial resistance and paracellular permeability, thus affecting the integrity of the barrier.

Because of its anti-inflammatory, analgesic, and anticonvulsant effects [28] without psychotropic properties [29], CBD has emerged as a promising candidate for the treatment of inflammatory disorders. The positive effects of CBD on the epithelial barrier function have been verified in various studies *in vitro*. In Caco-2 cells, CBD has been shown to prevent the decrease of transepithelial resistance (TER) and the increase of paracellular permeability caused by EDTA, oxidative stress, or inflammation [29–31]. Moreover, CBD has been demonstrated to impact TJs, although the exact mechanism remains unknown [31,32]. The positive effects of CBD have been confirmed *in vivo*. In a mouse model, CBD has been revealed to reduce colon injury induced by 2,4,6-dinitrobenzene sulfonic acid administration and to lower the expression of inducible nitric oxide synthase and the levels of interleukin-1 and -10 [33].

Tumor necrosis factor alpha (TNFα), an endogenous cytokine, is strongly involved in inflammation and the pathogenesis of various diseases, making it a relevant choice for representing inflammatory conditions [34]. The dysregulation of TNFα has been implicated in the development of autoimmune disorders, such as rheumatoid arthritis, inflammatory bowel disease, and psoriasis [35]. TNFα signaling is mediated by its two specific receptors, Tumor necrosis factor receptor 1 (TNFR-1) and Tumor necrosis factor receptor 2 (TNFR-2), which activate different downstream signaling pathways. TNFR-1 is the primary receptor for TNFα and is involved in the induction of apoptosis, inflammation, and immune responses, whereas TNFR-2 primarily regulates cell survival and proliferation [36]. Recently, TNFα-induced barrier disruption in IPEC-J2 cells has been shown to result in the decreased expression of sealing TJ proteins, leading to increased paracellular permeability and a reduction in TER [37].

The non-transformed porcine intestinal epithelial cell line IPEC-J2 used in our study serves as an appropriate *in vitro* model for investigating epithelial transport and barrier properties. An advantage of the IPEC-J2 cell line is its morphological and functional similarity to intestinal epithelial cells *in vivo* [38]. Among the non-human cell lines, IPEC-J2 mostly resembles the human physiology of the intestine [39,40].

The goal of our study has been to investigate the impact of CBD on the integrity and function of the intestinal barrier by utilizing the IPEC-J2 cell line as an *in vitro* model. Specifically, we sought to elucidate whether CBD has the potential to mitigate or prevent the barrier-disrupting effects of TNFα. By exposing IPEC-J2 cells to TNFα in the presence or absence of CBD, we have aimed to evaluate the therapeutic potential of CBD in ameliorating TNFα-induced barrier disturbances and to shed light on its underlying mechanisms of action for applications in health and disease.

2. Material and methods

2.1. Cell culture

The non-transformed intestinal porcine enterocyte cell line IPEC-J2 (DSMZ, Braunschweig, Germany) was used as a model to investigate the porcine epithelial barrier function. The cells were cultured in Dulbecco's MEM/Ham's F-12 (Biochrom, Berlin, Germany) containing L-glutamine, 10% fetal bovine serum, and 1% penicillin and streptomycin (Sigma Aldrich, Munich, Germany). Cells were incubated at 37 °C and under 5% CO_2 in a humified atmosphere. The medium was changed every second to third day. Every 7 days, the cells were passaged by trypsinization (0.05 g/L porcine trypsin, 0.02 g/L EDTA, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and split at a ratio of 1:3. For the experiments, cells were seeded on semipermeable Millicell® cell culture plate inserts with a diameter of 12 mm and 0.45 μm pore size (Millipore, Darmstadt, Germany). Cells were seeded at a density of 1×10^5 cells/insert and placed into 12-well cell culture plates. Incubation experiments started when the cells reached confluency and showed similar resistance values, which was generally between day 14–21 after seeding. Cell passages from 8 to 9 were used for the experiments.

2.2. Chemicals

Cannabidiol (CBD; Tocris, Bristol, United Kingdom) was dissolved in ethanol to give a stock concentration of 10 mM, 0.4% ethanol in culture medium was used as a control. As shown in the supplemental figure, transepithelial resistance (TER) is not affected by ethanol, indicating that the final concentration of 0.4% ethanol is not cytotoxic (Suppl. Fig. 1). Recombinant human tumor necrosis factor alpha (TNFα; PeproTech, Hamburg, Germany) was diluted in autoclaved H_2O to give a concentration of 0.1 mg/mL; 0.05% autoclaved H_2O in culture medium served as control application.

2.3. Incubation experiments

500 μL medium containing CBD at increasing concentrations (in μM: 2.5, 5, 10, 20, or 40) were added to the apical side of the cell culture inserts, with 1 mL medium without CBD being added to the basolateral side. These concentrations have been studied previously for their cytotoxicity (Suppl. Fig. 2). To study the effects of CBD under inflammatory conditions, IPEC-J2 cells were concurrently treated with 1000 U/mL TNFα basolaterally and CBD as described above for 48 h.

2.4. Transepithelial resistance measurements

Transepithelial resistance (TER), representing the epithelial barrier function, was measured using an Epithelial Volt/Ohm Meter (EVOM, World Precision Instruments, Sarasota, FL, USA). The TER was measured every hour for the first 10 h, and then again after 24 h and 48 h. The measurements were corrected to the resistance of the blank values of the filter supports and calculated with regard to the membrane area.

2.5. Protein extraction and quantification

The medium was removed at the end of the incubation experiments. The inserts were washed with PBS, following which the cells were lysed for protein extraction in RIPA buffer comprising 25 mM HEPES pH 7.6, 25 mM NaF, 2 mM EDTA, 1% sodium dodecyl sulfate (10%), H_2O, and enzymatic protease inhibitors (Complete EDTA-free, Boehringer, Mannheim, Germany). The RIPA buffer (150 μL) was pipetted onto each insert and the cells were carefully scraped off and transferred into Eppendorf tubes. After 30 min of incubation on ice, the samples were homogenized in an ultrasonic bath. Protein quantification was carried out by using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories GmbH, Munich, Germany).

2.6. Immunoblots and densitometry

Polyacrylamide gel electrophoresis (PAGE) was used to separate the isolated proteins according to their charge and molecular size. A sample containing 20 μg protein was mixed with Laemmli buffer (Bio-Rad Laboratories GmbH, Munich, Germany) and β-mercapthoethanol (AppliChem, Darmstadt, Germany) and heated at 95 °C for 5 min. The protein lysates were loaded onto 10% TGX Stain-Free FastCast gels (Bio-Rad Laboratories GmbH, Munich, Germany), and electrophoresis was carried out for 60 min at 150 V. The proteins were then transferred by electroblotting for 90 min at 100 V to PVDF membranes (Bio-Rad Laboratories GmbH, Munich, Germany) and then blocked in 5% milk (in Tris-buffered saline with 0.1% Tween 20) for 60 min. After incubation overnight at 4 °C with specific antibodies (all from Thermo Fisher Scientific, at concentrations according to the manufacturers

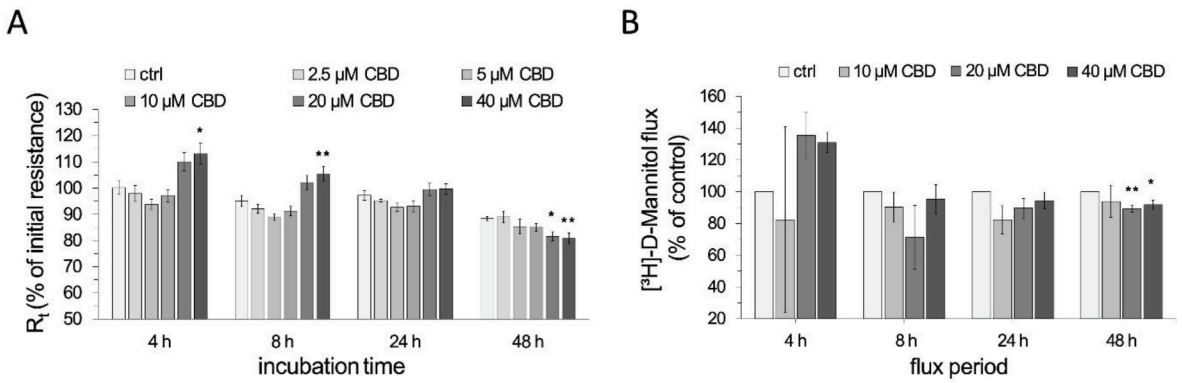

Fig. 1. Effects of CBD on transepithelial electrical resistance and paracellular permeability. **A** Transepithelial electrical resistance after incubation with various concentrations of CBD for 48 h (Kruskal-Wallis test; one-way ANOVA (Analysis of Variance); $n = 15–16$; $*p < 0.05$, $**p < 0.01$). **B** Measurement of paracellular permeability of [^{3}H]-D-Mannitol for 48 h, over four time periods (Kruskal-Wallis test; $n = 5–6$; $*p < 0.05$, $**p < 0.01$). Data are presented in mean ± SEM.

recommendations) against claudin-1 (cat. #51-9000), claudin-3 (cat. #34-1700), claudin-4 (cat. #32-9400), claudin-5 (cat. #34-1600), occludin (cat. #33-1500), or Zonula occludens 1 (ZO-1; cat. #33-9100), the membrane was then incubated with secondary horse anti-mouse IgG antibody conjugated to horseradish peroxidase (Cell Signaling Technology, cat. #7076) and goat anti-rabbit IgG (cat. #7074) at room temperature for 45 min. Subsequently, antibodies were detected by the Clarity Western ECL Blotting Substrate (Bio-Rad Laboratories GmbH, Munich, Germany) by using a ChemiDoc MP Luminescence imager (ChemiDoc MP, Munich, Germany). Densitometrical analysis of the immunoblotting bands was subsequently carried out using Image Lab, the software compatible with the imager (ImageLab, BioRad, Hercules, CA, USA). Bands were normalized based on the total protein amount and compared with the control group.

2.7. Immunocytochemistry

After the incubation experiments, the medium was removed, and the inserts were washed with PBS. The cells were fixed with ice-cold methanol for 10 min at -20 °C and then washed again with PBS. Permeabilization was performed in Triton X-100 (Roth, Karlsruhe, Germany) for 10 min at room temperature. After another washing step with PBS, the cells were blocked by incubation with a blocking solution (PBS with 1% bovine serum albumin and 5% goat serum) for 60 min at room temperature. The cells were next incubated with antibodies against claudin-1, -3, -4, -5, Zonula occludens 1 (ZO-1), and occludin (according to the manufacturer, as described above) for 60 min at 37 °C. Subsequently, cells were washed again and stained with secondary goat anti-rabbit Alexa Fluor-488 (1:1000, Thermo Fisher Scientific, cat. #A-11034) and goat anti-mouse Alexa Fluor-594 (cat. #A-11032) for 60 min at 37 °C. The cell nuclei were stained by DAPI (1:2000) for 5 min at room temperature followed by another wash with PBS. The filters were mounted on microscope slides by using ProTaqs Mount Fluor (Biocyc, Luckenwalde, Germany) and examined by means of a Zeiss 710 confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

2.8. Investigation of distribution and amount of cannabinoid and TNF receptors after incubation with TNFα and CBD

To study the effects of CBD and the co-incubation with TNFα on their specific receptors, the cells were stained with antibodies (from antibodies-online, if not declared otherwise) against Cannabinoid Receptor 1 (CBR-1; cat. #ABIN3183698), Cannabinoid Receptor 2 (CBR-2; cat. #ABIN680168), and TNF receptor 1 (TNFR-1; Abcam, cat. #AB19139) and 2 (TNFR-2; cat. #ABIN2789622) for immunoblotting and immunocytochemistry as described above.

2.9. Paracellular permeability

IPEC-J2 cells were treated with CBD alone or in combination with TNFα for 48 h as previously described. Mannitol was used as a paracellular flux marker from the apical to the basolateral side: 1 µM (6330 Bq) of [^{3}H]-D-Mannitol (PerkinElmer, Waltham, MA, USA) was added to the apical side of the semipermeable inserts. Aliquots of 50 µL from the apical side, representing "hot" samples, were taken at the beginning and at the end of the experiment. These samples were made up to 300 µL with the appropriate medium. Samples of 300 µL from the basolateral side were taken either after 4 h and 8 h or/and after 24 h and 48 h. This resulted in either two or four flux periods, respectively. Fresh medium with the appropriate concentration of CBD and/or TNFα was replenished directly. Samples were mixed with 300 µL Aquasafe 300 plus liquid scintillation cocktail (Zinsser Analytic, Frankfurt, Germany) for 10 min and then counted in a TriCarb 4910 TR liquid scintillation counter (PerkinElmer, Waltham, MA, USA).

2.10. ApoTox-Glo™ triplex assay

An ApoTox-Glo™ assay (Promega GmbH, Walldorf, Germany) was performed to investigate apoptosis (caspase-3/-7 activity), cytotoxicity, and the cell viability of IPEC-J2 after incubation with CBD. To accomplish this, 2×10^5 cells per mL were seeded onto 24 multiwell plates with polyester membrane inserts having a diameter of 6.5 mm and a pore size of 0.4 µm (Costar, Corning Incorporated, Kennebunk, ME, USA). Following a growing period of 14 days, cells were incubated for 48 h with CBD concentrations of 20 µM and 40 µM. After 48 h, 20 µL viability/cytotoxicity reagent containing GF-AFC substrate and bis-AFF-R110 substrate was added to each well and gently shaken for 30 s. Subsequently, after 30 min of incubation at 37 °C, the fluorescence was measured at 400EX/505 EM for viability and at 485EX/520 EM for cytotoxicity by means of an EnSpire Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA). Subsequently, 100 µL Caspase-Glo 3/7 reagent was added to each well and briefly mixed again for 30 s. The luminescence measurement of apoptosis was carried out after 30 min of incubation at room temperature.

2.11. Statistical analysis

Statistical analysis of the data was performed using JMP Pro 16. Experimental results are shown as average values ± standard error mean (SEM). The number of filters is specified as n. Normally distributed data were compared by using one-way ANOVA, and statistical significance was determined by Dunnett's post hoc test or Tukey Kramer. For non-normally distributed data, the Kruskal-Wallis test was applied as a non-parametric test with Tukey Kramer for the post hoc test or Dunnett's

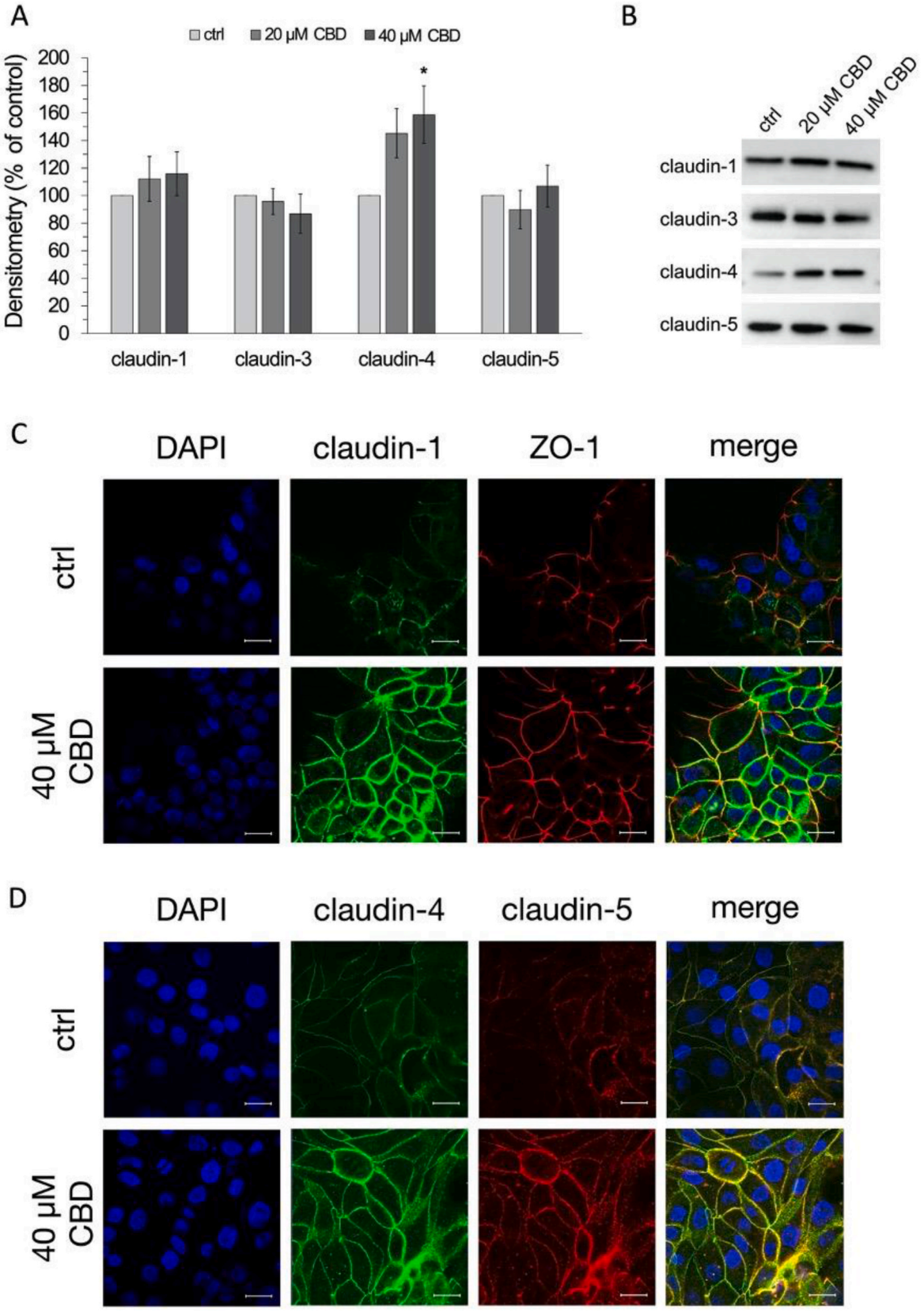

Fig. 2. Effects of CBD on the expression of tight junction proteins. **A** Densitometry and **B** Western blots of tight junction proteins after incubation with CBD for 48 h (one-way ANOVA, $n = 6$; $^*p < 0.05$). Immunofluorescent staining of **C** claudin-1 (green) and Zonula occludens 1 (ZO-1; red), **D** claudin-4 (green) and claudin-5 (red) after 48 h of incubation. Nuclei were stained in blue (DAPI; scale bar: 20 μm; $n = 4$; representative images).

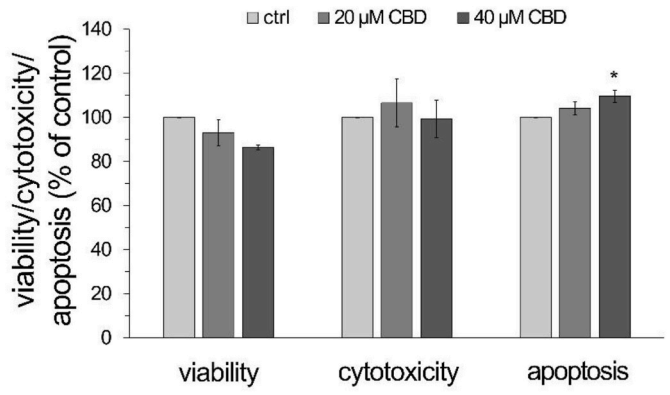

Fig. 3. Apotox-Glo™ assay. Impact of a 48 h CBD treatment on cellular viability, cytotoxicity, and apoptosis of IPEC-J2 cells (one-way ANOVA; $n = 4$; $^{*}p < 0.05$).

post hoc test. Statistical significance was assumed at values below $p = 0.05$.

3. Results

3.1. Effects of CBD on transepithelial barrier function

Measurements of transepithelial resistance (TER) and paracellular permeability of Mannitol were conducted to investigate the effects of cannabidiol (CBD) on the epithelial barrier of IPEC-J2 cells. After 4 h and 8 h of incubation, cells treated with 40 μM CBD showed increased TER values compared with controls (4 h: ctrl: 100.2 ± 2.64%; 40 μM: 113.12 ± 3.92%, $p < 0.05$; 8 h: ctrl: 95.07 ± 2.12%; 40 μM: 105.42 ± 2.92%, $p < 0.01$; $n = 16$; Fig. 1A). However, resistance at 20 and 40 μM decreased after 48 h (ctrl: 88.42 ± 0.80%; 20 μM: 81.58 ± 1.74%, $p < 0.05$; 40 μM: 80.83 ± 2.09%, $p < 0.01$; $n = 15$–16; Fig. 1A).

TER was not significantly affected by lower concentrations of CBD throughout the entire study period. As a result, paracellular permeability measurements, immunoblots, and immunocytochemical images were only undertaken for higher concentrations of CBD.

The paracellular permeability from the apical to basolateral sides was measured using the flux marker [^{3}H]-D-Mannitol. The flux rates of the control group were set to 100% and compared with those of the CBD-treated cells. Measurements were conducted at four different time periods (4, 8, 24, and 48 h). The results showed that the permeability of [^{3}H]-D-Mannitol was markedly lower following incubation of 20 and 40

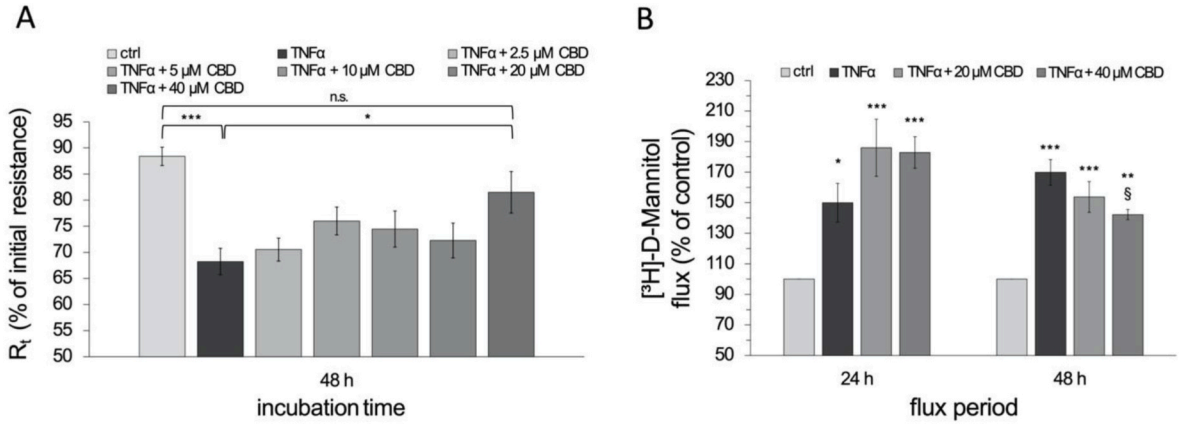

Fig. 4. Effects of CBD on the transepithelial barrier function under inflammatory conditions. **A** Transepithelial electrical resistance after incubation with 1000 U/mL TNFα and CBD for 48 h (Kruskal-Wallis test, $n = 20$; $^{*}p < 0.05$, $^{***}p < 0.001$). **B** Paracellular permeability of [^{3}H]-D-Mannitol for 48 h, over two time periods (one-way ANOVA; $n = 5$–6; $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control; §$p < 0.05$ compared with TNFα treated cells).

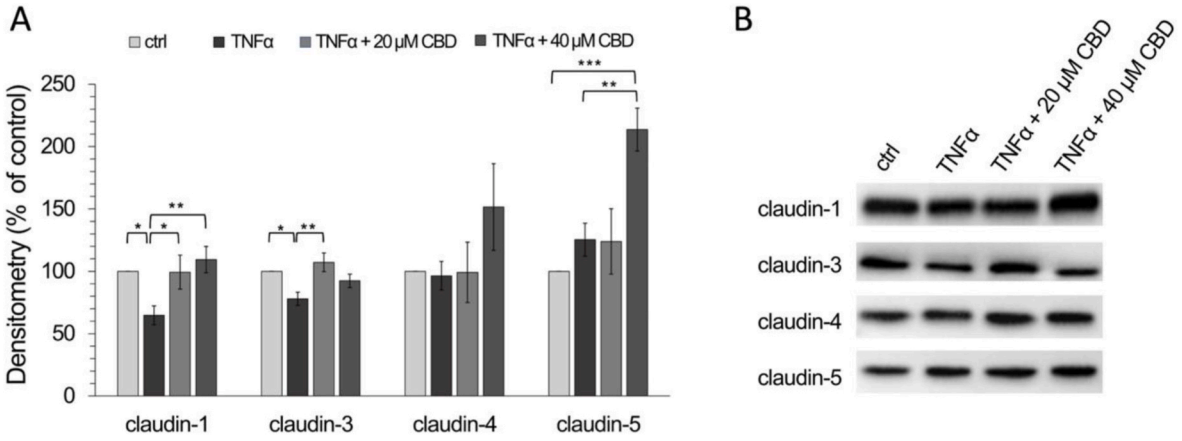

Fig. 5. Effects of co-incubation of TNFα and CBD on TJ protein expression. **A** Densitometry and **B** Western blots of tight junction proteins after incubation with 1000 U/mL TNFα and CBD for 48 h (one-way ANOVA; $n = 7$–8; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

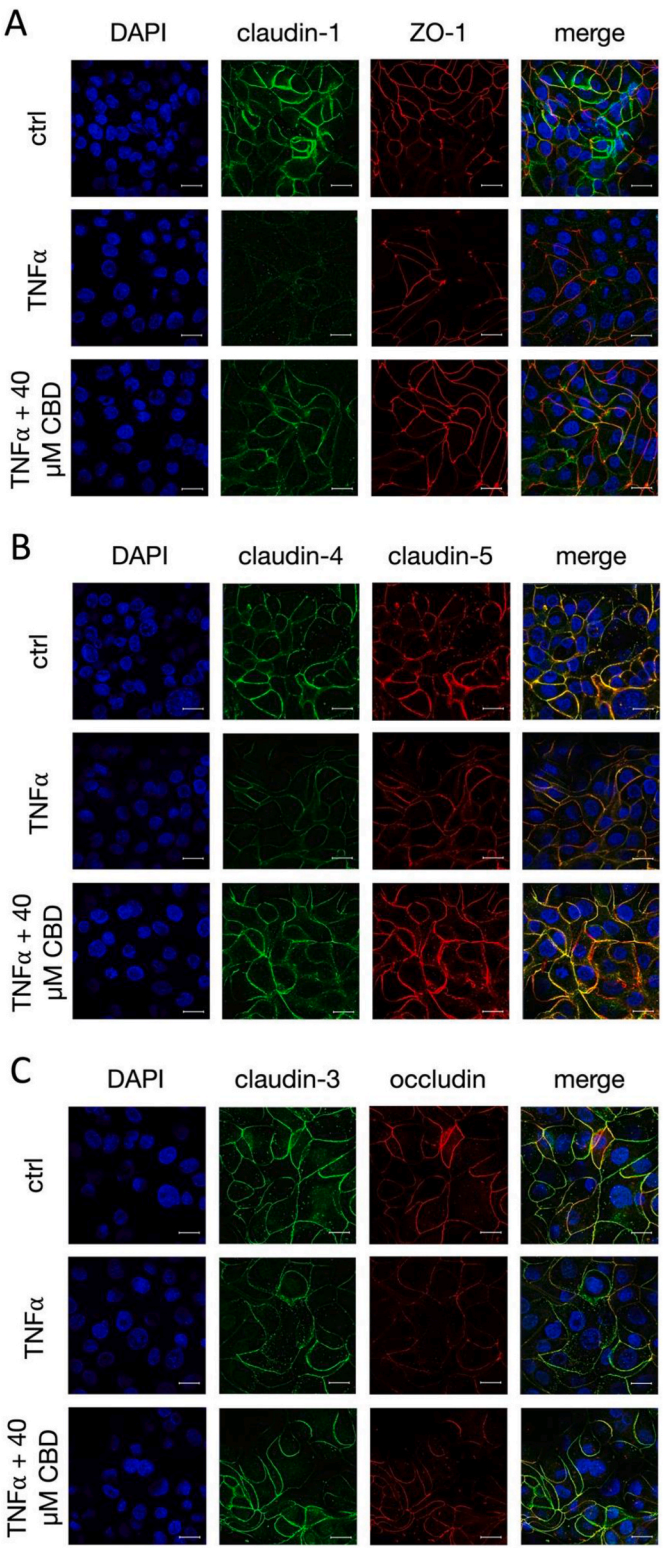

Fig. 6. Confocal laser microscopy studies of various tight junction proteins after co-incubation of TNFα and CBD. Immunofluorescent staining of **A** claudin-1 (green) and Zonula occludens 1 (ZO-1; red), **B** claudin-4 (green) and claudin-5 (red), **C** claudin-3 (green) and occludin (red) after 48 h of incubation. Nuclei were stained in blue (DAPI; scale bar: 20 µm; $n = 4$; representative images).

µM CBD compared with that of controls in the last flux period of 48 h (20 µM: 89.16 ± 1.95%, $p < 0.01$; 40 µM: 91.78 ± 2.79%, $p < 0.05$; $n =$ 5–6; Fig. 1B).

3.2. Immunoblotting

Immunoblots of tight junction (TJ) proteins were conducted to elucidate the underlying molecular mechanism of the alteration in transepithelial resistance and paracellular permeability. CBD at 40 µM markedly increased the expression of claudin-4 compared with that of the control group (ctrl: 100%; 40 µM CBD: 158.75 ± 20.94%, $p < 0.05$, $n = 6$), whereas no significant alteration was observed in the expression of the TJ proteins claudin-1, -3, and -5 (n = 6, respectively, Fig. 2A and B).

3.3. Confocal laser scanning microscope

Following the TER experiments, the cells were immunocytochemically stained for specific TJ proteins. Upon incubation with CBD, the immunofluorescence images exhibited an enhanced signal for claudin-1 (green) and ZO-1 (red; Fig. 2C) as compared with the control. Similar observations were made for claudin-4 (green) and claudin-5 (red; Fig. 2D) after incubation with CBD. The immunofluorescence images demonstrated a stronger signal for both claudins compared with the control (Fig. 2D).

3.4. Apotox-Glo™ assay

The effect of CBD on cell viability, cytotoxicity, and apoptosis was determined using the Apotox-Glo™ assay. CBD-treated cells were compared with control cells that were set at 100%. After 48 h of incubation with 40 µM CBD, the apoptosis rate was marginally increased (109.73 ± 2.77%, $p < 0.05$, $n = 4$; Fig. 3). On the other hand, 20 µM CBD did not show significant effects on apoptosis (104.32 ± 3.01%, $p =$ 0.272, $n = 4$; Fig. 3). Viability and cytotoxicity also did not differ significantly between the groups.

3.5. Effects of CBD on transepithelial barrier function under inflammatory conditions

Incubation with 1000 U/mL TNFα resulted in a significant decrease in transepithelial resistance over 48 h compared to control cells (ctrl: 88.37 ± 1.76%; TNFα: 68.24 ± 2.54%, $p < 0.001$, $n = 20$; Fig. 4A). However, when co-incubated with 40 µM CBD, the TNFα-induced effect was prevented (TNFα + 40 µM CBD: 81.51 ± 3.99%, $p < 0.05$, $n = 20$; Fig. 4A). Thus, after 48 h, no significant difference in terms of TER was observed between control cells and those treated with TNFα +40 µM CBD ($p = 0.649$).

Flux measurements of mannitol after 24 h revealed a significant increase in permeability for cells treated with TNFα and TNFα in combination with CBD compared to control cells (ctrl: 100%; TNFα: 149.97 ± 12.63%, $p < 0.05$; TNFα + 20 µM: 185.94 ± 18.74%, $p < 0.001$; TNFα + 40 µM: 182.8 ± 10.35%, $p < 0.001$, $n = 5$–6; Fig. 4B). Compared with TNFα alone, co-incubation with 40 µM CBD resulted in a significant lower permeability in the second flux period from 24 h to 48 h (ctrl: 100%; TNFα: 169.81 ± 8.41%, $p < 0.001$ compared with control; TNFα + 40 µM: 142.22 ± 3.30%, $p < 0.05$ compared with TNFα-treated cells; $n = 6$; Fig. 4B). However, co-incubation with CBD still resulted in increased permeability compared with control cells (ctrl: 100%, TNFα + 20 µM: 153.73 ± 10.01%, $p < 0.001$; TNFα + 40 µM: 142.22 ± 3.30%, $p < 0.01$, $n = 5$–6; Fig. 4B).

3.6. Influence on TJ protein expression by co-incubation with TNFα and CBD

The protein level of control groups was set to 100%. Values are the

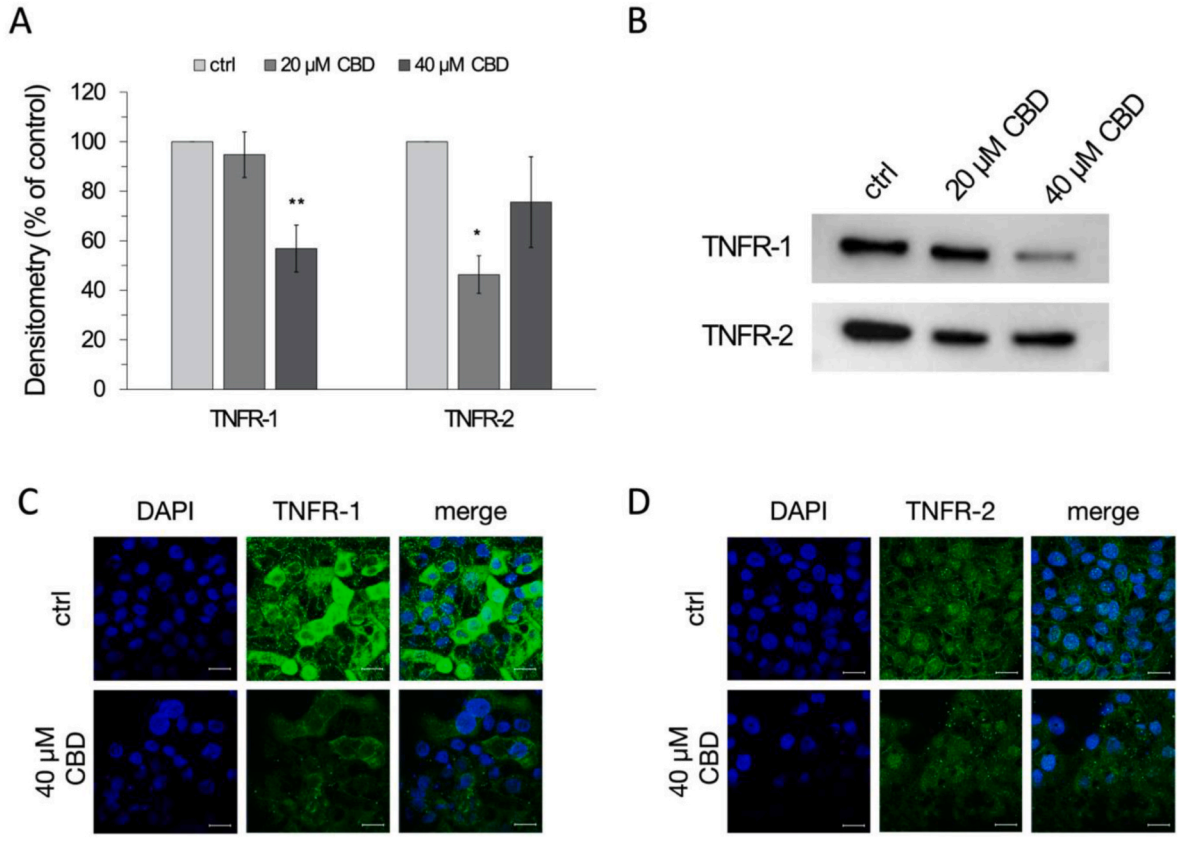

Fig. 7. Influence on TNF receptor expression by incubation with CBD. **A** Densitometry and **B** Western blots of TNFR-1 and TNFR-2 after 48 h of incubation with CBD (Kruskal-Wallis test; one-way ANOVA; $n = 5$–6; $^{*}p < 0.05$, $^{**}p < 0.01$). Immunofluorescent staining of **C** TNFR-1 (green), **D** TNFR-2 (green), nuclei were stained in blue (DAPI; scale bar: 20 μm; $n = 4$; representative images).

percentage of the control group. The cells treated with TNFα were statistically compared with control cells, whereas the cells co-incubated with TNFα and CBD were compared with TNFα treated cells. Sole TNFα incubation led to a significant decrease in the expression level of claudin-1 over 48 h compared to the controls (TNFα: 64.73 ± 7.61%, $p < 0.05$, $n = 6$; Fig. 5A and B). However, co-incubation of TNFα-treated cells with 20 μM or 40 μM CBD prevented the decrease in claudin-1 expression (TNFα + 20 μM CBD: 99.3 ± 11.15%, $p < 0.05$; TNFα + 40 μM CBD: 109.46 ± 10.53%, $p < 0.01$; $n = 6$; Fig. 5A and B). Similar results were observed for claudin-3 expression, with TNFα leading to a reduced expression (TNFα: 77.94 ± 5.31%, $p < 0.05$, $n = 8$; Fig. 5A and B), which was prevented by co-incubation with 20 μM CBD (TNFα + 20 μM CBD: 107.15 ± 7.51%, $p < 0.01$, $n = 8$; Fig. 5A and B). Claudin-4 was not significantly changed (TNFα: 96.42 ± 11.46%, $n = 7$; TNFα + 40 μM CBD: 151.55 ± 34.71%, $n = 8$). The expression of claudin-5 showed a marked increase in co-incubated cells with 40 μM CBD compared with controls and with sole TNFα incubation (TNFα: 125.28 ± 13.22%; TNFα + 40 μM CBD: 213.70 ± 17.22%, $p < 0.001$ compared with controls, $p < 0.01$ compared with TNFα-treated cells; Fig. 5A and B). However, the expression of ZO-1 and occludin was not significantly altered by TNFα or co-incubation with CBD (data not shown).

In addition, confocal laser microscopy studies were performed to investigate the distribution and localization of specific TJ proteins. After 48 h of TNFα treatment, a faint signal for claudin-1 (green; Fig. 6A) was observed, in contrast to the junctional signal observed in the control group (Fig. 6A). However, in combination with 40 μM CBD, a stronger paracellular signal could be seen for claudin-1. Similarly, a loss of signal for claudin-4 (green; Fig. 6B) and claudin-5 (red; Fig. 6B) was detected after TNFα treatment, which was protected by CBD, as shown by the preserved signal in co-incubation with CBD (Fig. 6B). Furthermore, TNFα treatment resulted in a loss of continuous signal for claudin-3 (green; Fig. 6C) at the apicolateral part of the cells, a loss that was also prevented by CBD (Fig. 6C). ZO-1 (red; Fig. 6A) and occludin (red; Fig. 6C) were not significantly affected.

3.7. Cannabinoid- and TNF receptor expression

To investigate whether CBD alone or in combination with TNFα affected the expression of specific TNF and CBD-receptors, Western blot and immunocytochemical staining analyses were performed. Incubation with 40 μM CBD resulted in a significant decrease in TNFR-1 expression compared with the control group (ctrl: 100%; 40 μM CBD: 56.85 ± 9.54%, $p < 0.01$, $n = 6$; Fig. 7A and B). On the other hand, TNFR-1 expression increased following the sole incubation of TNFα (TNFα: 147.67 ± 20.83%, $n = 12$; Fig. 8A and B) but decreased when cells had been co-incubated with CBD (TNFα + 20 μM CBD: 131.47 ± 23.35%, $n = 9$; TNFα + 40 μM CBD: 98.8 ± 15.13%, $n = 11$; Fig. 8A and B). The expression of TNFR-2 significantly decreased with incubation in 20 μM CBD compared with the control group (20 μM CBD: 46.36 ± 7.66%, $p < 0.05$, $n = 5$; Fig. 7A and B). However, the opposite effect was observed when CBD was co-incubated with TNFα (TNFα: 96.35 ± 17.68%, TNFα + 40 μM CBD: 117.98 ± 18.22%, $n = 12$; Fig. 8A. B).

Immunofluorescent staining, including z-stack images of TNFR-1, showed that the receptor was primarily located on the basolateral membrane under normal conditions. However, when cells were incubated with TNFα, a notable increase in signal intensity was observed throughout the cells. The co-incubation with CBD markedly attenuated this intense signal (Fig. 8C, E). In contrast, the signal for TNFR-2 was weaker upon incubation with TNFα alone, whereas co-incubation with CBD resulted in a marked increase in the signal (Fig. 8D, F).

Although CBD tended to increase the expression of CBR-2 slightly compared with that of the control group, as indicated by immunoblots

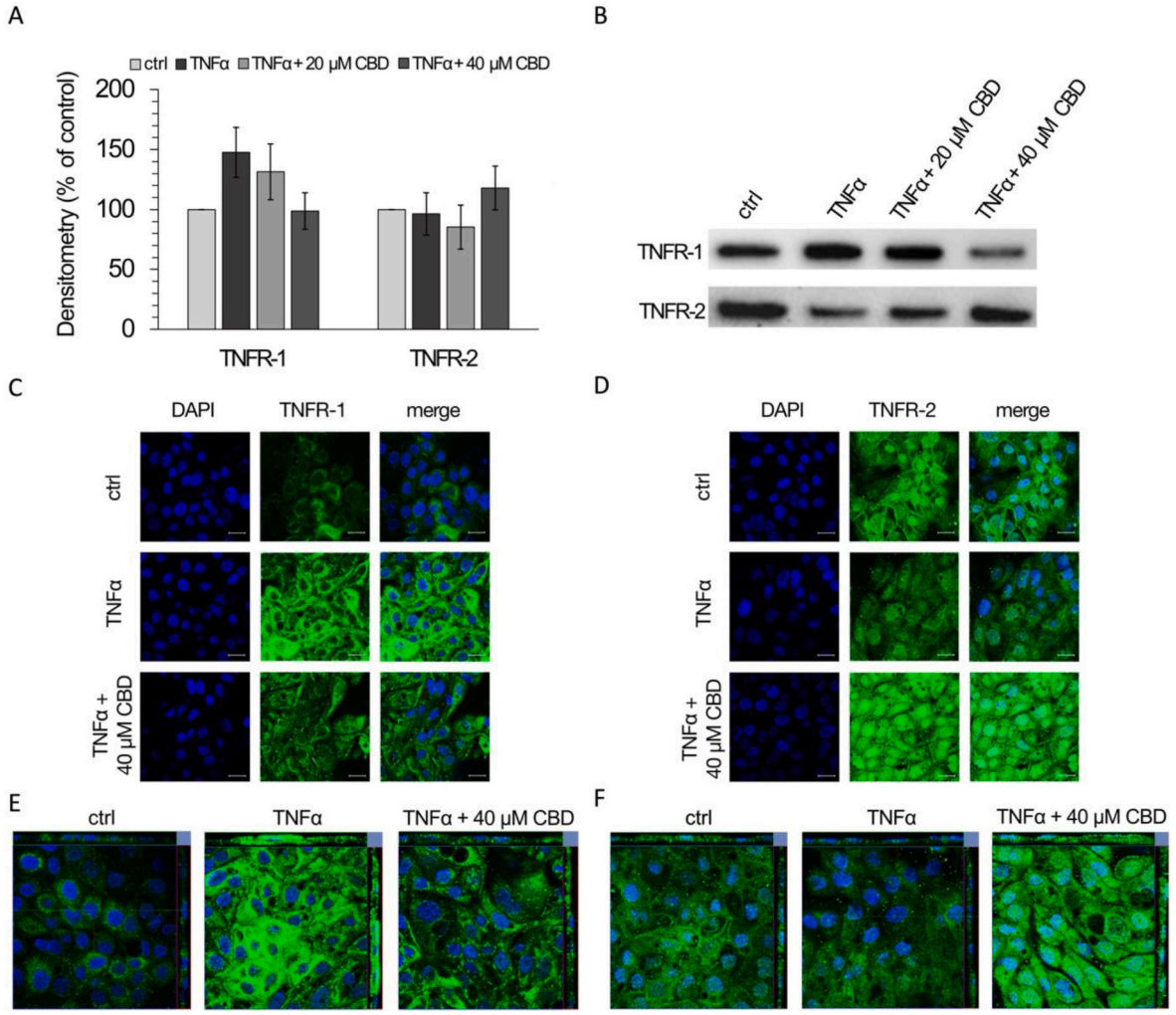

Fig. 8. Specific TNF receptor expression after co-incubation with 1000 U/mL TNFα and CBD. **A** Densitometry and **B** Western blots of TNFR-1 and TNFR-2 after 48 h of incubation with TNFα and CBD ($n = 9–12$). Immunofluorescent staining of **C** TNFR-1 (green), **D** TNFR-2 (green), nuclei were stained in blue (DAPI; scale bar: 20 μm; $n = 4$; representative images). Z-stack images of **E** TNFR-1(green) and **F** TNFR-2 (green) (DAPI (blue); scale bar: 20 μm; $n = 3$; representative images).

(ctrl: 100%, 20 μM: 156.68 ± 31.30%, 40 μM CBD: 150.69 ± 29.57%; Fig. 9A and B), and immunofluorescent staining (Fig. 9D), neither CBD nor TNFα significantly altered the expression of the cannabinoid receptors. Immunofluorescent staining revealed the different distributions of the cannabinoid receptors, with CBR-1 (green; Fig. 9C) being strongly expressed in individual cells, whereas CBR-2 (green; Fig. 9D) was more evenly distributed.

4. Discussion

Cannabidiol (CBD) has received growing attention in recent years because of its potential therapeutic properties for various ailments, including chronic pain, anxiety, and epilepsy [41]. It has been combined with other biomaterials such as chitosan and alginate-based hydrogel [4,5] and is a promising compound for the development of new therapeutic options aimed at restoring a disturbed barrier resulting from inflammatory conditions [42,43]. Despite its increasing popularity, much research is still needed if we are fully to understand the potential benefits and risks of using CBD to treat various health conditions. Our aim has therefore been to investigate the effects of CBD under normal *versus* inflammatory conditions in the non-tumorigenic intestinal epithelial cell line IPEC-J2.

In our study, we observed that CBD alone had the ability to increase transepithelial resistance (TER) in IPEC-J2 cells. Specifically, our findings indicated that higher concentrations of 20 μM and 40 μM CBD resulted in a significant increase in TER values at 4 h and 8 h. Additionally, we found that incubation with CBD led to a decrease in the paracellular permeability of [^{3}H]-D-Mannitol after 48 h, indicating still a barrier-strengthening effect of this cannabinoid. Although a decrease of TER after 48 h of CBD could also be observed, which is in accordance with the concept of independent regulation of the paracellular high-capacity pore pathway and the low capacity leak pathway [44,45]. Analysis of immunoblots revealed that CBD significantly induced the expression of claudin-4, a TJ protein known for its barrier-strengthening properties [46]. Claudin-4 plays a crucial role in regulating paracellular ion permeability and can be affected by various factors such as bacteria [47], bacterial toxins [48], and secondary plant compounds [25]. In a study conducted by Van Itallie et al., the induction of claudin-4 was demonstrated to lead to an increase in TER and a decrease in Na^+ permeability [49]. Furthermore, our immunofluorescence analysis revealed a stronger signal of claudin-1, claudin-4, and claudin-5 within TJ complexes, indicating the upregulation of these proteins by CBD.

The paracellular permeability of mannitol is based on the leak pathway and can be regulated by myosin light chain kinase 1 (MLCK1) [50,51]. Cytokines such as interleukin-1β and TNFα can activate the expression and enzymatic activity of MLCK1, which then initiates the endocytosis of occludin through a cascade, thereby increasing the permeability of TJs [52–54]. Activation of the NFkB signaling pathway

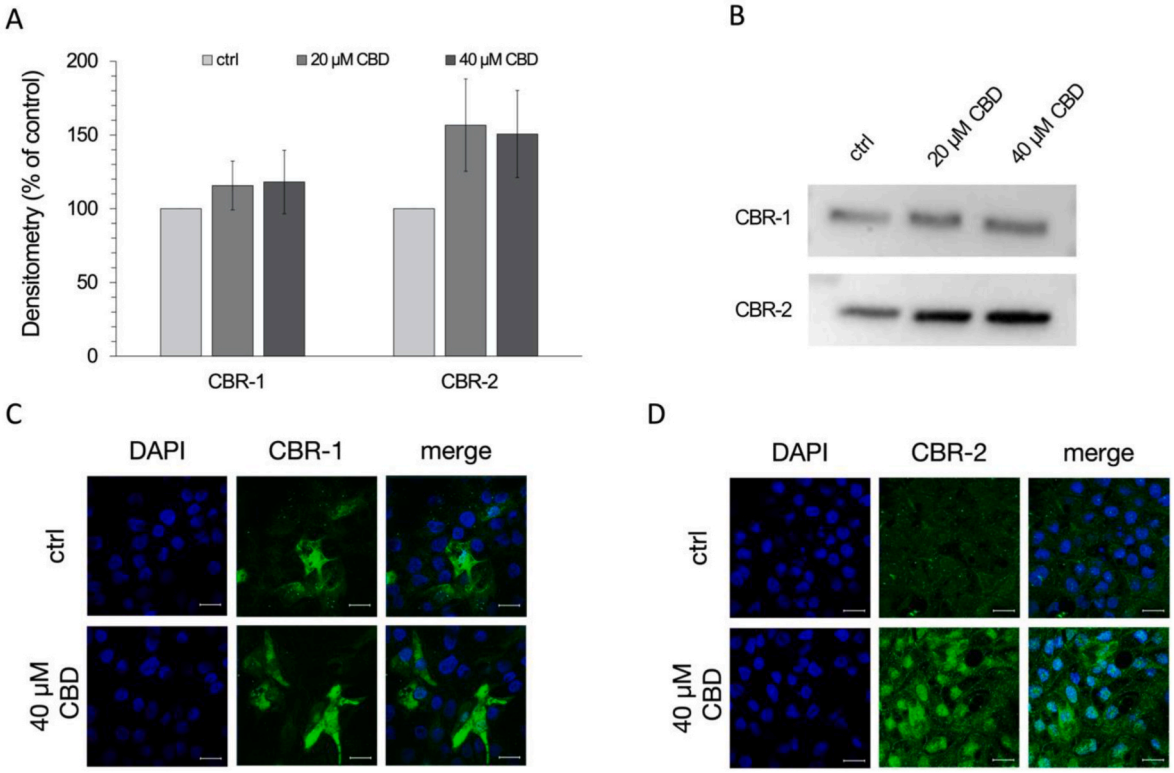

Fig. 9. Analysis of cannabinoid-receptor expression after incubation with CBD. **A** Densitometry and **B** Western blots of CBR-1 and CBR-2 after 48 h of incubation with CBD ($n = 5–6$). Immunofluorescent staining of **C** CBR-1 (green), **D** CBR-2 (green), nuclei were stained in blue (DAPI; scale bar: 20 µm; $n = 4$; representative images).

can induce the transcription of MLCK [52]. Several studies have demonstrated that CBD is capable of inhibiting the NFkB signaling pathway [55–57]. This might explain the observed decrease in paracellular permeability, as the inhibition of the NFkB pathway might lead to the reduced expression of MLCK.

To elucidate further the decrease of TER, we performed an Apotox-Glo assay to assess the potential impact of CBD on cell viability, apoptosis, and toxicity. Notably, the results revealed a slight increase in apoptotic activity at 40 µM CBD after 48 h compared with the control group. CBD is known to induce apoptosis through various mechanisms [58]. Several prior investigations have led to the proposal that the capacity of CBD to induce apoptotic cell death is linked to its anticancer properties [59,60]. Jeong et al. have demonstrated that CBD induces apoptosis in gastric cancer cell lines without affecting a normal gastric epithelial cell line [61]. One possible mechanism of apoptosis by CBD is the activation of CBR-1 and CBR-2, which can trigger a series of intracellular signaling pathways that lead to apoptosis [62]. Another possible mechanism might be the modulation of the expression of various proteins involved in apoptosis regulation, such as Bcl-2 and caspases [63]. The induction of apoptosis is unlikely to explain the decrease in TER with the paracellular permeability being unaffected, as apoptosis would also result in an increase of the leak pathway. Rather, the observed increase in caspase-3 and -7 levels indicates the beginning of apoptosis. Apoptosis comprises three phases: the initiation phase, the effector phase during which caspase-3 and -7 are activated, and the third phase during which morphological changes and DNA fragmentation occur [64]. In support of this hypothesis, we have not observed any loss of cells and gaps in the cell monolayer in the immunofluorescence images. Moreover, caspases have been shown also to be involved in non-apoptotic cellular mechanisms [65].

We also investigated the effects of CBD on the barrier function of cells under inflammatory conditions. The results showed that TNFα treatment led to a significant decrease in TER and increased permeability, whereas co-incubation with CBD mitigated or even prevented these barrier-disrupting effects. Prior research has shown that CBD has the potential to prevent the decline in resistance and the decrease in paracellular permeability resulting from inflammation [28,30]. In our study, CBD also prevented the decreased expression of claudin-1 and -3 caused by TNFα and increased the expression of claudin-5. This suggests that CBD can protect against the barrier-disturbing effects of TNFα on the distribution and localization of TJ proteins such as claudin-1, claudin-3, and claudin-4, as also observed in our confocal laser microscopy studies.

We further investigated the impact on the distribution and amount of CBD- and TNF receptors after incubation with TNFα and CBD. The results indicate that CBD, when used alone or in combination with TNFα, can affect the expression of TNF receptors (TNFR-1 and TNFR-2), as indicated by Western blot and immunocytochemical staining. In an *in vivo* study on mice subjected to bile-duct ligation, CBD demonstrated its potential to reduce TNFR-1 levels in the hippocampus [66,67]. Additionally, in another study involving an ischemic stroke model, CBD was found to exhibit similar effects on TNFR-1 levels [67]. Interestingly, TNFR-2 was found to be downregulated when cells were exposed to CBD alone, but upregulated when co-incubated with TNFα. No studies have been found so far that demonstrate an effect of CBD on TNFR-2. However, activation of TNFR-2 additionally has positive effects in terms of disease, as the receptor also has a proliferative and cell-survival effect [68].

TNF receptors have previously been identified as potential targets for CBD [69], although further research is needed to elucidate the underlying mechanisms and potential therapeutic implications of these findings.

Our study has demonstrated that CBD does not significantly impact CBD receptors, despite a trend towards an observed increase in CBR-2. CBD has however recently been shown to enhance CBR-2 on $CD4^+$ and regulatory T cells in the intestine [70]. We have been able to demonstrate a differential distribution of the receptors by means of immunofluorescence imaging. CBR-1 is expressed more strongly by

individual cells, whereas CBR-2 is more uniformly expressed by all cells.

IPEC-J2 cells, cultured with species-specific porcine serum, are a valuable model for studying the intestinal epithelium because of their ability to exhibit the morphological and functional characteristics of the small intestine [38,71]. Therefore, the use of IPEC-J2 cells provides important physiological insights into the effects of CBD on TNFα-induced inflammation in the intestinal epithelium. Since the systemic bioavailability of cannabinoids trough oral application is around 10–20% [72], it is reasonable to assume that this concentration is also achieved in the intestine. Additionally, it can be assumed that high concentrations of up to 40 μM of CBD in the intestine can be attainable with the administration of 120 mg/kg (381 μM) orally [73]. Whether these concentrations *in vivo* yield the same positive effects observed *in vitro* on IPEC-J2 cells should be the subject of further investigations. In our model, CBD might also counteract adverse effects of other recently characterized biomaterials, such as chitosan [17], and caprate [18], and generally can be regarded as a promising ingredient of anti-inflammatory applications.

5. Conclusions

This study highlights the potential of CBD-based applications in mitigating the barrier-disrupting effects of TNFα. Our results demonstrate that CBD enhances the presence of sealing TJ proteins, thereby strengthening the intestinal barrier. Additionally, CBD decreases the expression of TNFR-1, which plays a crucial role in the disruption of the intestinal barrier. Overall, these findings suggest that CBD will be a promising component in biomaterial-based therapeutic approaches for the treatment of inflammatory pathomechanisms.

Credit author statement

Elisa Boehm: Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Visualization. Linda Droessler: Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing. Salah Amasheh: Conceptualization, Writing – review & editing, Supervision, Funding acquisition, Project administration.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mtbio.2023.100808.

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