The non-euphoric phytocannabinoid cannabidivarin counteracts intestinal inflammation in mice and cytokine expression in biopsies from UC pediatric patients

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ARTICLE INFO

Keywords:
Inflammatory bowel disease
Cannabinoids
TRPA1 channels

ABSTRACT

Patients with ulcerative colitis (UC) using marijuana have been reported to experience symptomatic benefit. Cannabidivarin (CBDV) is a safe non-psychoactive phytocannabinoid able to activate and desensitize TRPA1, a member of the TRP channels superfamily, which plays a pivotal role in intestinal inflammation. Here, we have investigated the potential intestinal anti-inflammatory effect of CBDV in mice and in biopsies from pediatric patients with active UC. Colonic inflammation was induced in mice by dinitrobenzenesulfonic acid (DNBS). The effect of orally administered CBDV on macroscopic and microscopic damage, inflammatory parameters (*i.e.* myeloperoxidase activity, intestinal permeability and cytokine production) and faecal microbiota composition, was evaluated 3 days after DNBS administration. TRPA1 expression was studied by RT-PCR in inflamed colons of mice as well as in mucosal colonic biopsies of children with active UC, whose response to incubation with CBDV was also investigated. CBDV attenuates, in a TRPA1-antagonist sensitive manner, DNBS-induced signs of inflammation including neutrophil infiltration, intestinal permeability, and cytokine (*i.e.* IL-1β, IL-6 and the chemokine MCP-1) production. CBDV also alters the dysregulation of gut microbiota associated to colitis. Finally, CBDV lessens cytokine expression in colonic biopsies from pediatric patients with ulcerative colitis, a condition in which TRPA1 was up-regulated. Our preclinical study shows that CBDV exerts intestinal anti-inflammatory effects in mice *via* TRPA1, and in children with active UC. Since CBDV has a favorable safety profile in humans, it may be considered for possible clinical trials in patients with UC.

Abbreviations: ASD, autism spectrum disorder; CBDV, cannabidivarine; CD, crohn's disease; CMC, carboxymethylcellulose; DMSO, dimethylsulfoxide; DNBS, 2,4-dinitrobenzenesulfonic acid; DSS, Dextran Sulphate Sodium; EC50, half maximal effective concentration; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FITC, fluorescein isotiocyanate; GM, gut microbiota; H&E, haematoxylin and eosine; HC030031, TRPA1 selective antagonist; HPLC, high-performace liquid chromatography; i.p., intraperitoneal administration; IBD, inflammatory bowel disease; IC50, half maximal inhibitory concentration; IL, interleukin; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-10, interleukin-10; MCP-1, monocyte chemoattractant protein-1; MPO, myeloperoxidase; OTUs, operational taxonomic units; PBS, phosphate buffered saline; RPMI, roswell-park memorial institute; RT-PCR, real time-polymerase chain reaction; TRP, transient receptor potential; TRPA1, transient receptor potential ankirin-1; TRPV1, transient receptor potential vanillolide type-1; TRPV2, transient receptor potential vanillolide type-2; UC, ulcerative colitis

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Accepted 20 September 2019

1. Introduction

Inflammatory bowel diseases (IBDs), including ulcerative colitis (UC) and Crohn's disease (CD), are relapsing and lifelong disorders characterized by chronic inflammation of the gastrointestinal tract. IBDs represent a global health burden that affects millions of people with rising incidence and prevalence worldwide [1,2]. IBD results from a complex interaction between environmental, genetic and epigenetic risk factors that cause an inappropriate mucosal immune response leading to intestinal inflammation. Additionally, recent evidence suggests that gut microbiota dysbiosis is strictly linked to initiation and progression of IBD, although it is still unclear whether this is a primary or secondary occurrence [3].

The plant *Cannabis sativa* contains over 100 terpenophenolic constituents, known as phytocannabinoids [4,5]. A number of surveys and small clinical trials suggest that patients with IBD use cannabis preparations to alleviate symptoms such as diarrhea, abdominal pain, and loss of appetite [6–11]. Experimentally, single isolated cannabinoids such as delta-9-tetrahydrocannabinol [12], cannabidiol [12–15], cannabigerol [16], and cannabichromene [17] have been shown to attenuate intestinal inflammation.

Cannabidivarin (CBDV) is a non-psychoactive phytocannabionid that was identified by Vollner and coworkers in 1969, although little information on its pharmacology and mode of action has been reported in the subsequent forty years [18]. More recently, pharmacodynamic studies have shown that CBDV shows very little affinity for cannabinoid receptors, but is a potent agonist of the transient receptor potential (TRP) ankyrin type-1 (TRPA1) [19]. TRPA1, a cation-permeable channel, is a member of the TRP superfamily with a pivotal role in intestinal inflammation [20]. Specifically, TRPA1 is expressed in sensory fibers innervating the gastrointestinal tract, mediates gastrointestinal hypersensitivity to mechanical stimuli, and modulates the release of pro-inflammatory peptides [21]. Currently, CBDV is under clinical investigation for epilepsy (NCT02369471) and Autism Spectrum Disorder in children (NCT03202303).

Here, we report that oral CBDV exerts, *via* TRPA1, anti-inflammatory effects and changes the composition of gut microbiota in the murine model of colitis induced by DNBS. Additionally, CBDV reduces colonic inflammation in biopsies from pediatric patients with IBD, a condition in which we found an unprecedented example of TRPA1 upregulation.

2. Materials and methods

2.1. Drugs and reagents

2,4-dinitrobenzenesulfonic acid (DNBS) and myeloperoxidase (MPO) from human leucocytes, were purchased from Sigma Aldrich S.r.l. (Milan, Italy). CBDV [purity by high-performance liquid chromatography (HPLC), 99.0%], was kindly supplied by GW Research Ltd (Cambridge, UK). CBDV was dissolved in ethanol/Tween20/saline (1:1:8) for intraperitoneal (i.p.) injection (60 µl/mouse) and in carboxymethylcellulose (CMC 1%, 150 µl/mouse) for oral gavage administration. The vehicles had no significant effects on the responses under study.

2.2. Patients

Human studies were approved by Ethical Committee of the University of Trieste, Department of Medicine (protocol number 111/2015). Pediatric patients with a well-established diagnosis of ulcerative colitis (UC) were included. Human colon samples were obtained by colonoscopy as previous described [22]. The patients were clinically scored using the Geboes score [23]. A full description of the Geboes score as well as of the Geboes score allocated to each patient is reported in Supplementary Table S1. The Geboes score is divided in 6 grades: architectural changes [grade 0], chronic inflammatory infiltrate [grade 1], lamina propria neutrophils and eosinophils [grade 2], neutrophils in epithelium [grade 3], crypt destruction [grade 4] and erosions or ulcerations [grade 5] [23].

2.3. Human tissue culture

Colonic biopsies were collected from inflamed areas from pediatric patients with active UC. Biopsies of non-inflamed tissues were collected from areas proximal to the inflamed ones. Colonic biopsies were washed with cold phosphate buffer saline (PBS), divided in two pieces and put in culture in a 0.95 cm^2 plate with or without CBDV (10 µM) in RPMI-1640 medium [supplemented with 10% foetal bovine serum (FBS), 100U/ml penicillin and 100 µg/ml streptomycin and 2 mM l-glutamine] [24]. The concentration of CBDV was selected on the basis of in vitro studies reported in literature [25]. After 18 h, supernatants from colonic specimens were collected and stored at −80 °C for interleukin-1β detection.

2.4. Animals

The experimental protocol was evaluated and approved by the Institutional Animal Ethics Committee for the use of experimental animals and conformed to guidelines for the safe use and care of experimental animals in accordance with the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European Communities Council (86/609/ECC and 2010/63/UE).

Male adult CD1 mice (25–30 g) were purchased from Charles River Laboratories (Calco, Lecco, Italy) and maintained in the animal care facility at the University of Naples in polycarbonate cages under controlled temperature (23 ± 2 °C), constant humidity (60%) and with a 12-h light, 12-h dark cycle. The animals were acclimatised to their environment at least 1 week under standard conditions, with free access to tapwater and standard rodent diet; all mice were fasted overnight before the intracolonic injection of DNBS and for 2 h before the oral gavage of the drugs. Mice were randomly allocated to different experimental groups and outcome assessments were performed in single-blind.

2.5. Induction of experimental colitis and pharmacological treatments

2.5.1. DNBS-induced colitis

DNBS (150 mg/kg) was dissolved in 50% ethanol (150 µl/mouse) and was administrated into the rectum by a polyethylene catheter (1 mm in diameter) inserted approximately 4.5 cm proximal to the anus [15]. In the preliminary experiments this dose of DNBS was found to induce remarkable colonic damage associated with high reproducibility and low mortality. After 3 days, when intestinal inflammation can be easily assessed, all mice were euthanized by asphyxiation with CO_2, the mice abdomen was opened by a midline incision and the colon removed, isolated from surrounding tissues, length measured, opened along the antimesenteric border, rinsed and weighed, then processed [16]. Mice body weight was measured every day during the treatment period. The colon weight/colon length *ratio* (mg/cm) was used as an indirect marker of inflammation (n = 7 mice). For biochemistry analysis, tissues were kept at −80 °C until the use, while for histopathological analysis the colons were fixed in 10% formaldehyde.

In the preventive protocol, CBDV was given intraperitoneally (0.3–10 mg/kg) or by oral gavage (0.3–30 mg/kg) once a day for 7 consecutive days starting 3 days before DNBS administration, while in the curative protocol CBDV, at the same doses, was given for three consecutive days starting 24-h after DNBS administration. The last administration was given 1 h (for intraperitoneal administration) or 2 h (for oral gavage) before the euthanasia. The CBDV dose-range was selected on the basis of other phytocannabinoids previously evaluated in experimental colitis [15,16]. The TRPA1 antagonist, HC030031, was

dissolved in DMSO/Tween20/saline (1:1:8) and intraperitoneally injected (30 mg/kg) alone or 30 min before CBDV administration. The dose of HC030031 was selected on the basis of preliminary experiments showing that the antagonist, at 30 mg/kg dose, did not affect, per se, DNBS-induced intestinal inflammation. A higher dose of HC030031 (*i.e.* 50 mg/kg) reduced, per se, DNBS-induced colitis (data not shown).

2.5.2. DSS-induced colitis

Mice were administered 4% dextrane sulfate sodium (DSS, molecular weight 36,000–50,000; MP Biomedical, Illkirch, France) in drinking water ad libitum for 5 days; after that, the DSS solution was replaced with normal drinking water for additional 3 days. Control mice were given normal drinking water throughout the study. CBDV was administered by oral gavage [at dose of 3 mg/kg (dose found to be the most effective in the DNBS model of colitis)] once a day for 6 consecutive days starting 3 days after the first administration of DSS. The last administration of CBDV was given 2 h before the euthanasia. At day 8 all mice were euthanized, the mice abdomen was opened by a midline incision, the colon removed, isolated from surrounding tissues, length measured, rinsed and weighed (to measure colon weight/colon length ratio) and the spleen was collected and weighed. The DSS dose (4% w/v) and the time points (5-day treatment and sacrifice at day 8) were selected on the basis of our preliminary experiments showing a remarkable colonic damage associated to high reproducibility and low mortality.

2.6. Measurements of myeloperoxidase (MPO) activity

MPO activity, a peroxidase enzyme used to quantify the neutrophil infiltration in whole-tissue colons, was determined as previously described [15]. Full-thickness colons were mechanically homogenized in a lysis buffer composed of 0.5% hexadecyltrimethylammonium bromide in 3-(N morpholino) propanesulfonic acid (MOPS) 10 mM in the ratio of 50 mg tissue/ mL MOPS. The homogenates were centrifuged for 20 min at 15 000 × g at 4 °C and an aliquot of the supernatant was incubated with sodium phosphate buffer (NaPP pH 5.5) and tetra-methylbenzidine 16 mM. After 5 min, hydrogen peroxide (H_2O_2; 9.8 M in NaPP) was added and the reaction was stopped with acetic acid (2 M). The rate of change in absorbance was measured by a spectrophotometer at 650 nm. Different dilutions of human MPO enzyme of known concentration were used to obtain a standard curve. MPO activity was expressed as U/mg of tissue (n = 6 mice).

2.7. Histology

For histological analysis see online supplementary material (n = 3 mice).

2.8. Enzyme-linked immunosorbent assay

Interleukin (IL)-1β, IL-10 and IL-6 concentrations in homogenate

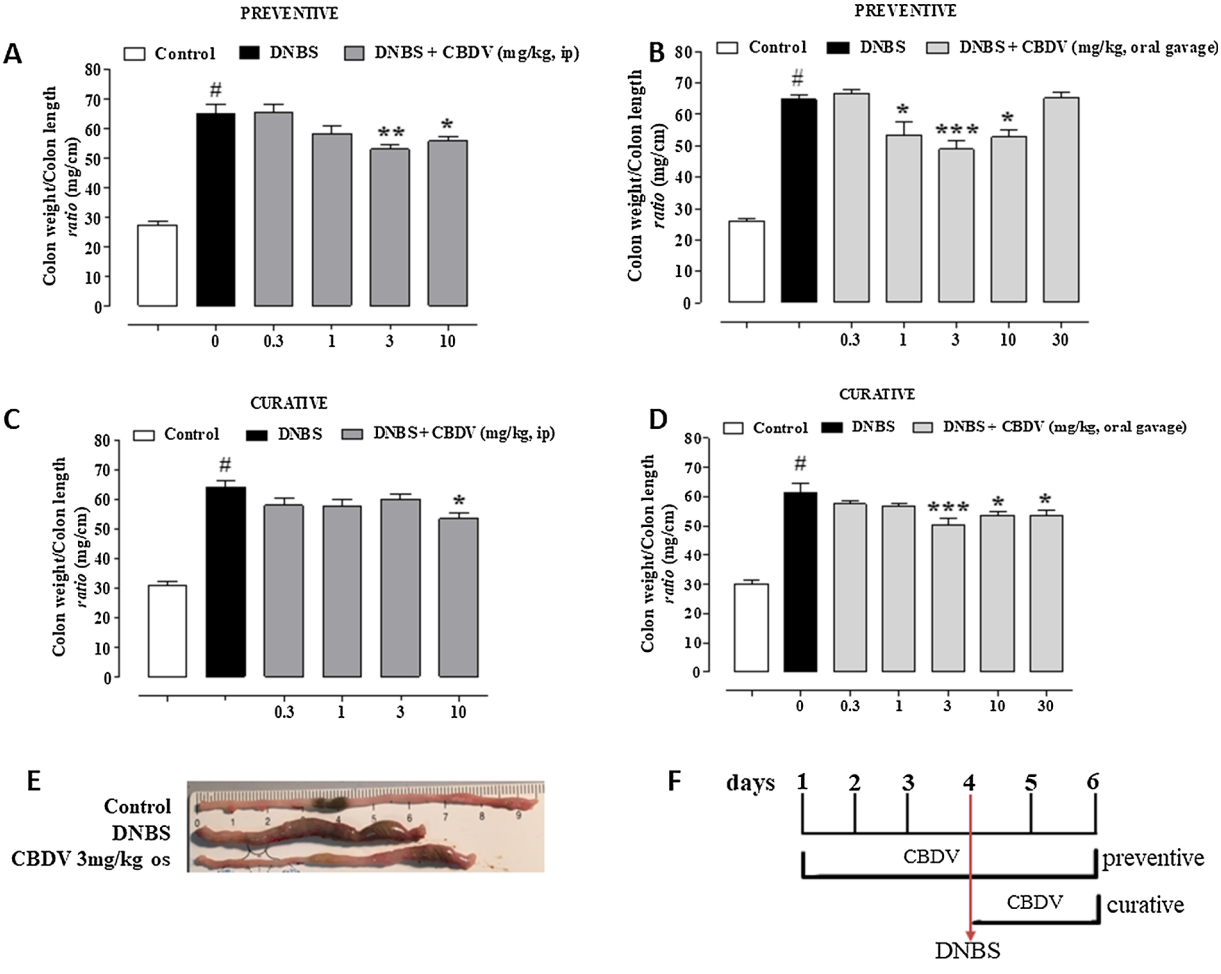

Fig. 1. Preventive and therapeutic effect of cannabidivarin (CBDV) on colon weight/colon length ratio in DNBS-induced acute colitis in mice. CBDV was administered once a day both in a preventive [*i.e.* starting three days before DNBS (A and B)] or therapeutic [*i.e.* starting 24 h after DNBS (C and D)] setting. Colons were collected three days after the induction of colitis by DNBS. Representative colon images of the indicated experimental groups in a curative protocol (E). Experimental protocol for DNBS-induced colitis (F). All data are represented as mean ± SEM of 7 mice for each experimental group. Statistical significance was calculated using one-way ANOVA test. $^{\#}p < 0.001$ *vs* control, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* DNBS alone.

obtained from full-thickness mice colonic tissues or from human mucosal colonic biopsies were determined using commercial ELISA kits (ThermoFisher Scientific, Milano) according to manufacturer's instructions (n = 5 mice).

2.9. In vivo epithelial barrier permeability

In vivo intestinal permeability was measured using a FITC-labelled dextran method, as previously described [26]. Mice were administered with 600 mg/kg of FITC-conjugated dextran (molecular mass 3–5 kDa) by oral gavage 2 days after DNBS-induced colitis. After 24 h, the blood was collected by cardiac puncture and the serum was immediately analysed for FITC-derived fluorescence (fluorescent microplate reader with 2104 EnVision Multilabel Plate Readers, PerkinElmer Instruments, Walthan, MA, USA) with an excitation wavelength of 485 ± 14 nm and emission wavelength of 520 ± 25 nm. Serial dilution of FITC dextran was made to generate a standard curve. Intestinal permeability was expressed as the concentrations of FITC (μM) detected in the serum (n = 6 mice).

2.10. Gene expression analysis by quantitative Real-Time (qRT)- PCR

Total RNA from murine and human tissues was purified, quantified, characterized and retrotranscribed as previously described by Iannotti and collaborators [27]. For more details see online supplementary material and Supplementary Table S2 (n = 3–6 mice).

2.11. Fecal microbiota analysis by 16S rRNA gene sequencing using Illumina technology

Fecal microbiota was analyzed on a subset of three mice randomly selected from each group. Fecal samples were collected at time points T0 (before the induction of the colitis) and T3 (three days after the induction of colitis by DNBS) from animals treated or not with CBDV (for more details see online supplementary material).

2.12. Metagenomic data analysis

For details see online supplementary material.

2.13. Statistics

Data are expressed as the mean ± SEM or SD of n experiments. As all data were normally distributed (normality was tested using the Anderson-Darling method, http://www.kevinotto.com/RSS/templates/Anderson-Darling Normality Test Calculator.xls), statistical analysis was performed using Student's *t*-test (for comparing a single treatment mean with a control mean) and a one-way ANOVA followed by a Tukey multiple comparisons test (for analysis of multiple treatment means). *P*-value < 0.05 was considered to be significant. G Power was used for sample size calculation.

3. Results

3.1. Preventive or curative CBDV (either oral or intraperitoneal) reduces colon weight/colon length ratio in experimental acute colitis

Intracolonic DNBS administration caused a number of signs of inflammation and disease, including increased colon weight/colon length *ratio*, neutrophil infiltration (as revealed by MPO activity), intestinal permeability, histological damage, up-regulation of pro-inflammatory cytokines and down-regulation of the anti-inflammatory cytokine IL-10

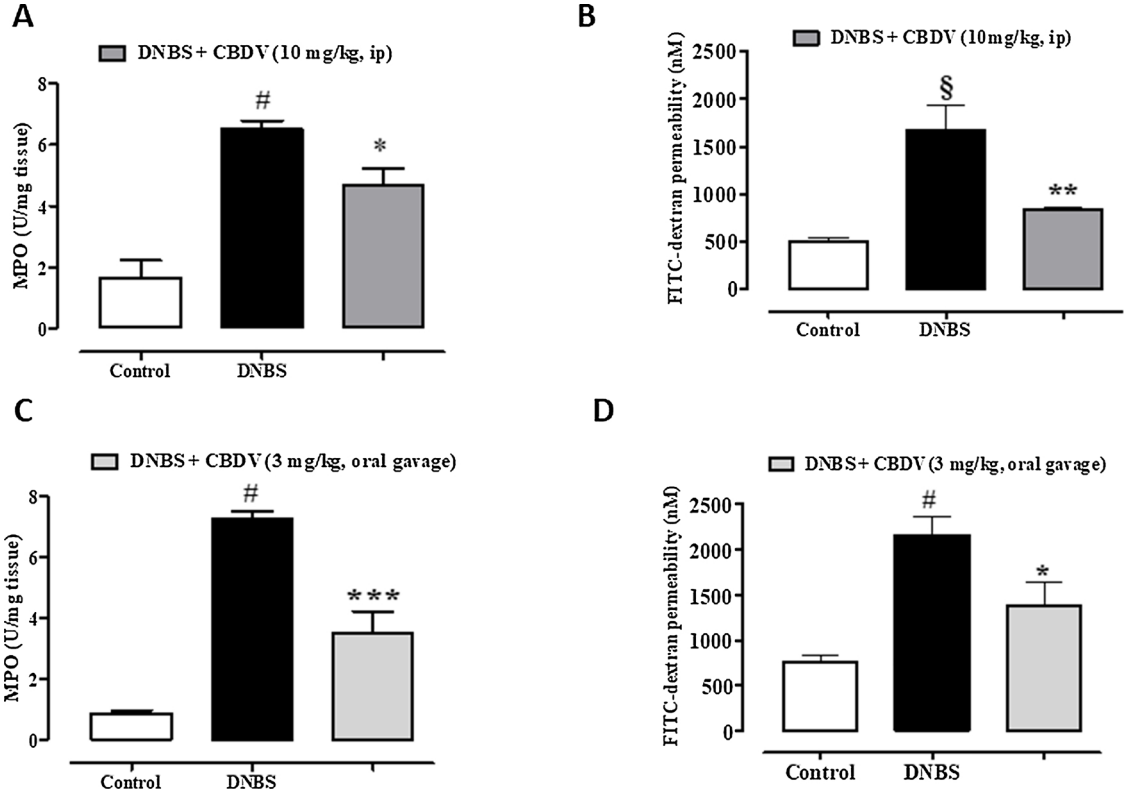

Fig. 2. Therapeutic effect of cannabidivarin (CBDV) on inflammatory parameters in DNBS-induced acute colitis. The effect of CBDV (10 mg/kg, i.p. or 3 mg/kg by oral gavage), given once a day for three consecutive days starting 24 h after DNBS, was evaluated on myeloperoxidase (MPO) activity (A and C) and intestinal permeability (B and D). Inflammatory parameters were assessed three days after the induction of colitis by DNBS. All data are represented as mean ± SEM of 6 mice for each experimental group. Statistical significance was calculated using one-way ANOVA test. §p < 0.01 and # < 0.001 *vs* control, *p < 0.05, **p < 0.01 and ***p < 0.001 *vs* DNBS alone.

(Fig. 1–3). Intraperitoneal or oral CBDV (0.3–10 mg/kg), given before (preventive protocol, Fig. 1A,B and F) or after (curative protocol, Fig. 1C,D and F) the inflammatory insult, significantly reduced colon weight/colon length ratio increased by DNBS. Significant effects were achieved starting from the 3 mg/kg (preventive protocol) and 10 mg/kg (curative protocol) doses when CBDV was given intraperitoneally (Fig. 1A and C). When CBDV was given orally, significant inhibitory effects on colon weight/colon length ratio were observed starting from 1 mg/kg (preventive protocol) or 3 mg/kg (curative protocol) dose (Fig. 1B and D). Because the main goal in IBD is to cure rather than to prevent, further studies were conducted using the curative protocol.

3.2. Oral and intraperitoneal CBDV decreases neutrophil infiltration and intestinal permeability in experimental acute colitis

The curative effect of CBDV at the dose of 10 mg/kg (intraperitoneal) and 3 mg/kg (oral gavage) on DNBS-induced colitis was confirmed by further analyses.

Neutrophil infiltration was biochemically monitored by measuring MPO activity in the colonic tissue. DNBS increased MPO activity, which was significantly reduced by either intraperitoneal (10 mg/kg) or oral gavage (3 mg/kg) administration of CBDV (Fig. 2A and C).

Experimental and clinical colitis involve abnormal intestinal permeability [28]. To investigate if CBDV affects intestinal barrier function, we determined the serum level of FITC-dextran after its oral administration. DNBS-treated mice displayed higher FITC-dextran serum concentrations than the control group and, importantly, CBDV reduced such increase when given either intraperitoneally or by oral gavage (Fig. 2B and D).

Because a significant anti-inflammatory effect of CBDV (as revealed by colon weight/colon length ratio, MPO activity and barrier function) was achieved at a lower dose (*i.e.* 3 mg/kg) after oral administration than after i.p. injection, and because the oral route of administration is more clinically relevant, we next focused our attention on the effect of CBDV given by oral gavage.

3.3. Oral CBDV attenuates DNBS-induced acute colonic damage and cytokine production

Histological evaluation of H&E-stained colon sections of the mice treated with DNBS showed an acute inflammation affecting the colonic mucosa characterized by erosion, ulceration, edema, hyperplasia, loss of goblet cells, disruption of crypt integrity, and massive inflammatory cell infiltration (Fig. 3A). Tissues from mice treated with oral CBDV (3 mg/kg) displayed a remarkable reduction in damage, mucosal ulcerations and mononuclear cell infiltration (Fig. 3A). CBDV-treated mice without DNBS were free of histological signs of colitis (data not shown).

The protective effect of CBDV was corroborated by quantification of a cytokines panel, which is dysregulated by DNBS administration, as assessed by quantitative (q)-PCR. Oral CBDV, at the dose of 3 mg/kg, decreased the colonic mRNA levels of pro-inflammatory cytokines (*i.e.* IL-1β and IL-6), but it did not modify the levels of IL-10, the main anti-inflammatory cytokine [Fig. 3B]. Furthermore, CBDV reduced colonic mRNA expression of the chemokine monocyte chemoattractant protein-1 (MCP-1), a chemokine responsible for recruiting macrophages into the inflamed tissue (Fig. 3B). Because in our experiments IL-1β increased much more (*i.e.* approximately 19-fold increase) compared to the other cytokines, we also measured its expression by ELISA. In agreement with the mRNA expression data, CBDV decreased by 78% IL-1β protein levels in the colon of DNBS-treated mice [IL-1β (pg/mL): control, 1283.2 ± 50.1; DNBS, 2828.3 ± 404.7$^{\#}$; DNBS + CBDV 3 mg/kg p.o., 1618.1 ± 128.3*; #p < 0.01 *vs* control and *p < 0.01 *vs* DNBS alone; mean ± SEM, n = 6].

3.4. Effect of oral CBDV on gut microbiota alterations induced by DNBS

Amplicon sequencing of the 16S rRNA gene (regions V3-V4) was conducted on a subset of animals randomly selected from each group (control, DNBS and DNBS plus CBDV at time points T0 and T3). The sequencing process generated 1,139,314 high quality sequences from 18 fecal samples, assigned to a total of 2968 operational taxonomic

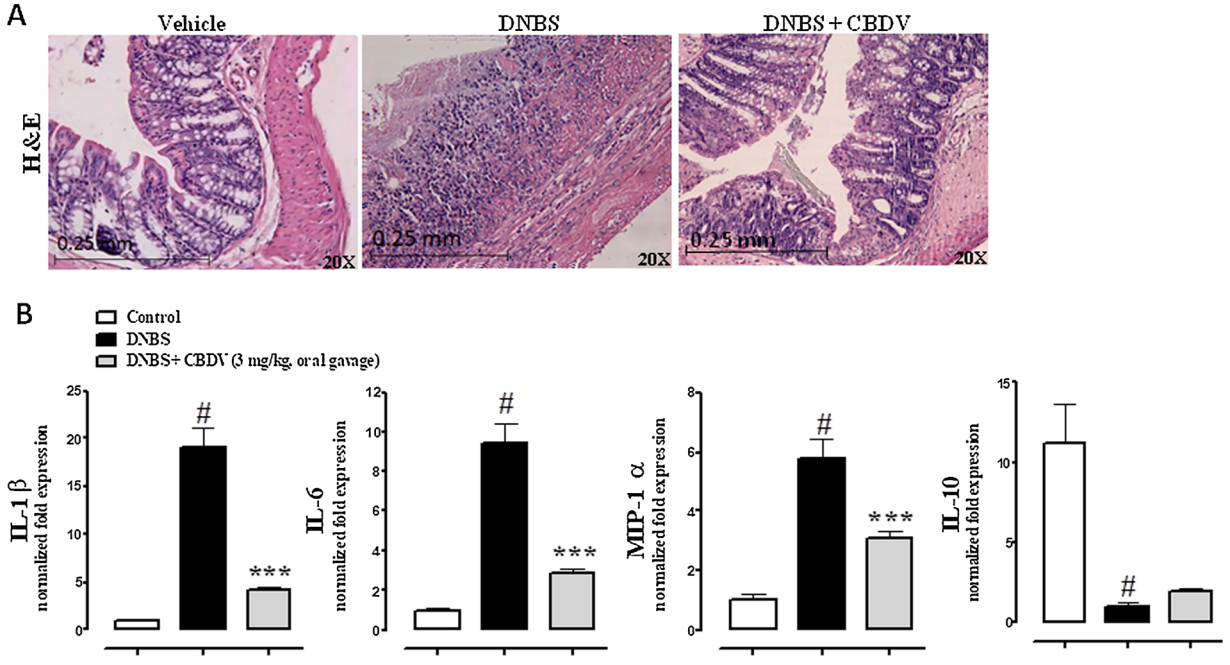

Fig. 3. Oral cannabidivarin (CBDV) reduces the inflammatory response and stimulates colonic regeneration following DNBS-induced histological damage. Representative H&E stained colon cross-sections of mice treated with vehicle, DNBS and DNBS plus CBDV (3 mg/kg by oral gavage), given once a day for three consecutive days starting 24 h after DNBS. Colons were collected three days after the induction of colitis by DNBS. Original magnification 20X (A).
IL-1β, IL-6, MCP-1α and IL-10 colonic expression, evaluated by qPCR analysis, in vehicle-, DNBS- or DNBS plus CBDV-treated mice (B). All data are represented as mean ± SD. #p < 0.001 *vs* control, ***p < 0.001 *vs* DNBS alone.

units (OTUs). A sequencing depth of 18,871 sequences/sample, with Good's coverage > 98%, was considered to describe and compare gut microbiota (GM) composition among the investigated groups. The total number of OTUs observed at sequencing depth was 2261. In order to evaluate species richness (number of observed species) and evenness (Shannon entropy) within each group, alpha diversity analyses were computed (supplementary Table S3). Comparison of alpha diversity results revealed no significant differences among groups, indicating no substantial deviations in species richness and distribution within communities upon treatments (supplementary Table S3). Phylogenetic distances among the samples of control, DNBS and DNBS plus CBDV at T0 and T3 were evaluated by unweighted beta diversity (OTUs presence or absence) and weighted beta diversity (abundance of all shared OTUs). Unweighted and weighted beta diversity analyses showed significant differences in GM composition among groups at time point T3 (Fig. 4A and B). After the inflammatory insult (DNBS T3) the microbial fecal composition showed a significant increase of phylogenetic distance compared to control samples, indicating a shift in the type of species and a strong alteration in relative abundances of shared species (Fig. 4A and B). The observed increased distance represents the dysbiosis induced by DNBS treatment. CBDV treatment showed a trend to decrease the Unifrac distances in terms of type and relative abundance of species with respect to mice treated with DNBS alone, producing a microbiota profile more similar to controls (Fig. 4A and B).

Profiling and comparison of GM in the different groups were carried out at phylum at T3 time point (Fig. 4C). Firmicutes and Bacteroidetes were the most abundant phyla detected in all groups. A striking increase in Proteobacteria upon DNBS treatment was identified with respect to untreated mice; short term oral CBDV administration produced a trend to restore the abundance of this phylum to control levels. Furthermore, DNBS plus CBDV treatment for 3 days induced a trend reduction of Firmicutes with respect to mice treated with DNBS alone (Fig. 4C). To find alterations at species level, the Greengene taxonomic classification of bacterial genera was implemented with SPINGO package (Table 1). Among significant genera, U.g. of Bacteroidales was significantly increased by CBDV in DNBS-induced colitis (Table 1 and supplementary Table S3). We found several bacterial species assigned to *Clostridium* cluster XIVa and IV decreased by DNBS treatment. Among these, *Clostridium* aldenense, was increased by oral CBDV treatment and partially restored to control levels (Table 1).

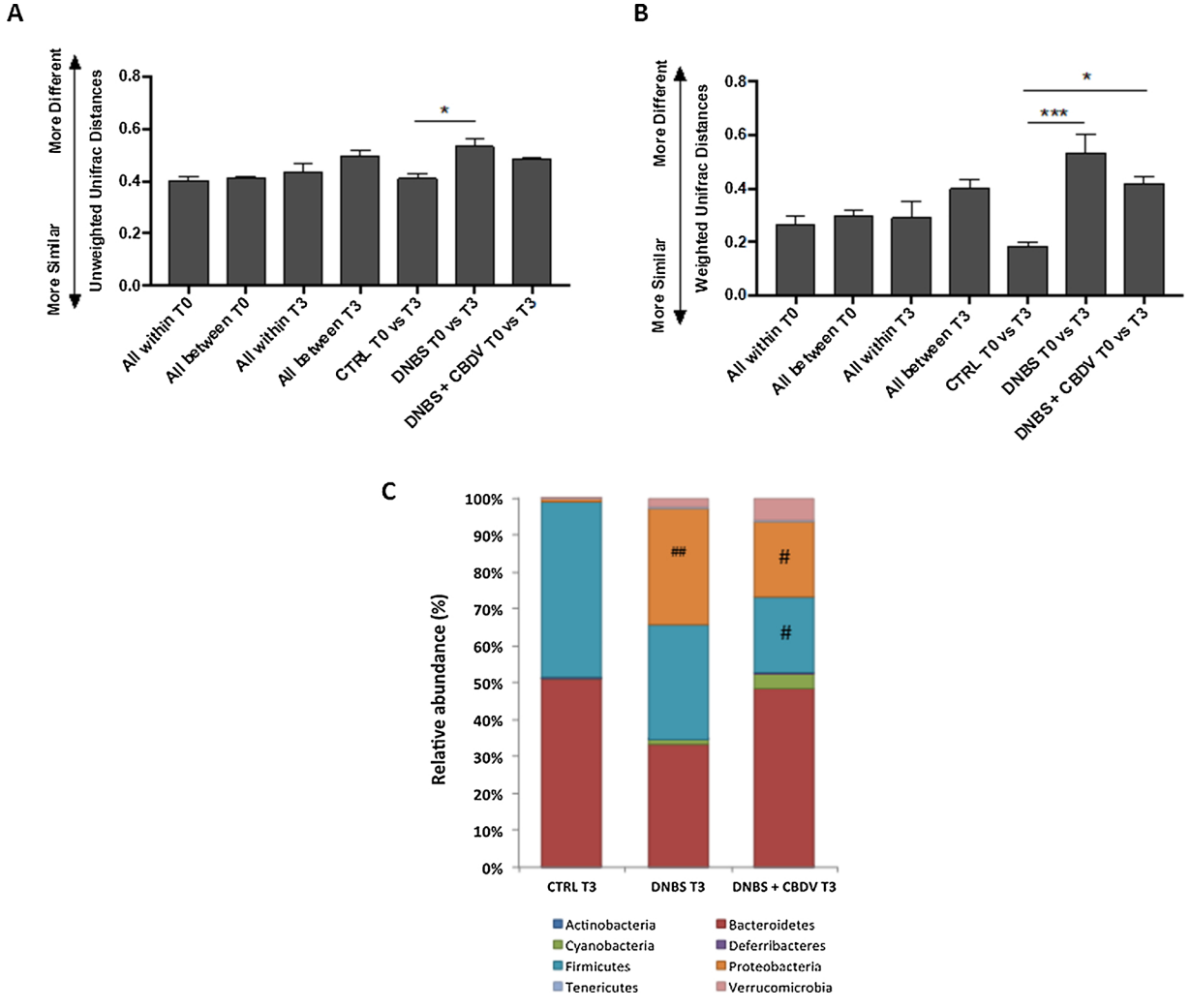

Fig. 4. Fecal microbiota composition upon DNBS treatment. Differences in fecal microbiota composition among vehicle-, DNBS- and DNBS plus CBDV-treated mice before (at time points T0) or three days after (at time point T3) the induction of colitis. Comparison of mean (± SEM) of unweighted (A) and weighted (B) Unifrac phylogenetic distances.

Percentage distribution of all bacterial phyla identified with 16S rRNA gene sequencing at time point T3 (three day after the induction of colitis and CBDV treatment (C).

Statistical significance was calculated by using one-way ANOVA test, followed by Tukey multiple comparison post-hoc test. *$p < 0.05$ and ***$p < 0.001$; $^{\#}P < 0.05$ and $^{\#\#}p < 0.01$ *vs* CTRL T3 (n = 3 mice for each experimental group).

3.5. CBDV reduces the upregulation of TRPA1 in the murine inflamed colon

In order to investigate the mode of action of CBDV, we focused on the possible involvement of TRPA1. Indeed, CBDV is able to potently activate (EC_{50} = 0.42 ± 0.01 μM,) and subsequently desensitize (IC_{50} = 1.29 ± 0.38 μM) the TRPA1 channel [19]. Therefore, we firstly investigated the effect of CBDV on TRPA1 expression during experimental acute colitis.

In line with previous studies [20], the intestinal inflammation induced by DNBS caused a significant up-regulation of TRPA1 channel. Interestingly, oral administration of CBDV completely counteracted the changes in DNBS-induced TRPA1 expression (Fig. 5A). Additionally, in order to show if the CBDV effect was selective for TRPA1 we also measured the expression of two different members of TRP family of ion channels (*i.e.* TRPV1 and TRPV2), both known to be targeted by higher concentration of CBDV and dysregulated by the colitis [19,29–31]. DNBS administration caused a significant down-regulation of TRPV1, with no changes in TRPV2 expression (Fig. 5B and C). CBDV treatment did not modify either TRPV1 or TRPV2 expression in DNBS-treated mice (Fig. 5B and C).

3.6. TRPA1 receptor is involved in the intestinal anti-inflammatory effect of CBDV

To further confirm the possible TRPA1 involvement in the intestinal anti-inflammatory effect of CBDV, we tested the phytocannabinoid in presence of HC030031, a selective TRPA1 antagonist. HC030031 reduced the anti-inflammatory effect of CBDV, as assessed by MPO activity (Fig. 5D). Moreover, CBDV was not able to significantly affect inflammation, as revealed by colon weight/colon length ratio, in mice treated with HC030031. In such experiments, the reversion by the TRPA1 antagonist was not statistically significant (Fig. 5E). The TRPA1 antagonist, HC030031, at the dose (30 mg/kg) used in the present investigation, did not modify, per se, DNBS-induced colitis [MPO (U/mg of tissue) DNBS 7.03 ± 0.72; HC030031 (30 mg/kg) 6.85 ± 0.97; mean ± SEM, n = 6].

3.7. TRPA1 is upregulated in the colonic mucosa of children with active UC

While the pro-inflammatory role of TRPA1 in colitis and its function in visceral pain is well-established in adult IBD patients [32], its expression in pediatric IBD patients has been not investigated to date. Consistent with our results in murine colitis samples (see above), TRPA1 was found to be expressed at significant higher levels in inflamed colons collected from children with active UC compared to non-inflamed tissues (Fig. 6A).

3.8. CBDV reduces colonic IL-1β production in biopsies from children with active UC

To verify if CBDV is a potential anti-inflammatory agent in humans, we investigated its effect in colonic mucosal biopsies obtained from children with active UC. The patients were clinically scored using the Geboes Score (Supplementary Table S1). IL-1β quantification by ELISA showed a significant reduction of the pro-inflammatory cytokine levels in the supernatant collected from CBDV (10 μM)-treated human colonic tissues compared with inflamed untreated biopsies (Fig. 6B).

3.9. Oral CBDV administration ameliorates DSS-induced colitis

Finally, we evaluated the effect of single oral dose of CBDV in another model of murine colitis. In the DSS-induced colitis model, CBDV, given by oral gavage at the 3 mg/kg dose, exerted a beneficial effect on colitis, reducing the colon weight/length ratio as well as DSS-induced splenomegaly (Fig. 7A and B). Furthermore, CBDV at 3 mg/kg, significantly reduced the MPO activity and IL-1β levels in colon of DSS-treated mice and produced a complete reduction – although statistically non-significant - in IL-6 levels (Fig. 7C and D). According to the DNBS model, CBDV did not affect the expression of the anti-inflammatory cytokine IL-10 (Fig. 7D).

4. Discussion

Despite *Cannabis* treatment being used in the USA by IBD patients to reduce their IBD symptoms [33,34], our study represents the first evidence of CBDV intestinal anti-inflammatory effects. CBDV is the propyl analogue of cannabidiol, the main non-psychoactive cannabinoid isolated from *Cannabis sativa* [35]. Recently, CBDV has emerged among cannabinoids for its possible clinical use as an antiepileptic drug. In 2015, a Phase 2, double blind, randomized, placebo-controlled study has shown that CBDV was generally well tolerated (GWP42006) in patients with inadequately controlled focal seizures (NCT02369471; https://www.gwpharm.com/about-us/news/gw-pharmaceuticals-announces-preliminary-results-phase-2a-study-its-pipeline-compound).

Table 1
Significant bacterial species belonging to key genera discriminating T3 groups.

Significant genera	Species °°	control (T3)	DNBS (T3)	DNBS plus CBDV (T3)
U.g. Bacteroidales	Cytophaga fermentans (Ambiguous)	0.04 ± 0.03	0.05 ± 0.03	0.70 ± 0.26*
U.g Clostridiales	AMBIGUOUS	2.37 ± 0.51	0.31 ± 0.18*	0.32 ± 0.22*
	Anaerosporobacter mobilis	0.08 ± 0.03	0*	0*
	Clostridium aldenense	3.30 ± 0.32	0.25 ± 0.14*	1.20 ± 0.87
	Clostridium asparagiforme	2.81 ± 0.80	0.01 ± 0.01*	0.05 ± 0.04*
	Clostridium celerecrescens	1.19 ± 0.40	0.05 ± 0.03*	0.05 ± 0.04*
	Clostridium hylemonae	0.11 ± 0.03	0.01 ± 0.01*	0.01 ± 0.00*
	Clostridium saccharolyticum	2.89 ± 0.37	0.18 ± 0.10***	0.39 ± 0.16***
	Filifactor villosus	0.22 ± 0.08	0.01 ± 0.01*	0.01 ± 0.00*
	Oscillibacter valericigenes	0.12 ± 0.03	0.02 ± 0.01*	0.02 ± 0.01*
	Oscillospira guilliermondii	0.13 ± 0.02	0.05 ± 0.02*	0.02 ± 0.01*
U.g Lachnospiraceae	Clostridium asparagiforme	0.36 ± 0.08	3.5E-05 ± 1.8E-05**	0.02 ± 0.01**
U.g Ruminococcaceae	Butyricicoccus pullicaecorum	0.69 ± 0.20	0.13 ± 0.05*	0.07 ± 0.04*
	Flavonifractor plautii	0.25 ± 0.06	0.04 ± 0.02*	0.05 ± 0.03*
	Intestinimonas butyriciproducens	0.22 ± 0.04	0.03 ± 0.01**	0.02 ± 0.01**
	Oscillibacter valericigenes	3.51 ± 0.65	0.65 ± 0.35**	0.62 ± 0.16**
	Oscillospira guilliermondii	0.17 ± 0.02	0.02 ± 0.01**	0.03 ± 0.01**

SPINGO classification of significant bacterial species according to Ribosomal Database Project taxonomy (80% minimum bootstrap cutoff). Bacterial species with relative abundance > 0.1% in at least one of the 3 groups are shown (mean ± SEM). Variations in species' relative abundances among control T3, DNBS T3 and DNBS plus CBDV T3 and groups were assessed by one-way ANOVA followed by Tukey's multiple comparison post-hoc test (*$p < 0.05$ *vs* control T3). °°Species are as reported by SPINGO at 80% minimum bootstrap cutoff, using a general database, thus they should be considered as indicative and in some cases ambiguous.

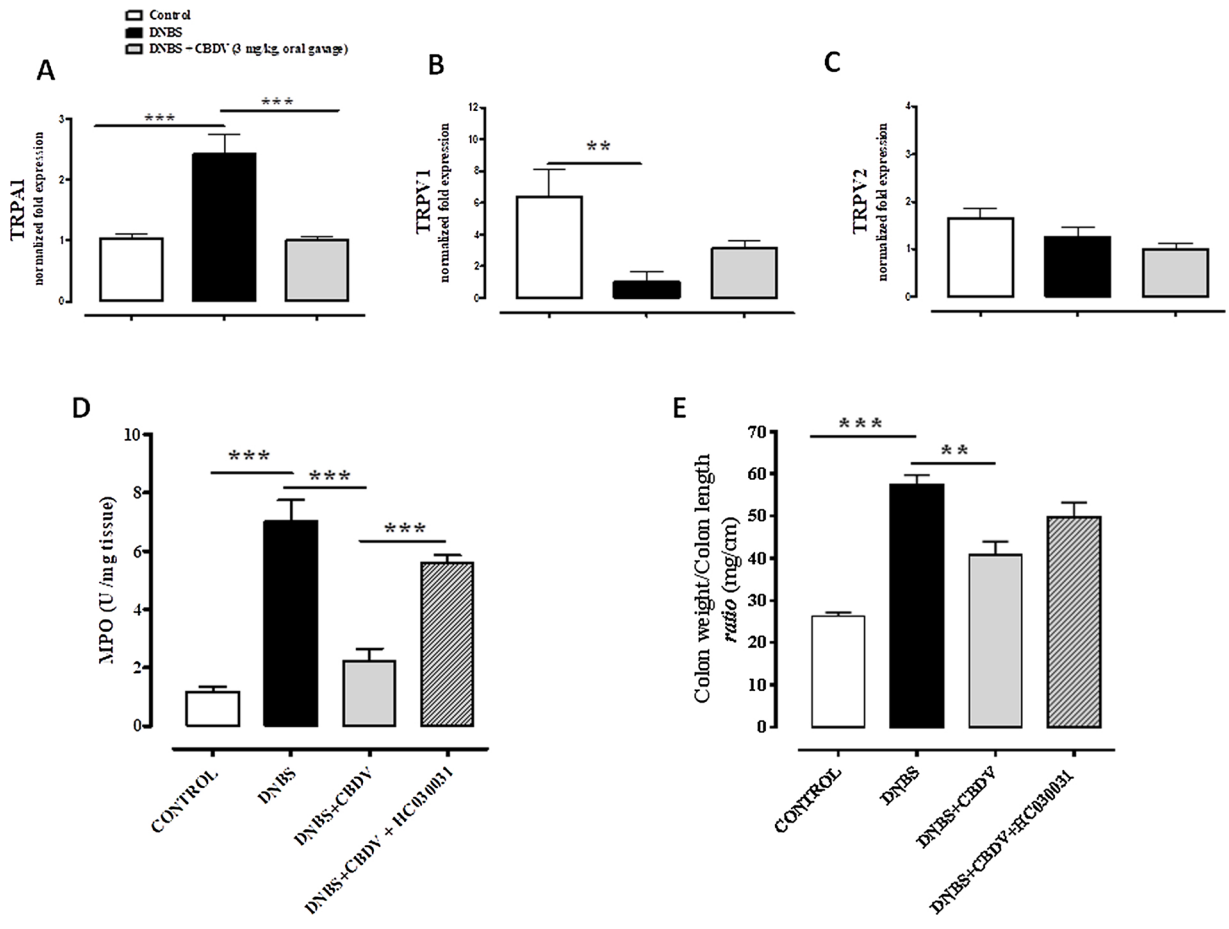

Fig. 5. TRPA1 is involved in the anti-inflammatory effect of cannabidivarin (CBDV) in DNBS-treated mice. Expression (by qPCR) of TRPA1 (A) and, for comparison, of TRPV1 (B) and TRPV2 (C) in mice colon treated with vehicle-, DNBS- or DNBS plus CBDV. Target expression was evaluated 3 days after the induction of colitis by DNBS (n = 3 mice for each experimental group).

Effect of CBDV alone or in the presence of the TRPA1 receptor antagonist HC030031 (30 mg/kg, i.p.) on MPO activity (D) and colon weight/colon length ratio (E) in mice with experimental colitis induced by DNBS. CBDV (3 mg/kg, by oral gavage) was administered once a day for three consecutive days starting 24 h after the inflammatory insult. The TRPA1 antagonist was given 30 min before CBDV administration. Tissues were analyzed three days after vehicle or DNBS administration. All data are represented as mean ± SEM. Statistical significance was calculated using one-way ANOVA test. $^{**}p < 0.01$ and $^{***}p < 0.001$ (n = 6 mice for each experimental group).

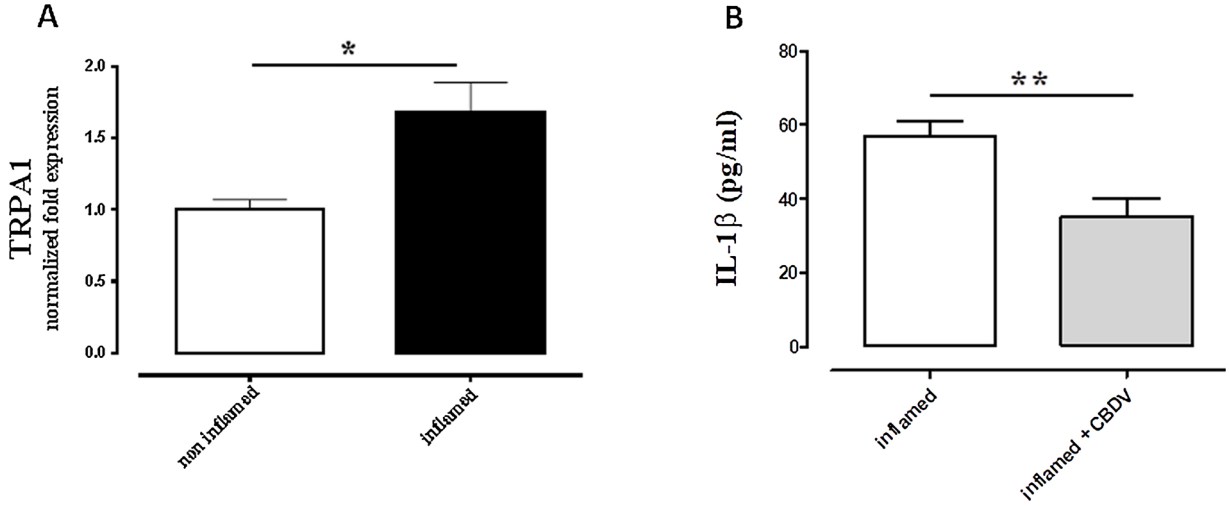

Fig. 6. Effect of cannabidivarin (CBDV) on the colonic mucosa of pediatric patients with active UC. Colonic mucosa TRPA1 gene expression in specimens taken from non-inflamed and inflamed area of pediatric patients with active UC (A). All data are represented as mean ± SD. Statistical significance was calculated using Student's *t*-test. $^{*}p < 0.05$ (n = 6 for each experimental group).

The effect of CBDV (10 μM) was evaluated on IL-1β production in inflamed colonic biopsies (B). Cytokine levels were evaluated in the supernatant collected after 24-hs incubation with vehicle or CBDV. All data are represented as mean ± SEM. Statistical significance was calculated using Student's *t*-test $^{**}p < 0.01$ (n = 4 for each experimental group).

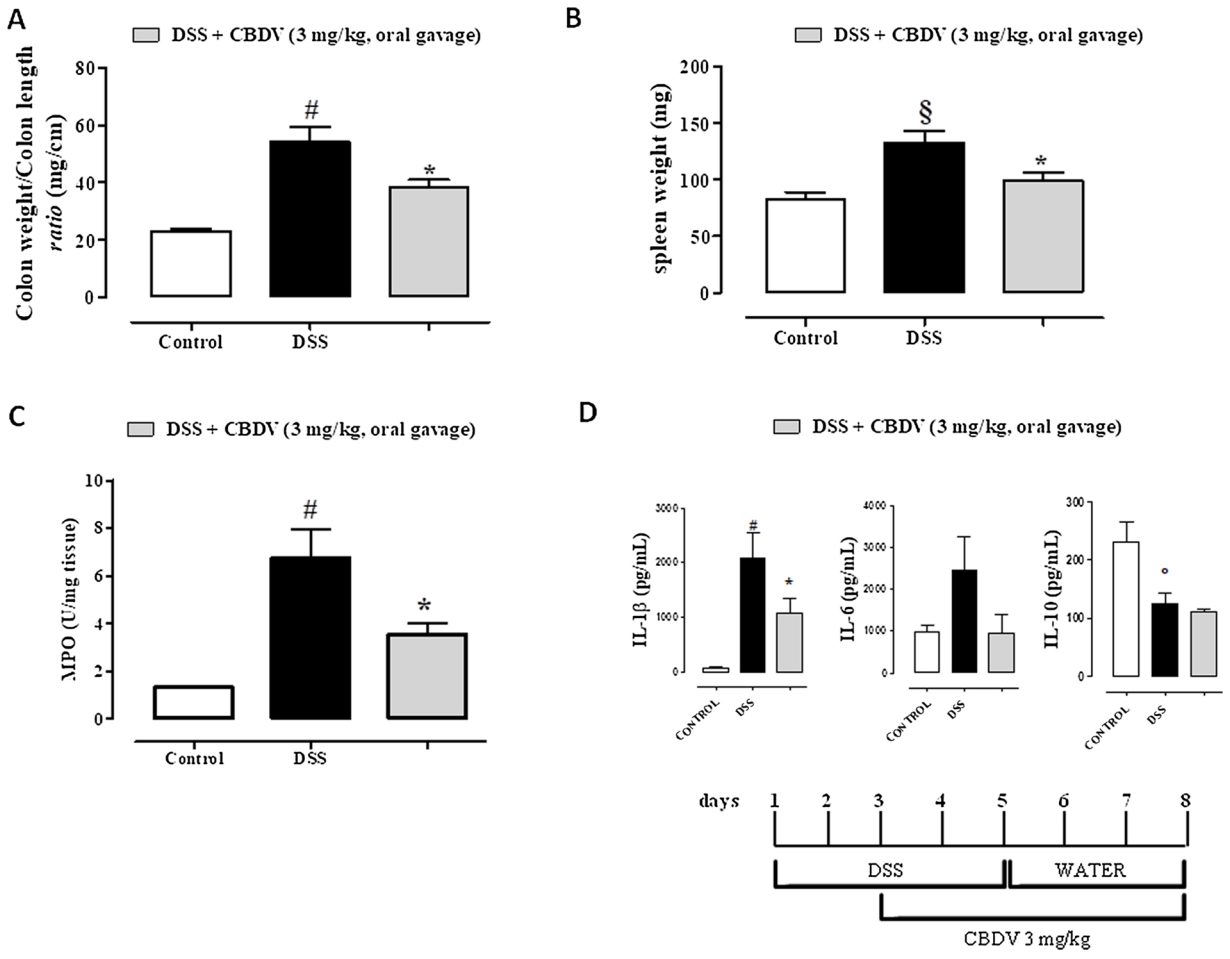

Fig. 7. Therapeutic effect of cannabidivarin (CBDV) on colon weight/colon length ratio and splenomegaly in DSS-induced colitis. CBDV (3 mg/kg, by oral gavage) was administered once a day, starting three days after DSS for 6 consecutive days. Colons (A) and spleens (B) were collected 8 days after the induction of colitis by DSS. All data are represented as mean ± SEM of 7 mice for each experimental group.
MPO colonic activity (C), IL-1β, IL-6, and IL-10 colonic levels (D) were evaluated in vehicle-, DSS- or DSS plus CBDV-treated mice. All data are mean ± SEM of 5 mice for each experimental group.
Experimental protocol for the DSS-induced colitis (bottom right). Statistical significance was calculated using one-way ANOVA test. °$p < 0.05$, §$p < 0.01$ and $^{\#}p < 0.001$ *vs* control, *$p < 0.05$ *vs* DSS alone.

Furthermore, a Phase 2 clinical trial, is planned to establish the efficacy and safety of CBDV in children with Autism Spectrum Disorder (NCT03202303).

We proved that oral or intraperitoneal CBDV attenuated inflammation in the experimental model of colitis induced by DNBS. Specifically, CBDV was able to: i) reduce the colon weight/colon length *ratio* of the inflamed colonic tissues, a gross parameter of the severity of experimental colitis [36]; ii) partially restore the impairment of intestinal permeability induced by the inflammatory stimulus; iii) counteract the massive inflammatory cell infiltration, as shown by the reduction of MPO activity and MCP-1 expression [37,38]; iv) decrease the colonic histological damage and v) lessen pro-inflammatory cytokine production.

Interestingly, a full dose response was not observed in the dose-range evaluated (0.3–30 mg/kg), with a higher effect at the 3 mg/kg dose following oral administration. The lack of a full dose-response curve is not uncommon among phytocannabinoids, such as cannabidiol [12], and it likely reflects the multi-targets action of such phytochemicals [39]. Remarkably, the 3 mg/kg CBDV oral dose was about 70-fold less than the dose reducing seizure severity in rodents [25]. Surprisingly, we found that at the 3 mg/kg dose, CBDV displayed a significant effect after oral but not after IP administration. Therefore, the possibility that a more active CBDV metabolite is produced after oral, but not after IP, administration – although not proven - cannot be excluded.

DSS-induced colitis is another largely used model of colitis that, in contrast to the DNBS model which acts as hapten, causes the entry of luminal bacteria into the mucosa through the disruption of the intestinal epithelial barrier, thereby activating the innate immune response [41]. Therefore, in order to verify if CBDV was effective in a different pathogenetic model of colitis, we evaluated the CBDV 3 mg/kg dose in the DSS model of colitis. We found that CBDV significantly reduced the increase in colon weight/colon length ratio, MPO and splenomegaly induced by DSS, thus confirming its intestinal anti-inflammatory effects. Furthermore, and similarly to the DNBS model, CBDV displayed a reduction of pro-inflammatory cytokines (*i.e.* IL-1β and IL-6), with no effect on the anti-inflammatory cytokine IL-10. We have not investigated the possibility that CBDV attenuates visceral pain, *i.e.* a common symptom of IBD patients, and there is no information in the literature that CBDV exerts such pharmacological action. It should be noted that other TRPA1 agonists have been shown to exerts analgesic effect in experimental model of both somatic [40] and visceral pain [41,42]. On the other hand, a study reported that other non-euphoric phytocannabinoids, namely cannabidiol and cannabichromene, were not effective in the acetic acid model of visceral nociception [43].

Although the precise role of intestinal bacteria remains to be clarified, there is strong evidence that alterations in gut microbiota

composition contribute to IBD pathogenesis [3,44]. IBD patients have an abnormal or unbalanced microbiome that undergoes considerable fluctuation over time, although no single microorganism has been identified as cause of the disease [45]. We have shown that intracolonic DNBS caused a shift in both abundance and type of bacterial taxa and, remarkably, CBDV was able to partially modify DNBS-induced changes, producing a microbiota profile more similar to that of non-inflamed mice, particularly for what concerns the reduction of the colon inflammation-related phylum of Proteobacteria [46]. Interestingly, CBDV caused an approximately 8-fold increase of an u.g. of Bacteroidales. Members of this order have been recently suggested to play an important role in gut homeostasis by recruiting IL-6 producing lymphocytes with intestinal barrier promoting action [47]. Notably, we did not observe IL-6 increase upon concomitant DNBS and CBDV treatment, thus we could speculate that protective action of u.g. of Bacteroidales may occur also *via* an IL-6-independent mechanism. This hypothesis is also in agreement with the recent suggestion that different Bacteroidales species may play opposing role in colonic inflammation [48]. DNBS causes the reduction of *Clostridium* cluster XIVa and IV known to play an important role in gut function and homeostasis. Of note, CBDV positively affects *Clostridium* aldenense fecal levels, one of the main species of Clostridial cluster reduced by DNBS treatment. To our knowledge this is the first time that this bacterial species is detected to be modulated in murine model of colitis. In brief, short term treatment with CBDV induces a partial reversion of DNBS induced dysbiosis. However, from our results, a causal role in the microbiota related to the effects of CBDV in this model cannot be assumed.

In order to delineate the mode of CBDV action, we hypothesized a possible involvement of TRPA1. TRPA1 is a Ca^{2+}-permeable cation channel belonging to the TRP superfamily of ion channels [49] crucially involved in IBD [50]. TRPA1 activation, possibly *via* release of substance P, contributes to the pathogenesis of colitis [20] and pharmacological blockade or genetic ablation of TRPA1 ameliorates experimental colitis whilst decreasing visceral hypersensitivity [20,51,52] Moreover, TRPA1 agonists, *via* receptor desensitization, has been associated to intestinal analgesic and anti-inflammatory effects in an experimental model of colitis [42]. Relevant for our results, CBDV has been shown to potently activate (EC_{50} = 0.42 ± 0.01 μM,) and subsequently desensitize (IC_{50} = 1.29 ± 0.38 μM) TRPA1 channels [19].

The possible role of TRPA1 in CBDV mode of action was explored by investigating the effect of CBDV on TRPA1 expression in the inflamed gut and by evaluating its anti-inflammatory action in mice treated with HC030031, a selective TRPA1 antagonist. We found that DNBS administration caused a significant up-regulation of TRPA1 and that CBDV completely counteracted this effect. Although we did not prove that the effect on TRPA1 expression was due to TRPA1 activation by CBDV, the effect of the phytocannabinoid was specific for the TRPA1 channel, since the phytocannabinoid did not affect the mRNA expression of TRPV1 and TRPV2, two other members of the TRP channels superfamily which are known i) to be targeted by CBDV, although at higher concentrations (range 3.6–7.3 μM) and ii) to be involved in colitis [19,30,31]. Furthermore, and more importantly, pharmacological experiments revealed that HC030031 reduced the anti-inflammatory effect of CBDV, as assessed by MPO activity. The TRPA1 antagonist, HC030031, at the dose (30 mg/kg) used in the present investigation, did not modify, per se, DNBSinduced colitis. A higher dose (300 mg/kg) of the antagonist has been shown to be protective in both acute and chronic models of experimental colitis [20]. These data indicate that the anti-inflammatory effect of CBDV against DNBS-induce colitis is dependent on the presence of an active TRPA1, which is probably one of the major causes of colonic inflammation in mice, and is likely due to the desensitization of this channel, which is overexpressed under our experimental conditions.

TRPA1 expression has been shown to be increased in experimental colitis as well as in adult patients with IBD [32]. However, no information is available on its expression in the pediatric population. IBD is increasing in incidence in young patients and approximately 20%–30% of them have onset of symptoms before age 18 years [53]. Moreover, children often present a more aggressive form of the disease and a more rapid progression compared to adults [54,55]. We observed that TPRA1 is up-regulated – compared to control tissues - in colonic samples collected from children with active UC. Based on the mouse data in experimental colitis showing anti-inflammatory effects following TRPA1 blockade [20], we hypothesize that the increase in TRPA1 is causative in initiating or perpetuating the intestinal inflammatory process, and that CBDV's capability of desensitizing this channel may therefore be of therapeutic value for this population of patients with UC.

Finally, in order to verify this latter hypothesis, we tested the effect of CBDV on IL-1 β production in mucosal biopsies from UC pediatric patients. By using an in vitro system to culture and treat colonic biopsies from IBD pediatric patients [24], we found that CBDV reduced the production of the proinflammatory cytokine in these tissues. By using the same protocol, others have shown that infliximab, a monoclonal antibody against tumor necrosis factor–α (TNF-α), reduces proinflammatory cytokines and the number of immune cells thus contributing to the mucosal healing in the intestinal biopsies [24].

In conclusion, we have shown that the phytocannabionid CBDV attenuates intestinal inflammation in mice with a mechanism likely involving TRPA1. The anti-inflammatory effect of CBDV was associated with changes in the gut microbiota, which might contribute to, or result from, its beneficial effects in experimental colitis. The pros for a possible clinical application of CBDV include its therapeutic activity at very low doses after oral administration and its ability to attenuate inflammation in biopsies from pediatric patients with active UC. Moreover, considering that TRPA1 is upregulated in colonic biopsies of these children, it will be important to explore the possibility of evaluating its potential prognostic role in pediatric IBD, in which the diagnosis is even more challenging compared to the adult population [56,57].

Contributors

E.P. was responsible for acquisition, analysis and interpretation of data; conception and design; and redaction of the manuscript. B.R., F.I., O.A.P., M.D.A., T.V., S.P., L.C., M.L., G.S., F.L., P.O. and R.C. carried out acquisition, analysis and interpretation of data. A.A.I. and V.D. were responsible for conception and design, analysis and interpretation of data, and critical reading of the manuscript. F.B. performed conception and design, analysis and interpretation of data, and redaction of the manuscript.

Funding

This work was supported by GW Research Ltd., UK.

Patient consent

Obtained.

Declaration of Competing Interest

AI, VD and FAI receive research grants from GW Research Ltd., UK.

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