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MICE LACKING CANNABINOID CB1-, CB2-RECEPTORS OR BOTH RECEPTORS SHOW INCREASED SUSCEPTIBILITY TO TRINITROBENZENE SULFONIC ACID (TNBS)-INDUCED COLITIS

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This study was performed to assess whether mice lacking the cannabinoid receptor CB1, CB2 or both receptors show increased susceptibility to TNBS colitis in comparison to wildtype mice. Previously, activation of CB1 and CB2 receptors showed attenuation of TNBS colitis in mice. The aim of the study was to investigate the susceptibility of three mouse strains CB1-, CB2- and CB1+2 double knockout mice in the model of TNBS colitis. The different knockout mice were given each a single enema with TNBS 7 mg, volume 150 µl (in 50% ethanol solution) on day 1. Control group (C57BL/6 mice) received the same concentration of TNBS enema and each strain received vehicle application of 150 µl 50% ethanol solution. After a 3-day period, the animals were sacrificed and their colon excised. A scoring system was used to describe macroscopical and histological changes. Messenger RNA-expression of TNF-α and IL-1β as pro-inflammatory markers was measured by RT-PCR. All three knockout strains showed increased susceptibility to TNBS colitis quantified by macroscopical and histological scoring systems and pro-inflammatory cytokine expression in comparison to the TNBS control group (wild type C57BL/6 animals). Mice lacking the CB1-, CB2-receptor or both receptors showed aggravation of inflammation in the model of TNBS colitis. Lacking of both cannabinoid receptors did not result in potentiation of colitis severity compared to lacking of each CB1 or CB2, respectively. These results suggest that the endocannabinoid system may have tonic inhibitory effects on inflammatory responses in the colon.

Key words: *endocannabinoid system, trinitrobenzene sulfonic acid colitis, proinflammatory cytokines, CB1 receptors, anandamide*

INTRODUCTION

A variety of natural and synthetic cannabinoids have been shown to possess anti-nociceptive and anti-inflammatory activity also in the gut (1-5). The endocannabinoid system (ECS) which is expressed throughout the gastrointestinal tract consists of endocannabinoids, cannabinoid receptors CB1 and CB2, carrier proteins in the cell membrane (endocannabinoid membrane transporter, EMT) and microsomal enzymes of degradation (fatty acid amide hydrolase, FAAH; monoacylglycerol lipase, MAGL) (1, 5-11). The ECS exerts multiple effects on the gastrointestinal tract. *In vivo* pharmacological studies have shown that activation of CB1 receptors reduces emesis (12, 13), produces inhibition of gastric acid secretion (14) and relaxation of the lower oesophageal sphincter (15) and inhibits intestinal motility and secretion (16). CB1 receptors are located on intrinsic (enteric) (17) and extrinsic neurons (18) such as cell bodies of sensory neurons in the dorsal root ganglia, nodose ganglion and vagal efferents (19-24) and CB2 receptors are expressed mainly on immune cells, although there is also recent evidence for neuronal expression (25). Recently, CB2 receptor expression has been observed in the enteric nervous system in rodents and human ileum (18, 26). The inflammatory process in inflammatory bowel disease (IBD) consists of a complex network of interactions between the nervous system and the immune system. Mast cells, lymphocytes, granulocytes and macrophages are under the permanent influence of peptidergic neurons in the gut (27, 28). Increased epithelial CB1 and CB2 immunoreactivity was observed at sites of mucosal ulceration in inflammatory bowel diseases (29-33). Elevation of anandamide levels and an increase in CB1 and CB2 receptor expression was reported in the colon of tri- (TNBS) and dinitrobenzene sulfonic acid (DNBS) -treated mice and in biopsies of patients with ulcerative colitis (26, 29, 33). Furthermore FAAH activity was found to be reduced in the inflamed mouse colon (26). Taken together, this suggests a reactive overactivity of the ECS in inflammatory states. ECS overactivity subsequently results in anti-inflammatory effects, which is supported by a large body of evidence. Our group recently showed that intraperitoneal application of anandamide attenuated TNBS colitis and decreased levels of the pro-inflammatory cytokines TNF-α and IL-1β (34). Systemical administration of a CB2-selective receptor agonist improved dextrane sulphate sodium (DSS) and mustard oil colitis, however was less effective than treatment with a CB1-selective agonist (32). Genetic ablation or pharmacological inhibition of FAAH as well as systemic administration of an inhibitor of anandamide cellular reuptake caused amelioration of experimental DNBS and TNBS colitis (26, 33).

Interestingly, to date data on inhibition of the ECS in inflammatory states is rare. Massa *et al*. (31) showed that genetic or pharmacological blockade of CB1 receptors causes worsening of DNBS and DSS colitis. To identify whether the CB1 or CB2 receptor plays the predominant role in experimental colitis we used double knockout mice CB1+2(-/-). This study was aimed to elucidate whether mice lacking both cannabinoid receptors demonstrated a potentiation of increased susceptibility to TNBS colitis in comparison to mice lacking only one of both receptors.

MATERIALS AND METHODS

Animals

All experiments were performed in accordance with the local standing committee for ethical experimentation on animals approved by the Animal Protection Authority of the District Government (Regierung von Mittelfranken, Wurzburg, Germany). We used wild type (WT) C57BL/6 mice (20-30 g) and colonies of CB1(-/-), CB2(-/-) and CB1+2(-/-) mice (15-30 g) (generously provided by Prof. Dr. A. Zimmer, University of Bonn, Germany). All mice were conventionally genotyped using commercially available primers (Metabion).

Induction of colitis and dosing regimens

All mouse strains were housed under standard conditions and supplied with drinking water and food ad libitum. After 24 hours of starving, colitis was induced in mice by application of a single enema with 7 mg TNBS (2,4,6-trinitrobenzene sulfonic acid; Sigma-Aldrich laboratories, Seelze, Germany) *via* a polyethylene catheter (outer diameter 2 mm) 4 cm from the anus. In pilot experiments, this dose of TNBS was found to induce reproducible colitis in C57BL/6 mice. Noteworthy, our previous study showed that C57BL/6 WT mice were less susceptible towards TNBS-induced colitis than AKR mice (34). Interestingly, inflammation severity was increased in the middle and distal parts of the colon in all mouse strains. Animals were lightly anesthetized with ketamine (40 mg/kg, i.p.) immediately before the procedure. TNBS was dissolved in 50% ethanol solution and the total volume of the enema was 150 µl. Ethanol is required to break the mucosal barrier, whereas TNBS is believed to haptenize colonic autologous or microbiotic proteins rendering them immunogenic to the host immune system (35). Similarly, as observed in inflammatory bowel diseases in humans, this experimental model involves the immune and neuroendocrine systems and leads to long-lasting ulcerative damages of the colonic mucosa (36). Vehicle control (50% ethanol solution 150 µl) was performed in all strains (each n=4). For 3 days mice were monitored for colitis and then were killed in a 100% carbon dioxide atmosphere and their colons excised and collected for further analysis. This time point was chosen because maximal acute TNBS-induced inflammation has been reported in mice after 3 days (37). At 3 cm *ab ano* colonic segments were dissected (slice thickness 1-3 mm) and placed in 10% neutral buffered formalin for subsequent histological analysis.

Scoring systems

Different scoring systems were used to describe clinical and macroscopical changes before tissue samples were collected. All scoring systems were used previously with slight modifications and are accepted scoring systems in colitis models (36, 39). Among the macroscopical parameters analysed were length of the colon starting from 5 mm above the anus to the top of the caecum, colon weight and the consistency of any stools found within the colon. The summation of these parameters resulted in the Macroscopical Score (MS) with a maximum of 12 points (*Table 1*). A semiquantitative score was used to evaluate severity of histological changes in the inflamed colon (Histopathological Colitis Score, HCS). The HCS featured the parameters inflammation extent, crypt architecture, hyperemia/edema and infiltration with inflammatory cells, with a maximum of 11 points (*Table 2*). The scoring system used to describe the changes for each of these parameters is detailed in *Table 2*. The two scores MS and HCS were added together to provide the Major Colitis Score (MCS) with a maximum of 23 points graded in mild, moderate and severe colitis (*Table 3*).

Histology evaluations

Cross-sectioned segments from the colon of each animal were removed, rinsed in saline and then fixed in 10% neutral-buffered formalin. They were embedded in paraffin, sectioned and stained with haematoxylin/eosin. The sections were examined by light microscopy (Olympus Model BX-50 microscope, Olympus Instruments, Melville, NY, USA) and were scored by an investigator, blinded to the experimental groups tested. Scoring method was described before (*Table 2*). Photomicrographs were taken with a Leitz Laborlux S microscope (Wild Leitz, Wetzlar, Germany) using a 4x and 10x

Table 1. Macroscopical score (MS).

Stool score: 0= normal (well formed faecal pellets); 1= loosely shaped, moist pellets; 2= amorphous, moist, sticky pellets; 3= diarrhea + 1 occult blood in stool. Colon weight score (weight gain): 0= < 10%; 1= 10 – 50%; 2= >50 – 100%; 3= >100 – 150%; 4= > 150%. Colon length score (shortening): 0= < 5%; 1= 5 – 14%; 2= 15 – 24%; 3= 25 – 35%; 4= > 35%. The macroscopical characterisation of the severity of TNBS colitis is measured through these criteria with a total maximum of 12 points.

Table 2. Histopathological colitis score (HCS) (ulceration and/or crypt abscess respectively +1). The Histopathological colitis score is derived from the features listed above with a maximum of 11 points. These features describe inflammation criteria to determine colitis severity.

Feature	**Description**	**Score**
Inflammation extent	none	0
	mucosa	1
	mucosa + submucosa	2
Damage in crypt architecture	none	0
	regeneration	1
	destruction	2
Hyperemia / Edema	without	0
	mild	1
	moderate	2
	severe	3
Infiltration with inflammatory cells	without	0
	mild	1
	moderate	2
	severe	3

Table 3. Major colitis score MCS (= MS + HCS). The major colitis score (MCS) is derived from the macroscopical and microscopical (histological) features listed above with a total maximum of 23 points. Severity is graded according to the following table:

Major colitis score MCS
0 (no signs of colitis)
≤ 7 (mild colitis)
≤14 (moderate colitis)
>14 (severe colitis)

objective and a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Reverse transcription polymerase chain reaction (RT-PCR)

The expression of TNF-α and IL-1β was analysed by means of RT-PCR. Mucosal specimens were scraped off on ice using slide glass and immediately snap frozen in liquid nitrogen and stored at -80°C until analysis. Total RNA was extracted from mucosal samples using a guanidium isothiocyanate phenol chloroform single step extraction kit from Stratagene (Heidelberg, Germany) based on the method described by Chomczynski and Sacchi (40). Following precipitation, RNA was resuspended in RNAse-free TE buffer and its concentration was estimated by absorbance at 260 nm wavelength. Furthermore, the quality of each RNA sample was determined by running the agarose-formaldehyde electrophoresis. RNA samples were stored at -80°C until analysis. Single stranded cDNA was generated from 5 µg of total cellular RNA using Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) (Stratagene, Heidelberg, Germany) and oligo-(dT)-primers (Stratagene, Heidelberg, Germany). Briefly, 5 µg of total RNA was uncoiled by heating (65°C for 5 min) and then reverse transcribed into complementary DNA (cDNA) in a 50 µl reaction mixture that contained 50 U MMLV-RT, 0.3 µg oligo-(dT)-primer, 1 µl RNAse block ribonuclease inhibitor (40 U/µl), 2 µl of a 100 mM/l mixture of deoxyadenosine triphosphate (dATP), deoxythymydine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP), 10 mM/l Tris-HCl (pH=8.3), 50 mM KCl, 5 mM $MgCl_2$. The resultant cDNA (2 µl) was amplified in a 50 µl reaction volume containing 2 U Taq polymerase, dNTP (200 µM each) (Pharmacia, Germany), 1.5 mM $MgCl_2$, 5 µl 10x polymerase chain reaction buffer (50 mM KCl, 10 mM Tris-HCL, pH=8.3) and specific primers for β-actin, TNF-α and IL-1β used at final concentration of 1 mM (all reagents from Takara, Shiga, Japan). The mixture was overlaid with 25 µl of mineral oil to prevent evaporation. The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT) and the incubation and thermal cycling conditions were as followed: denaturation at 94°C for 1 min, annealing at 60°C for 45 sec and extension 72°C for 2 min. The number of cycles was 30 for β-actin, TNF-α and 29 for IL-1β. The nucleotide sequences of the primers were as follows: β-actin sense: AGACCTCTATGCCAACACAGTG, antisense: TCCTGCTTGCTGATCCACATC, TNF-α sense: AGCAAGCAGCCAACCAGG, antisense: GCCACAAGCAGGAATGAGAAG, IL-1β sense: CTCGCAGCAGCACATCAAC, antisense: TGTTCATCTCGGAGCCTGTAG. The primer sequences for β-actin, TNF-α and IL-1β were based on the sequences of the published cDNAs. Polymerase chain reactions products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Location of predicted products was confirmed by using a 100-bp ladder (Takara, Shiga, Japan) as a standard size marker. The gel was then photographed under UV-transillumination. The intensity of PCR products was measured using the video image analysis system (Kodak Digital Science) as described earlier (41). The signal for investigated mRNA was standardised against that of the β-actin mRNA from each sample and the results were expressed as analysed mRNA/β-actin mRNA ratio as described earlier (42).

Statistical analysis

Results are presented as means±standard error of the mean (SEM). The number (n) that is quoted throughout the manuscript refers to the number of animals. Accordingly for inter-individual statistical comparison the Mann-Whitney U test was used (Statistica®, StatSoft, USA). Asterisks in the figures indicate significance: *$p<0.05$.

RESULTS

Intracolonic administration of 150 µl of 50% ethanol did not induce detectable inflammation in all vehicle groups (each n=4) after 3 days. Colons of these mice macroscopically and histologically had only minor changes (*Fig.1-3* show vehicle-treated C57BL/6 MS 0.3±0.3, HCS 0.8±0.8, MCS 1±1, n=4). There were no significant differences between vehicle-treated C57BL/6, CB1(-/-), CB2(-/-) and CB1+2(-/-) mice (data not shown). After intracolonic administration of TNBS (7 mg/150 µl), macroscopical and histopathological evaluation of all knockout strains colons revealed stronger inflammation as compared to the C57BL/6 control group (*Fig. 1-3*). All CB knockout strains demonstrated higher stool score, increased colon weight gain and a shorter colon which resulted in a higher MS in comparison to control C57BL/6 treated with TNBS. Interestingly, double knockout mice showed a significant higher MS than did CB1(-/-) or CB2(-/-) respectively (C57BL/6 3.9±0.5, n=9; CB1(-/-) 6.4±0.6, n=9; CB2(-/-) 6.7±0.6, n=11; CB1+2(-/-) 8.6±0.5, n=10, $p<0.05$) (*Fig. 1*). However, summed up with the HCS (*Fig. 2*) the MCS (*Fig. 3*) of CB double knockout mice did not reach statistical significance compared to CB1(-/-) or CB2(-/-). Histopathological colitis score (HCS) consisted of five criteria as described in methods. All three knockout strains showed increased inflammation extent, loss of crypt architecture, hyperemia/edema and degree of infiltration with inflammatory cells (*Table 2*) in comparison to wildtype C57BL/6 control animals. Quantitatively the HCS was increased in CB1(-/-) (6.9±0.8, n=9, $p<0.05$), CB2(-/-) (8.2±0.5, n=11, $p<0.05$) and CB1 + 2 (-/-) (7.4±0.8, n=10, $p<0.05$) compared to C57BL/6 (4.7±0.6, n=9) (*Fig. 2*). However, the HCS showed no differences between CB1(-/-), CB2(-/-) or CB1+2 double knockout mice. All strains of null mutant animals revealed severe transmural colitis with severe loss of epithelium, thickening of the bowel wall and inflammatory infiltrates compared to C57BL/6 mice (*Fig. 4A-J*). Summed up the Major Colitis Score (MCS) was increased in all three CB knockout strains compared to WT (C57BL/6 8.6±0.9, n=9; CB1(-/-) 13.6±1.4, n=9; CB2(-/-) 14.6±0.8, n=11; CB1+2(-/-) 16.0±1.0, n=10, $p<0.05$) (*Fig. 3*). According to our scoring system, all three knockout strains showed severe colitis, whereas in the wild type group colitis had a significant lower extent and severity with a moderate degree.

RT-PCR analysis

No differences between all vehicle-treated strains were observed for both cytokines (data not shown). Particularly,

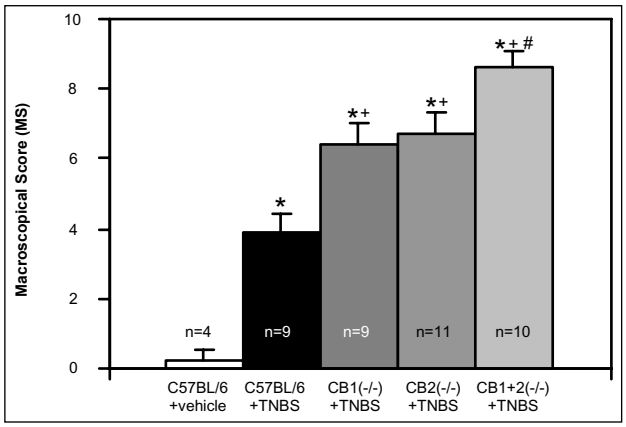

Fig. 1. Macroscopical score (MS) in the four different mouse strains. MS was significantly higher in TNBS-treated mice compared to vehicle-treated C57BL/6 (indicated with "*"). All TNBS-treated CB KO strains showed an increased MS compared to TNBS controls (C57BL/6) (indicated with "+"). CB1+2(-/-) double knockout mice showed an increased MS compared to mice lacking either CB1- or CB2 receptor (indicated with "#"). Data are mean values±SEM. Differences are statistically significant at $p<0.05$ indicated with "*", "+" and "#", Mann-Whitney U test. All values were generated from an interindividual comparison between each group.

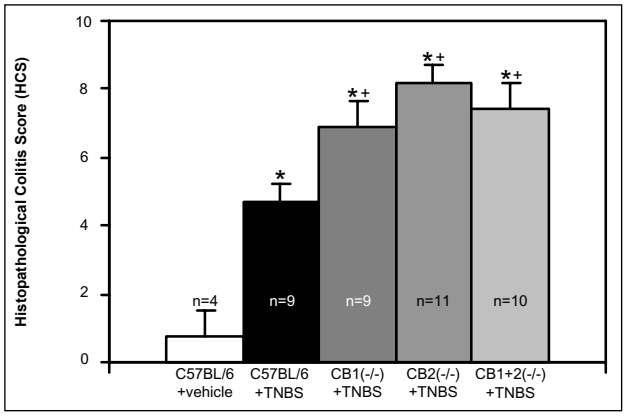

Fig. 2. Histopathological colitis score (HCS) in the four different mouse strains. HCS was significantly higher in TNBS-treated mice compared to vehicle-treated C57BL/6 (indicated with "*"). All TNBS-treated CB KO strains showed an increased HCS compared to TNBS controls (C57BL/6) (indicated with "+"). The HCS was not significantly different between the TNBS-treated CB KO strains. Data are mean values±SEM. Differences are statistically significant at $p<0.05$ indicated with "*" and "+", Mann-Whitney U test. All values were generated from an interindividual comparison between each group.

knockout animals (vehicle group) did not show increased levels of mRNA for the pro-inflammatory cytokines IL-1β and TNF-α per se (data not shown). The exposure to TNBS and induction of colitis was associated with a significant increase in the mRNA expression for IL-1β and TNF-α in colonic tissue of all mice strains. TNBS treatment of either CB1(-/-) (n=9), CB2(-/-) (n=11) or double knockout mice (n=10) resulted in a significant increase in the mRNA expression for IL-1β and TNF-α compared to the TNBS control group (*Fig. 5*). However, there was no difference in IL-1β and TNF-α mRNA expression between each CB knockout strain in an interindividual comparison.

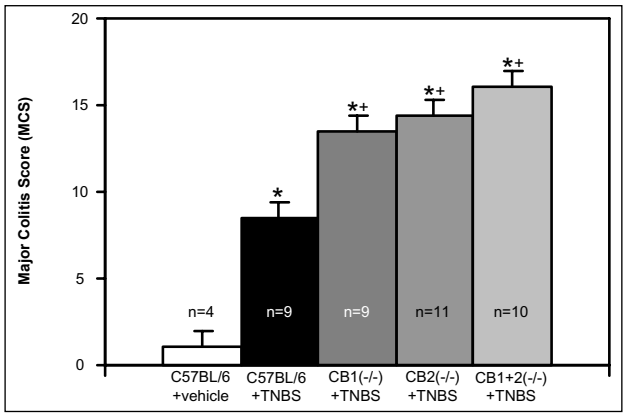

Fig. 3. Major colitis score (MCS) in the four different mouse strains. TNBS-treated C57BL/6 mice showed moderate colonic inflammation, whereas colons of all three KO strains were severely inflamed. "*" indicates significance *vs*. vehicle-treated C57BL/6 and "+" indicates significance *vs*. TNBS-treated C57BL/6 control group. The MCS was not significantly different between the TNBS-treated CB KO strains. Data are mean values±SEM. Differences are statistically significant at $p<0.05$ indicated with "*" and "+", Mann-Whitney U test. All values were generated from an interindividual comparison between each group.

DISCUSSION

Lacking of cannabinoid receptors results in increased susceptibility to experimental colitis

Here we demonstrate for the first time that lacking of either CB1, CB2 or both receptors results in aggravation of experimental colitis in mice. This increase in inflammatory activity resulted in higher levels of mRNA expression of the pro-inflammatory mediators TNF-α and IL-1β and in increased macro- and miocroscopical parameters.

To date, inhibition of the ECS in experimental colitis has not been as extensively studied as activation. One reason for that might be that therapeutic effects correlate with activation of the ECS. However, for our understanding of the pathophysiology of colitis and the role of the ECS under normal and pathologic conditions it is of importance to evaluate whether perturbance of the ECS aggravates/disinhibits colonic inflammation. Previously, Massa *et al*. reported stronger inflammation in CB1(-/-) littermates and in mice treated with a selective CB1 receptor antagonist in DNBS and DSS colitis (31). In addition to their findings we report aggravation of colitis in the model of TNBS-colitis in CB1(-/-) littermates. For the first time we demonstrate here that deletion of the CB2 receptor or both CB1 and CB2 results in increased susceptibility to TNBS colitis to a degree that is comparable to mice lacking only the CB1 receptor. Worsening of macroscopic and microscopic scores was mirrored in increased expression of the pro-inflammatory cytokines TNF-α and IL-1β in all three KO strains to a comparable degree.

Cannabinoid receptor activation inhibits experimental colitis

Previously, a multiplicity of studies focused on ECS activation and its role in inflammation. Activation of both cannabinoid receptors showed inhibition of inflammation in the gut, although different colitis models produced discrepant results with regard to the question which receptor CB1 or CB2 played the predominant role.

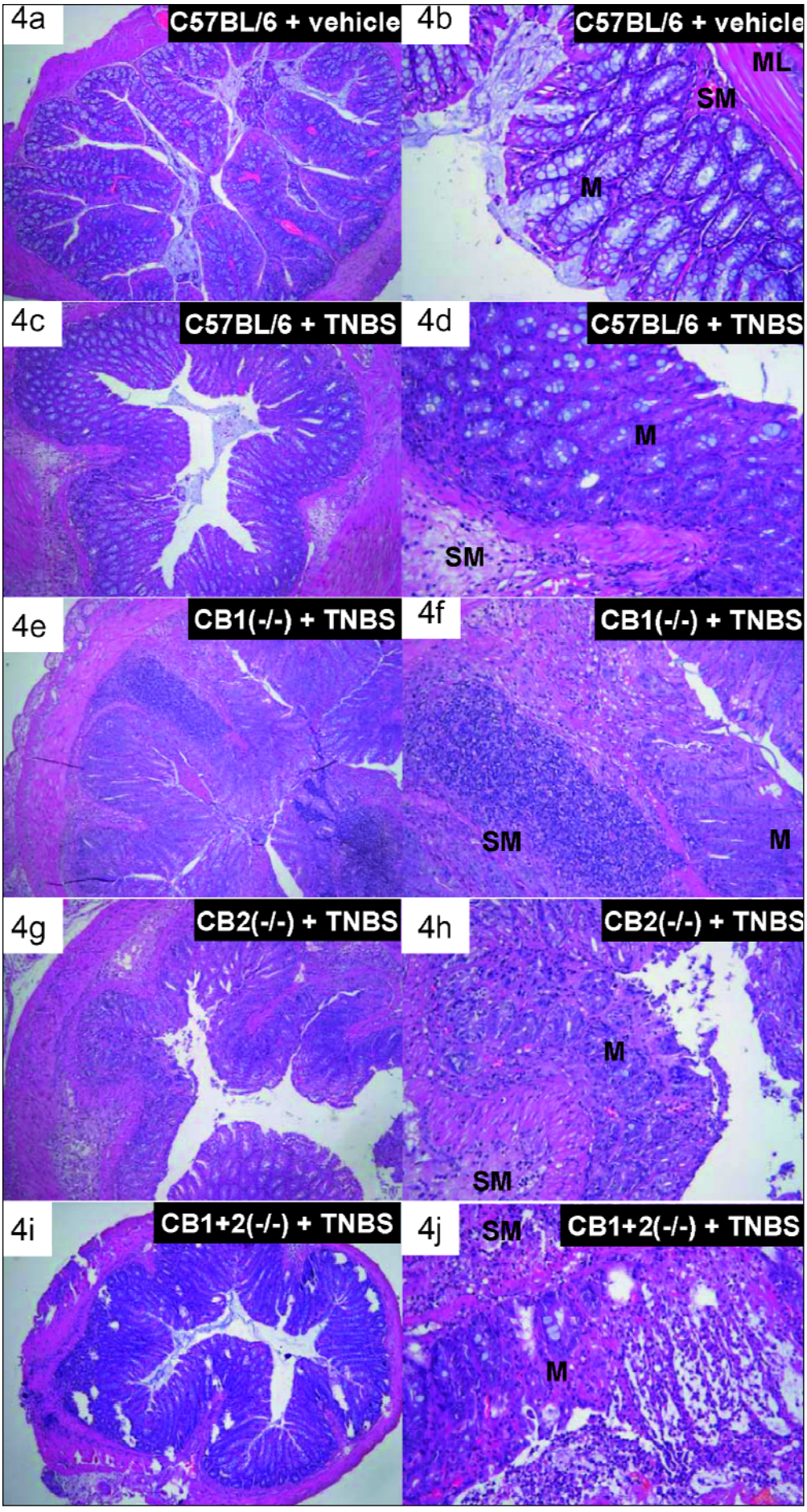

Fig. 4A-J. Vehicle group (C57BL/6) showed minor changes in epithelial structure (*Fig. 4 A,B*). TNBS-treated C57BL/6 control animals showed moderate disruption of the epithelial structure and infiltration of neutrophils with acute inflammation extending into the colonic submucosa (*Fig. 4 C,D*). CB1(-/-) (*Fig. 4E,F*), CB2(-/-) (*Fig. 4G,H*) and CB1+2(-/-) (*Fig. 4I,J*) knockout mice showed severe colonic inflammation with necrosis and increased infiltration by inflammatory cells in comparison to TNBS-treated C57BL/6. HCS did not reach statistical significance in an interindividual comparison between the knockout strains (see *Fig. 2*). M, mucosa; SM, submucosa; ML, muscle layer.

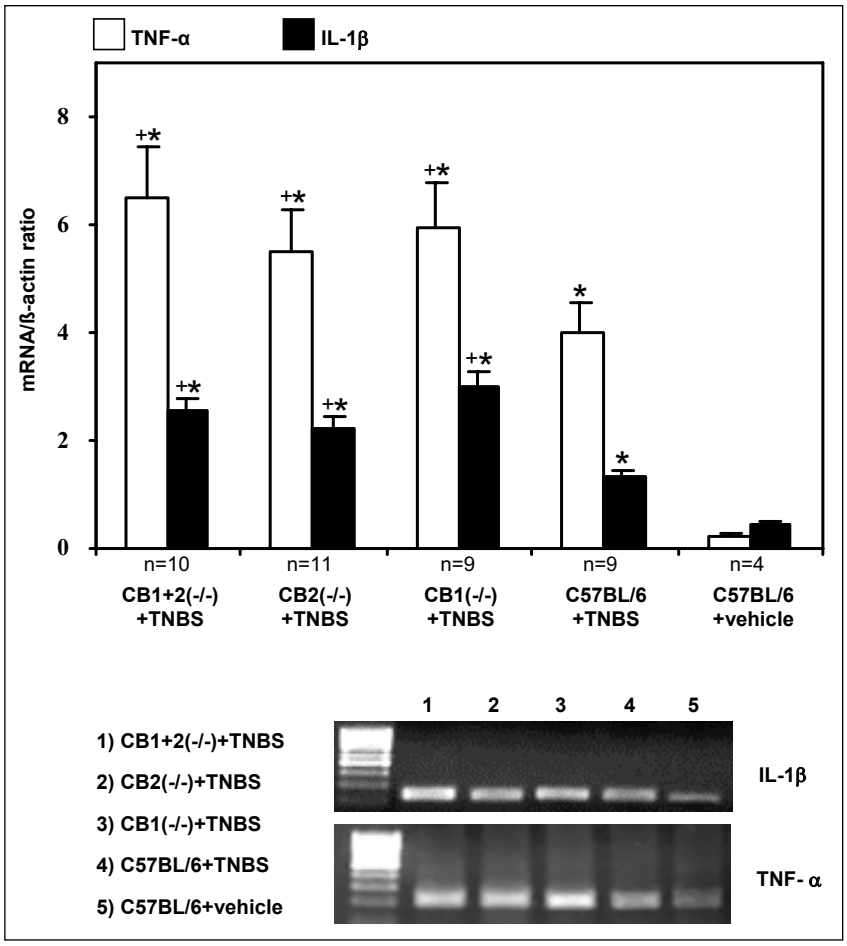

Fig. 5. Expression of the pro-inflammatory cytokines IL-1β (black columns) and TNF-α (white columns) in mouse colon of all three TNBS-treated knockout mice, TNBS-treated C57BL/6 control mice and vehicle-treated mice (C57BL/6) (representative RT-PCR). TNBS control mice (C57BL/6) and all CB KO strains showed significant increase of IL-1β and TNF-α mRNA expression compared to vehicle-treated animals (C57BL/6) (indicated with "*") and mRNA expression of both cytokines was increased in all CB KO strains in comparison to TNBS-treated C57BL/6 controls (indicated with "+"). "*" and "+" indicate significance at $p<0.05$. TNF-α, respectively IL-1β levels were not significantly different between the KO strains.

It was shown that endocannabinoids might regulate response to gut inflammation at different levels (47). Synthetic and endogenous cannabinoids were found to reduce release of neurotransmitters that affect intestinal motility and secretion at inflammatory states and by that reduced diarrhea. Systemic application of anandamide reduced colonic levels of TNF-α and IL-1β and improved macroscopical and microscopical scores of colitis (34). Additionally, it has been demonstrated that CB1 receptors promoted colonic epithelial wound healing (28).

Recently, Storr *et al*. showed that protective effects of FAAH or EMT blockade were mediated by both CB1 and CB2 receptors in an acute model of TNBS colitis (25). There is substantial evidence that under different *in vitro* and *in vivo* inflammatory conditions, both endogenous and synthetic cannabinoid receptor agonists downregulate mast cells and granulocytes, and reduce cytokine release by acting mainly at CB2 receptors (43-46). CB2 receptor activation in TNBS- and DSS-induced colitis limited immune cell recruitment, decreased cytokine and chemokine production and improved macroscopic and histological scores (47). Previously it was found that a CB2-selective agonist was unable to protect mice from acute DSS colitis, but was able to protect animals from an immune-mediated colitis ($G\alpha i2^{-/-}$ T-cell transfer model of colitis) (48). Another study from the same group found that CB2 receptor deficient mice had profound deficiencies in splenic marginal zone, peritoneal B1a cells, splenic memory CD4+ T cells, and intestinal natural killer cells and natural killer T cells and it was suggested that these findings indicate that CB2-selective agonists may modulate the development and activity of immunoregulatory cell subsets and that the endocannabinoid system is required for the formation of T- and B-cell subsets involved in immune homeostasis (49). Recently, data obtained from colonic epithelial cell lines (HT-29) strengthened the role of CB2 receptors in protection against gut inflammation. A selective CB2 receptor but not a selective CB1 receptor agonist inhibited TNF-α induced release of IL-8 in a CB2 antagonist-sensitive manner in HT-29 cells (50).

Summarized, CB2 receptor activity seems to be responsible for an appropriate immune cell function both in the adaptive and innate immune system. Hence, our results suggest that deletion of the CB2 receptor may lead to a disinhibition/disregulation of the cellular inflammatory response. Interestingly, the remaining CB1 receptor activity in CB2(-/-) was not sufficient to suppress colonic inflammation.

Non-cannabinoid receptor mediated effects of anandamide in experimental colitis

We assumed that endocannabinoid synthesis and release were still abundant in all knockout strains. Since anti-inflammatory effects in colitis were described before for each CB1 or CB2-mediated pathways one would have expected that colonic inflammation would be increased in double knockout mice in comparison to mice lacking one of both CB receptors - but this was not the case in our experiments. Non-cannabinoid receptor mediated anti-inflammatory effects of anandamide in experimental colitis were described previously and may explain our observation (36, 51-56). Anandamide is an agonist at the transient receptor potential of the vanilloid type 1 (TRPV1), expressed on primary afferent neurons and causing neurogenic inflammation *in vivo* when activated (57). Massa *et al*. propagated a protective role of TRPV1 receptors in mouse DNBS colitis (36) as TRPV1(-/-) mice showed aggravation of colitis.

Topical capsaicin administration partly protected against TNBS-induced colitis in the rat (51), was shown to induce hyperaemia in the colon (52) and to protect against colon injury elicited by acetic acid (53). It was furthermore hypothesized that the protective effect was mediated by the local release of vasoactive neuropeptides, such as calcitonin gene-related peptide (CGRP) from nerve endings (54, 55); consistently, Reinshagen *et al*. demonstrated worsening of TNBS colitis in rats after subcutaneous application of a CGRP but not substance P (SP) receptor antagonist or after intraperitoneal instillation of a CGRP antibody (56). However, neurogenic inflammation in the gut has also been proposed to exert aggressive mechanisms (58-60).

For the protective effects of anandamide on TNBS colitis vasodilatatory effects might as well be responsible. Anandamide was shown to increase microcirculation (61) and cause vasodilatation by activation of presynaptic neuronal and postsynaptic vascular CB1 receptors (62) or by induction of CGRP release from sensory nerve endings *via* TRPV1 receptor activation (63). Furthermore vasodilatation in response to anandamide was demonstrated to be mediated *via* the EP4 receptor in vascular smooth muscle cells or the induction of nitric oxide (NO) synthesis. However, data on the role of the NO pathway in inflammation remains controversial since numerous animal studies have described an improvement of experimental colitis with inducible NO synthase (iNOS) inhibition (64, 65), but other reports showed ineffectiveness (66, 67) or detrimental effects of those inhibitors (68, 69). Finally anandamide acts on the orphan G-protein-coupled receptor GPR55 (70, 71), the transient receptor potential ankyrin type 1 channel (TRPA1) and inhibits the transient receptor potential channel of melastatin type 8 (TRPM 8) receptor (72). The role of these receptors in experimental colitis remains to be elucidated in future. Previously, the endocannabinoid system was identified to control food intake and energy balance (73, 74). Since we did not measure possible alterations in levels of other hormones that take part in control circuits of energy homeostasis in our knockout mice, we cannot exclude hormonal induced changes of colitis susceptibility in these animals. Particularly, it has been suggested that the endocannabinoid tone was linked to ghrelin levels. This inter-relation might have contributed to a disinhibition of colonic inflammation, as we recently showed that exogenous application of ghrelin accelerated healing of colonic lesions in chemically-induced colitis (75).

We conclude that in CB1+2 double knockout mice anti-inflammatory effects of non-cannabinoid receptors or possible alterations in hormonal levels were not sufficient to inhibit colonic inflammation, yet may be responsible for the absence of an exaggeration of the inflammatory response in comparison to mice lacking only CB1 or CB2. Finally, this observation suggests that cannabinoid receptor mediated effects of endocannabinoids play the predominant role in tonic suppression of inflammatory reactions in the gut.

CONCLUSION

Overactivity of the endocannabinoid system has become a well established concept in human intestinal conditions with an inflammatory component (37). This concept can be expanded now as we showed here that deletion of each, respectively both known cannabinoid receptors led to aggravation of colonic inflammation. The inflammatory response to TNBS challenge involved both cannabinoid receptors to a comparable degree. Disintegration of the ECS resulted in a profound disinhibition of an obviously tonic anti-inflammatory effect of the ECS. Interestingly, cannabinoid receptor mediated effects of endocannabinoids play the predominant role in tonic suppression of inflammatory reactions in the gut. However, the net effects of a supposed sustained endocannabinoid action *via* non-CB1 and non-CB2 receptors (*e.g. via* TRPV1 or TRPA1) functionally might have impeded the potentiation of an inflammatory reaction in the gut *in vivo*.

Abbreviations: CB: cannabinoid receptor; CGRP: calcitonin gene-related peptide; DNBS: dinitrobenzene sulfonic acid; DSS: dextrane sulphate sodium; EMT: endocannabinoid membrane transporter; EP4: subclass of PGE_2 receptors; FAAH: fatty acid amide hydrolase; IBD: inflammatory bowel disease; MAGL: monoacyl glycerol lipase; NO: nitric oxide; SP: substance P; TNBS: trinitrobenzene sulfonic acid; TRPA1: transient receptor potential channel of ankyrin type 1; TRPM 8: transient receptor potential melastatin type 8; TRPV1: transient receptor potential vanilloid 1.

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REFERENCES

1. Di Carlo G, Izzo AA. Cannabinoids for gastrointestinal diseases: potential therapeutic applications. *Expert Opin Investig Drugs* 2003; 12(1): 39-49.
2. Zurier RB. Prospects for cannabinoids as anti-inflammatory agents. *J Cell Biochem* 2003; 88(3): 462-466.
3. Richardson JD, Kilo S, Hargreaves KM. Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral CB1 receptors. *Pain* 1998; 75(1): 111-119.
4. Dembinski A, Warzecha Z, Ceranowicz P, *et al*. Cannabinoids in acute gastric damage and pancreatitis. *J Physiol Pharmacol* 2006; 57(Suppl 5): 137-54.
5. Pertwee RG. Cannabinoids and the gastrointestinal tract. *Gut* 2001; 48(6): 859-867.
6. Coutts AA, Irving AJ, Mackie K, Pertwee RG, Anavi-Goffer S. Localisation of cannabinoid CB1 receptor immunoreactivity in the guinea pig and rat myenteric plexus. *J Comp Neurol* 2002; 448(4): 410-22.
7. Pinto L, Izzo AA, Cascio MG, *et al*. Endocannabinoids as physiological regulators of colonic propulsion in mice. *Gastroenterology* 2002; 123(1): 227-234.
8. Jaggar SI, Sellaturay S, Rice AS. The endogenous cannabinoid anandamide, but not the CB2 ligand palmitoylethanolamide, prevents the viscero-visceral hyper-reflexia associated with inflammation of the rat urinary bladder. *Neurosci Lett* 1998; 253(2): 123-126.
9. Izzo AA, Fezza F, Capasso R, *et al*. Cannabinoid CB1-receptor mediated regulation of gastrointestinal motility in mice in a model of intestinal inflammation. *Br J Pharmacol* 2001; 134(3): 563-570.
10. Izzo, AA, Mascolo N, Capasso F. The gastrointestinal pharmacology of cannabinoids. *Curr Opin Pharmacol* 2001; 1(6): 597-603.
11. Storr M, Sibaev A, Marsicano G, *et al*. Cannabinoid receptor type 1 modulates excitatory and inhibitory neurotransmission in mouse colon. *Am J Physiol Gastrointest* Liver *Physiol* 2004; 286(1): 110-117.
12. Darmani NA. Delta(9)-tetrahydrocannabinol and synthetic cannabinoids prevent emesis produced by the cannabinoid CB(1) receptor antagonist/ inverse agonist SR 141716A. *Neuropsychopharmacology* 2001; 24: 198-203.

13. Van Sickle MD, Oland LD, Ho W, *et al*. Cannabinoids inhibit emesis through CB1 receptors in the brainstem of the ferret. *Gastroenterology* 2001; 121: 767-774.
14. Adami M, Frati P, Bestini S, *et al*. Gastric antisecretory role and immunohistochemical localization of cannabinoid receptors in the rat stomach. *Br J Pharmacol* 2002; 135: 1598-1606.
15. Lehmann A, Blackshaw LA, Branden L, *et al*. Cannabinoid receptor agonism inhibits transient lower esophageal sphincter relaxations and reflux in dogs. *Gastroenterology* 2002; 123: 1129-1134.
16. Pinto L, Izzo AA, Cascio MG, *et al*. Endocannabinoids as physiological regulators of colonic propulsion in mice. *Gastroenterology* 2002; 123: 227-234.
17. Casu MA, Porcella A, Ruiu S, *et al*. Differential distribution of functional cannabinoid CB1 receptors in the mouse gastroenteric tract. *Eur J Pharmacol* 2003; 459: 97-105.
18. Duncan M, Davison JS, Sharkey KA. Endocannabinoids and their receptors in the enteric nervous system. *Aliment Pharmacol Ther* 2005; 22: 667-683.
19. Derbenev AV, Stuart TC, Smith BN. Cannabinoids suppress synaptic input to neurones of the rat dorsal motor nucleus of the vagus nerve. *J Physiol* 2004; 559: 923-938.
20. Burdyga G, Varro A, Dimaline R, *et al*. Ghrelin receptors in rat and human nodose ganglia: putative role in regulating CB-1 and MCH receptor abundance. *Am J Physiol Gastrointest Liver Physiol* 2006; 290: 1289-1297.
21. Kulkarni-Narla A, Brown DR. Localization of CB1-cannabinoid receptor immunoreactivity in the porcine enteric nervous system. *Cell Tissue Res* 2000; 302: 73-80.
22. MacNaughton WK, Van Sickle MD, Keenan CM, *et al*. Distribution and function of the cannabinoid-1 receptor in the modulation of ion transport in the guinea pig ileum: relationship to capsaicin-sensitive nerves. *Am J Physiol Gastrointest Liver Physiol* 2004; 286: 863-871.
23. Mascolo N, Izzo AA, Ligresti A, *et al*. The endocannabinoid system and the molecular basis of paralytic ileus in mice. *FASEB J* 2002; 16: 1973-1975.
24. Pinto L, Izzo AA, Cascio MG, *et al*. Endocannabinoids as physiological regulators of colonic propulsion in mice. *Gastroenterology* 2002; 123: 227-234.
25. Wotherspoon G, Fox A, McIntyre P, Colley S, Bevan S, Winter J. Peripheral nerve injury induces cannabinoid receptor 2 protein expression in rat sensory neurons. *Neuroscience* 2005; 135(1): 235-245.
26. Storr MA, Keenan CM, Emmerdinger D, *et al*. Targeting endocannabinoid degradation protects against experimental colitis in mice: involvement of CB1 and CB2 receptors. *J Mol Med* 2008; 86(8): 925-936.
27. Maggi CA. The effects of tachykinins on inflammatory and immune cells. *Regul Pept* 1997; 70: 75-90.
28. Holzer P. Implications of tachykinins and calcitonin gene-related peptide in inflammatory bowel disease. *Digestion* 1998; 59(4): 269-283.
29. Wright K, Rooney N, Feeney M, *et al*. Differential expression of cannabinoid receptors in the human colon: cannabinoids promote epithelial wound healing. *Gastroenterology* 2005; 129: 437-453.
30. Wright KL, Duncan M, Sharkey KA. Cannabinoid CB(2) receptors in the gastrointestinal tract: a regulatory system in states of inflammation. *Br J Pharmacol* 2008; 153: 263-270.
31. Massa F, Marsicano G, Hermann H, *et al*. The endogenous cannabinoid system protects against colonic inflammation. *J Clin Invest* 2004; 113: 1202-1209.
32. Kimball ES, Schneider CR, Wallace NH, *et al*. Agonists of cannabinoid receptor 1 and 2 inhibit experimental colitis induced by oil of mustard and by dextran sulfate sodium. *Am J Physiol Gastrointest Liver Physiol* 2006; 291: 364-371.
33. D'Argenio G, Valenti M, Scaglione G, *et al*. Up-regulation of anandamide levels as an endogenous mechanism and a pharmacological strategy to limit colon inflammation. *FASEB J* 2006; 20: 568-570.
34. Engel MA, Kellermann CA, Rau T, Hahn EG, Konturek PC. Ulcerative colitis in AKR mice is attenuated by intraperitoneally administered anandamide. *J Physiol Pharmacol* 2008; 59(4): 673-689.
35. Wirtz S, Neufert C, Weigmann B, Neurath MF. Chemically induced mouse models of intestinal inflammation. *Nat Protoc* 2007; 2(3): 541-546.
36. Massa F, Sibaev A, Marsicano G, Blaudzun H, Storr M, Lutz B. Vanilloid receptor (TRPV1)-deficient mice show increased susceptibility to dinitrobenzene sulfonic acid induced colitis. *J Mol Med* 2006; 84(2): 142-146.
37. Di Marzo V, Izzo AA. Endocannabinoid overactivity and intestinal inflammation. *Gut* 2006; 55(10): 1373-1376.
38. Keates AC, Castagliuolo I, Cruickshank WW, *et al*. Interleukin 16 is up-regulated in Crohn's disease and participates in TNBS colitis in mice. *Gastroenterology* 2000; 119(4): 972-982.
39. Kimball ES, Wallace NH, Schneider CR, D'Andrea MR, Hornby PJ. Vanilloid receptor 1 antagonists attenuate disease severity in dextran sulphate sodium-induced colitis in mice. *Neurogastroenterol Motil* 2004; 16(6): 811-818.
40. Chomczynski P, Sacchi N. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. *Nat Protoc* 2006; 1(2): 581-585.
41. Harsch IA, Brzozowski T, Bazela K, *et al*. Impaired gastric ulcer healing in diabetic rats: role of heat shock protein, growth factors, prostaglandins and proinflammatory cytokines. *Eur J Pharmacol* 2003; 481: 249-260.
42. Konturek PC, Brzozowski T, Sulekova Z, Meixner H, Hahn EG, Konturek SJ. Enhanced expression of leptin following acute gastric injury in rat. *J Physiol Pharmacol* 1999; 50(4): 587-595.
43. Mazzari S, Canella R, Petrelli L, Marcolongo G, Leon A. N-(2 hydroxyethyl) hexadecanamide is orally active in reducing edema formation and inflammatory hyperalgesia by down-modulating mast cell activation. *Eur J Pharmacol* 1996; 300: 227-236.
44. Parolaro D. Presence and functional regulation of cannabinoid receptors in immune cells. *Life Sci* 1999; 65: 637-644.
45. Massi P, Fuzio, D, Vigano D, Sacerdote P, Parolaro D. Relative involvement of cannabinoid CB1 and CB2 receptors in the D9-tetrahydrocannabinol-induced inhibition of natural killer activity. *Eur J Pharmacol* 2000; 387: 343-347.
46. Sacerdote P, Massi P, Panerai AE, Parolaro D. In vivo and in vitro treatment with the synthetic cannabinoid CP55,940 decreases the in vitro migration of macrophages in the rat: involvement of both CB1 and CB2 receptors. *J Neuroimmunol* 2000; 109: 155-163.
47. Thuru X, Chamaillard M, Karsak M, *et al*. Cannabinoid receptor 2 is required for homeostatic control of intestinal inflammation. *Gastroenterology* 2007; 132(Suppl 2): 228.
48. Ziring DA, Braun J. AM1241, a CB2-specific agonist protects against immune but not acute colitis. *Gastroenterology* 2007; 132(Suppl 2): 232.
49. Ziring D, Wei B, Velazquez P, Schrage M, Buckley NE, Braun J. Formation of B and T cell subsets require the cannabinoid receptor CB2. *Immunogenetics* 2006; 58: 714-725.
50. Ihenetu K, Molleman A, Parsons ME, *et al*. Inhibition of interleukin-8 release in the human colonic epithelial cell line HT-29 by cannabinoids. *Eur J Pharmacol* 2003; 458: 207-215.
51. Goso C, Evangelista S, Tramontana M, *et al*. Topical capsaicin administration protects against trinitrobenzene

sulfonic acid-induced colitis in the rat. *Eur J Pharmacol* 1993; 249(2): 185-190.
52. Leung FW. Role of capsaicin-sensitive afferent nerves in mucosal injury and injury-induced hyperemia in rat colon. *Am J Physiol* 1992; 262: 332.
53. Endoh K, Leung FW. Topical capsaicin protects the distal but not the proximal colon against acetic acid injury. *Gastroenterology* 1990; 98: A446.
54. Eysselein VE, Reinshagen M, Cominelli F, *et al*. Calcitonin gene-related peptide and substance P decrease in the rabbit colon during colitis. *Gastroenterology* 1991; 101: 1211.
55. Renzi D, Tramontana M, Panerai C, *et al*. Decrease of calcitonin gene-related peptide (CGRP), but not of vasoactive intestinal polypeptide (VIP) and substance P (SP) in the TNB-induced experimental colitis in rats. *Neuropeptides* 1992; 22: 56.
56. Reinshagen M, Flamig G, Ernst S, *et al*. Calcitonin gene-related peptide mediates the protective effect of sensory nerves in a model of colonic injury. *J Pharmacol Exp Ther* 1998; 286(2): 657-661.
57. Ahluwalia J, Urban L, Bevan S, Nagy I. Anandamide regulates neuropeptide release from capsaicin-sensitive primary sensory neurons by activating both the cannabinoid 1 receptor and the vanilloid receptor 1 in vitro. *Eur J Neurosci* 2003; 17(12): 2611-2618.
58. Fujino K, Takami Y, de la Fuente SG, *et al*. Inhibition of the vanilloid receptor subtype-1 attenuates TNBS-colitis. *J Gastrointest Surg* 2004; 8(7): 842-827.
59. Kihara N, de la Fuente SG, Fujino K, *et al*. Vanilloid receptor-1 containing primary sensory neurones mediate dextran sulphate sodium induced colitis in rats. *Gut* 2003; 52(5): 713-719.
60. Holzer P, Gamse R, Lembeck F. Distribution of substance P in the rat gastrointestinal tract-lack of effect of capsaicin pretreatment. *Eur J Pharmacol* 1980; 61(3): 303-307.
61. Movahed P, Evilevitch V, Andersson TL. Vascular effects of anandamide and N-acylvanillylamines in the human and skin microcirculation. *Br J Pharmacol* 2005; 146: 179-179.
62. Malinowska B, Lupinski S, Godlewski G, Baranowska U, Schlicker E. Role of endocannabinoids in cardiovascular shock. *J Physiol Pharmacol* 2008; 59(Suppl 8): 91-107.
63. Zygmunt PM, Petersson J, Andersson DA, *et al*. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* 1999; 400: 452-457.
64. Menchen LA, Colon AL, Moro MA, *et al*. N-(3-(aminomethyl)benzyl)acetamidine, an inducible nitric oxide synthase inhibitor, decreases colonic inflammation induced by trinitrobenzene sulfonic acid in rats. *Life Sci* 2001; 69(4): 479-491.
65. Kankuri E, Vaali K, Knowles RG, *et al*. Supression of acute experimental colitis by a highly selective inducible nitric oxide synthase inhibitor, N-{3-(aminomethyl)benzyl}acetamidine. *J Pharmacol Exp Ther* 2001; 298: 1128-1132.
66. Vardareli E, Dundar E, Angin K, Saricam T, Inal M. Effects of intrarectal and intraperitoneal NG-nitro-L-arginine methyl ester treatment in 2,4,6-trinitrobenzene sulfonic acid induced colitis in rats. *Exp Toxicol Pathol* 2003; 55(4): 271-276.
67. Armstrong AM, Campbell GR, Gannon C, Kirk SJ, Gardiner KR. Oral administration of inducible nitric oxide synthase inhibitors reduces nitric oxide synthesis but has no effect on the severity of experimental colitis. *Scand J Gastroenterol* 2000; 35(8): 832-838.
68. Dikopoulos N, Nussler AK, Liptay S, *et al*. Inhibition of nitric oxide synthesis by aminoguanidine increases intestinal damage in the acute phase of rat TNBS-colitis. *Eur J Clin Invest* 2001; 31(3): 234-239.
69. Yoshida Y, Iwai A, Itoh K, *et al*. Role of inducible nitric oxide synthase in dextran sulfate sodium-induced colitis. *Aliment Pharmacol Ther* 2000; 14(Suppl 1): 26-32.
70. Pertwee RG. GPR55: a new member of the cannabinoid receptor clan? *Br J Pharmacol* 2007; 152: 984-946.
71. Brown AJ. Novel cannabinoid receptors. *Br J Pharmacol* 2007; 152: 567-575.
72. De Petrocellis L, Vellani V, Schiano-Moriello A, *et al*. Plant-derived cannabinoids modulate the activity of transient receptor potential channels of ankyrin type-1 and melastatin type-8. *J Pharmacol Exp Ther* 2008; 325(3): 1007-1015.
73. Fogel WA, Stasiak A, Lewinski A, Maksymowicz M, Jochem J. Satiety signalling histaminergic system and brain-gut peptides in regulation of food intake in rats with portocaval anastomosis. *J Physiol Pharmacol* 2008; 59(Suppl 2): 135-144.
74. Di Marzo V, Matias I. Endocannabinoid control of food intake and energy balance. *Nat Neurosci* 2005; 8(5): 585-589.
75. Konturek PC, Brzozowski T, Engel M, *et al.* Ghrelin ameliorates colonic inflammation. Role of nitric oxide and sensory nerves. *J Physiol Pharmacol* 2009; 60(2): 41-47.

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