

Increasing endogenous 2-arachidonoylglycerol levels counteracts colitis and related systemic inflammation

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ABSTRACT Inflammatory bowel diseases (IBDs) are chronic inflammatory conditions for which new therapeutic approaches are needed. Genetic and pharmacological data point to a protective role of CB₁ and CB₂ cannabinoid receptor activation in IBD experimental models. Therefore, increasing the endogenous levels of 2-arachidonoylglycerol, the main full agonist of these receptors, should have beneficial effects on colitis. 2-Arachidonoylglycerol levels were raised in the trinitrobenzene sulfonic acid (TNBS)-induced colitis mouse model by inhibiting monoacylglycerol lipase (MAGL), the primary enzyme responsible for hydrolysis of 2-arachidonoylglycerol, using the selective inhibitor JZL184. MAGL inhibition in diseased mice increased 2-arachidonoylglycerol levels, leading to a reduction of macroscopic and histological colon alterations, as well as of colonic expression of proinflammatory cytokines. The restored integrity of the intestinal barrier function after MAGL inhibition resulted in reduced endotoxemia as well as reduced peripheral and brain inflammation. Coadministration of either CB₁ (SR141716A) or CB₂ (AM630) selective antagonists with JZL184 completely abolished the protective effect of MAGL inhibition on TNBS-induced colon alterations, thus demonstrating the involvement of both cannabinoid receptors. In conclusion, increasing 2-arachidonoylglycerol levels resulted in a dramatic reduction of colitis and of the related systemic and central inflammation. This could offer a novel pharmacological approach for the treatment of IBD based on the new protective role of 2-arachidonoylglycerol described here.—Alhouayek, M., Lambert, D. M., Delzenne, N. M., Cani, P. D., Muccioli, G. G. Increasing endogenous 2-arachidonoylglycerol levels counteracts colitis and related systemic inflammation. *FASEB J.* 25, 2711–2721 (2011). www.fasebj.org

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INFLAMMATORY BOWEL DISEASES (IBDs) are persistent inflammatory conditions of the gastrointestinal tract that deeply affect patients' well-being. The two major forms of IBD are Crohn's disease and ulcerative colitis. Although the etiology of IBD is not fully understood, it

is currently presumed to result from the interplay of genetic predisposition with environmental factors affecting the immune system and resulting in an aberrant inflammatory response (1–3). Until recently, treatments for IBD were essentially 5-aminosalicylic acid derivatives, steroids, and immunoregulatory agents such as azathioprine and methotrexate. The success of TNF- α blockade in treating patients with Crohn's disease launched the era of biological therapy for IBD (4, 5). However, these available therapies remain inadequate for maintaining remission in the long term and are associated with a high rate of adverse events (6, 7). Consequently, there is an undeniable need for new pharmacological strategies, and with the recent advances in the understanding of mucosal immunology, numerous therapies are being investigated, ranging from targeted antibodies to less conventional treatments, such as probiotics (6, 8).

The endocannabinoid system has been implicated in human IBD as well as experimental models of colitis (9–11). Indeed, activation of CB₁ or CB₂ cannabinoid receptors resulted in the attenuation of experimental colitis in mice; moreover, genetic blockade of one or both cannabinoid receptors resulted in increased susceptibility to experimental colitis (12, 13). These data point to a protective role of the activation of both CB₁ and CB₂ receptors in colitis; therefore, targeting both receptors should be more effective in protecting against colitis than a receptor-specific approach. Because the endocannabinoid 2-arachidonoylglycerol (2-AG) is the main endogenous full agonist at both CB₁ and CB₂ receptors (14), increasing its levels should result in greater activation of cannabinoid receptors and thus have a beneficial effect. Therefore, raising endogenous 2-AG levels by inhibiting monoacylglycerol lipase (MAGL), the main enzyme responsible for hydrolysis of 2-AG (15, 16), could be a valuable strategy.

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In the present study, we investigated the effect of raising 2-AG endogenous levels using JZL184, a potent and selective MAGL inhibitor (17), on trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice. This form of experimental colitis displays Crohn's disease-like features, notably a predominantly Th1-driven immunological response (2, 18, 19). Using selective CB₁ and CB₂ cannabinoid receptors antagonists, we also sought to determine whether the effects observed after MAGL inhibition were actually due to activation of the cannabinoid receptors.

MATERIALS AND METHODS

Animals

Eight- to 9-wk-old male C57BL/6 mice (Charles River Laboratories, Brussels, Belgium) were housed under standard conditions and supplied with drinking water and food *ad libitum*. Protocols were approved by the Université Catholique de Louvain animal committee (UCL/MD/2009-010).

Induction of colitis and study design

Food (but not water) was withdrawn 12 h before administration of TNBS (Sigma-Aldrich, Bornem, Belgium). Mice (10/group) were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). TNBS (100 mg/kg, in 0.9% NaCl-ethanol, 50:50, v/v) was intrarectally administered (50 μ l) into the colon using a cannula inserted 4 cm from the anus. Control mice received 50 μ l of a 0.9% NaCl-ethanol (50:50, v/v) solution. To ensure a homogeneous distribution and retention of TNBS (or vehicle) within the colon, mice were held by the tail, in a vertical position, for 1 min after administration (20). JZL184 (Cayman Europe, Talin, Estonia), in a mixture of saline-ethanol-Tween 80 (18:1:1, v/v/v), or the vehicle alone was administered i.p. 12 h before the induction of colitis and then twice daily until sacrifice. JZL184 dose and administration scheme were based on the reported activity and half-life of the drug (17). Cannabinoid receptor antagonists, SR141716A and AM630 (Tocris Bioscience, Bristol, UK), for the CB₁ and the CB₂ receptors, respectively, were administered in the same vehicle as JZL184, but once a day. SR141716A and AM630 were used at 3 and 10 mg/kg, respectively, based on previous studies (9, 20, 21). Mice were monitored daily for body weight loss and survival and sacrificed 3 d after TNBS administration.

Macroscopic and histological grading of colitis

Macroscopic colonic damage was assessed 3 d after colitis induction, based on two main characteristics of the pathological state: colon length shortening and colon weight gain. Thus, at the time of sacrifice, the excised colons were measured and weighed. We then determined the colon weight/colon length ratio, which is considered a reliable and sensitive indicator of the severity and extent of the inflammatory response in colitis (22). For histological scoring, small segments of the colon were fixed in 10% buffered formalin overnight and embedded in paraffin. Two sets of 3 serially cut sections (5 μ m) were cut at a distance of 100 μ m, and the 6 sections were evaluated for each mouse. Sections were stained with hematoxylin and eosin, and histological scores were determined in a blind procedure according to a widely used

scoring system for colitis, with slight modifications (23–25). The histological score represents the sum of an infiltration score and a tissue damage score. Infiltration was graded semiquantitatively from 0 to 4 as follows: 0, no infiltrate; 1, infiltrate around crypt basis; 2, infiltrate extending in the submucosa; 3, extensive infiltration reaching the submucosa with edema and/or slight infiltration of the muscularis propria; and 4, extensive infiltration of the muscularis propria. Tissue damage was graded from 0 to 3 as follows: 0, normal morphology; 1, punctuate mucosal erosions, muscularis mucosa intact; 2, mucosal erosions in large areas and/or deeper areas of ulcerations; and 3, extensive mucosal damage and extension into deeper structures of the bowel wall, *i.e.*, the muscularis propria.

Real-time quantitative PCR (qPCR)

For mRNA analysis, tissues were excised at the time of sacrifice, snap-frozen in liquid nitrogen, and stored at -80°C . Total RNA from tissues was extracted using TriPure reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. cDNA was synthesized using an RT kit (Promega, Madison, WI, USA) from 1 μ g of total RNA. qPCR was performed with a StepOnePlus instrument and software (Applied Biosystems, Foster City, CA, USA). PCR reactions were run with 2 μ l of cDNA in a total volume of 25 μ l using a SYBR Green mix (MESA FAST qPCR MasterMix Plus for SYBR Assay; Eurogentec, Verviers, Belgium). Each sample was measured in duplicate in the same run. The following conditions were used for amplification: an initial holding stage of 10 min at 95°C , then 45 cycles consisting of denaturation at 95°C for 3 s, annealing at 60°C for 26 s, and extension at 72°C for 10 s. Products were analyzed by performing a melting curve at the end of the PCR reaction. Data are normalized to the 60S ribosomal protein L19 (RPL19) mRNA expression. Primer sequences are given in Supplemental Table S1.

MAGL and fatty acid amide hydrolase (FAAH) activity

Colons were homogenized in TE buffer (50 mM Tris and 1 mM EDTA, pH 7.4), and the resulting homogenates were centrifuged at 30,000 *g* for 30 min. The supernatant was collected, and the pellet was homogenized once more in TE buffer before centrifugation (30,000 *g*, 30 min). The supernatant fractions were pooled, and the pellet fraction was resuspended in 150 μ l of TE buffer before storage at -80°C (26, 27). MAGL activity was assayed in the supernatant fraction (10 μ g of protein/tube) using 2-oleoylglycerol (2-OG) as substrate (10 μ M, Sigma-Aldrich; and 20,000 dpm of [^3H]2-OG, American Radiolabeled Chemicals, St. Louis, MO, USA). In brief, after 15 min of incubation (37°C), 400 μ l of ice-cold CHCl_3 -methanol (CHCl_3 -MeOH; 1:1, v/v) was added to the incubation medium, and the tubes were subsequently centrifuged at 800 *g*. The radioactivity ([^3H]glycerol) present in the aqueous layer was counted using Aqualuma (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) as scintillation medium. Blanks, accounting for the chemical degradation of the substrate, were made for each test, and the radioactivity found was subtracted from all the other tubes. FAAH activity was determined in the pellet fraction (10 μ g protein/tube) with a similar protocol but using anandamide as substrate (2 μ M; Cayman Chemical, Ann Arbor, MI, USA; and 75,000 dpm of [^3H]anandamide; American Radiolabeled Chemicals).

2-AG and anandamide quantification

Tissues were homogenized in CHCl_3 (10 ml), and deuterated standards (d_5 -2-arachidonoylglycerol and d_4 -anandamide,

200 pmol) were added. Then MeOH (5 ml) and H₂O (2.5 ml) were added, the lipids were extracted by vigorous mixing, and the organic layer was recovered and dried under N₂. The resulting lipid fraction was prepurified by solid-phase extraction over silica, and 2-AG and anandamide were eluted with ethyl acetate-acetone (1:1, v/v) (28, 29). The resulting lipid fraction was analyzed by HPLC-MS using a LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an Accela HPLC system (Thermo Fisher Scientific). Analyte separation was achieved using a C-18 Supelguard precolumn and a Supelcosil LC-18 column (3 μ M, 4 $\times$ 150 mm) (both from Sigma-Aldrich). Mobile phases A and B were composed of MeOH-H₂O-acetic acid (75:25:0.1, v/v/v) and MeOH-acetic acid (100:0.1, v/v), respectively. The gradient (0.5 ml/min) was as follows: from 100% A to 100% B in 15 min, followed by 10 min at 100% B and subsequent reequilibration at 100% A. Mass spectrometry analysis in the positive mode was performed with an atmospheric pressure chemical ionization (APCI) source. Capillary and APCI vaporizer temperatures were set at 250 and 400°C, respectively (30). 2-AG and anandamide were quantified by isotope dilution using their respective deuterated standard (showing identical retention times). The calibration curves were generated as described, and the data were normalized by tissue sample weight (28).

Endotoxemia measurement

Lipopolysaccharide (LPS) concentration was measured with Endosafe-MCS (Charles River Laboratories, Lyon, France) based on the Limulus amoebocyte lysate (LAL) kinetic chromogenic methodology that measures color intensity directly related to the endotoxin concentration in a sample. Serum was diluted 1:10 with endotoxin-free buffer to minimize interference in the reaction (inhibition or enhancement) and heated 15 min at 70°C (31). Each sample was diluted 1:200 with endotoxin-free LAL reagent water (Charles River Laboratories) and treated in duplicate, and 2 spikes for each sample were included in the determination. All samples have been validated for the recovery and the coefficient of variation. The lower limit of detection was 0.01 endotoxin units (EU)/ml.

Plasma cytokine level quantification

Plasma cytokines were determined in duplicate by using a Bio-Plex Multiplex Kit (Bio-Rad, Nazareth, Belgium) as described previously (31).

Tissue cytokine quantification by ELISA

Concentrations of proinflammatory cytokines (IL-1 β and IL-6) in tissues were determined by a sandwich-type ELISA technique using the Ready-Set-Go! Kit (eBioscience, Vienna, Austria) following the manufacturer's instructions. For experiments in the colon, proteins were isolated from the same tissue samples used for RT-qPCR using TriPure reagent after RNA extraction according to the manufacturer's instructions. For cytokine quantification in the liver, homogenates were directly prepared from frozen tissue samples. In brief, 100 mg of liver tissue was homogenized in 1 ml of extraction buffer [1% PBS and 1% SDS with 1 tablet of complete protease inhibitor cocktail (Roche), per 50 ml of solution]. Homogenates were then sonicated for 10 min in a water bath sonicator and centrifuged at 24,000 g for 30 min at 4°C. Supernatants were collected, and several aliquots were measured and stored at -80°C for later assessment. Total protein concentration was determined using the DC protein assay (Bio-Rad) before the ELISA assays were run.

Statistical analysis

Data are expressed as means $\pm$ SE. Differences between groups were assessed by 1-way ANOVA followed by a Bonferroni *post hoc* test. Data were analyzed using GraphPad Prism 5.0 for Windows (GraphPad Software Inc., San Diego, CA, USA) and JMP 8.0.1 (SAS, Cary, NC, USA).

RESULTS

MAGL inhibition prevents TNBS-induced colitis

Colitis induced by TNBS is a well-established experimental model of human IBD, characterized by altered colon structure and histology (Fig. 1A, B) (2, 18). We postulated that increasing the endogenous levels of 2-AG would result in decreased colitis in mice. Because MAGL is the main enzyme responsible for 2-AG degradation, we administered the highly selective MAGL inhibitor JZL184. Administration of JZL184 (16 mg/kg i.p., b.i.d.) to mice completely prevented macroscopic colon injury in TNBS-induced colitis, with colon weight over length ratio, a

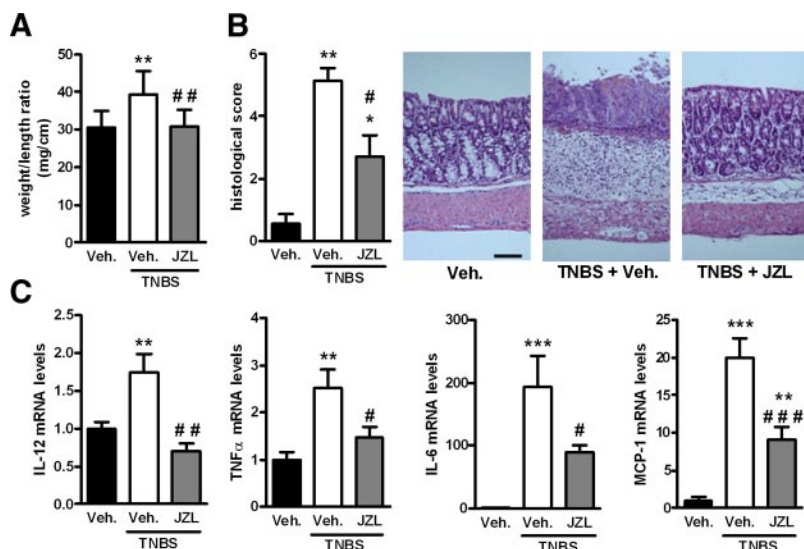


Figure 1. MAGL inhibition prevents TNBS-induced colitis. Colonic inflammation was induced by intrarectal administration of TNBS to mice treated with vehicle (saline-ethanol-Tween 80 i.p., b.i.d.; TNBS+Veh.) or JZL184 (16 mg/kg i.p., b.i.d.; TNBS+JZL). Control mice (Veh.) received i.p. injections of vehicle (b.i.d.) and an intrarectal administration of 50% ethanol. A) Colon weight/length ratio for control ($n=10$), TNBS-treated ($n=8$), and JZL184-treated TNBS mice ($n=10$). B) Histological score obtained by blind evaluation of 2×3 colon sections/mouse and representative photomicrographs of hematoxylin and eosin-stained paraffin sections of colons. Scale bars = 100 μ m. C) Proinflammatory cytokines IL-12, TNF- α , and IL-6 and chemokine MCP-1 colonic mRNA expression analysis by RT-qPCR (data are normalized to RPL19 mRNA expression). Values are means $\pm$ SE. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control; # $P < 0.05$, ## $P < 0.01$ vs. TNBS.

measure of colon alteration, similar to that of the control group (Fig. 1A). Histological analysis demonstrated a remarkable reduction of the submucosa edema, a normalized mucosa structure, and reduced leukocyte infiltration for the treated group (Fig. 1B). These reductions were associated with decreased mucosal mRNA levels of proinflammatory cytokines associated with TNBS-induced colitis; namely, IL-12, IL-6, and TNF- α . mRNA expression of the chemokine monocyte chemoattractant protein-1 (MCP-1), responsible for recruiting macrophages into inflamed tissues, was also significantly reduced in the colon, following MAGL inhibition (Fig. 1C). In addition to mRNA expression, cytokine quantification in the colon by ELISA displayed the same trend, with decreased levels of IL-1 β (5.26 ± 1.27 and 1.88 ± 0.40 pg/mg tissue, $P < 0.05$, for TNBS+vehicle and TNBS+JZL184 groups, respectively) and IL-6 (3.59 ± 0.57 and 2.14 ± 0.28 pg/mg tissue, $P < 0.05$, for TNBS+vehicle and TNBS+JZL184 groups, respectively) in the treated group compared with the untreated colitis group.

Effect of TNBS-induced colitis on the endocannabinoid system

Several constituents of the endocannabinoid system (*e.g.*, FAAH mRNA expression, anandamide levels) are known to be affected by the colitis (12, 20). Here, cannabinoid

receptor expression was not affected by colon inflammation (Fig. 2C). However, we found decreased colon mRNA expression levels for all four enzymes, MAGL, α/β -hydrolase domain (ABHD) 6 and ABHD12, and FAAH, reported to hydrolyze 2-AG (Fig. 2A). Nevertheless, these reductions in mRNA expressions were not followed by decreased FAAH and MAGL activities (Fig. 2B), and thus not by increased 2-AG levels (Fig. 2D). Inhibition of MAGL by JZL184 had no effect on its expression (Fig. 2A), but, as expected, significantly reduced MAGL activity (Fig. 2B) and strongly increased 2-AG levels (by ~ 3 -fold) compared to TNBS-treated mice (Fig. 2D). Note that although JZL184 treatment reduced FAAH activity (Fig. 2B), it had no effect on the levels of the endocannabinoid anandamide (176 ± 46 and 201 ± 40 pmol/g, $P > 0.05$, for TNBS+vehicle and TNBS+JZL184 groups, respectively), of which increased levels were reported to reduce colitis (12, 32, 33).

MAGL inhibition prevents colitis-induced endotoxemia and subsequent increase of circulating inflammatory markers

IBD-induced colon alterations result in impaired gut barrier function (34, 35) and consequently increased plasma LPS levels (*i.e.*, endotoxemia) (36, 37). Accordingly, we found that LPS plasma levels were increased in the TNBS-induced colitis group, whereas MAGL inhibition markedly reduced endotoxemia (Fig. 3A). A multiple

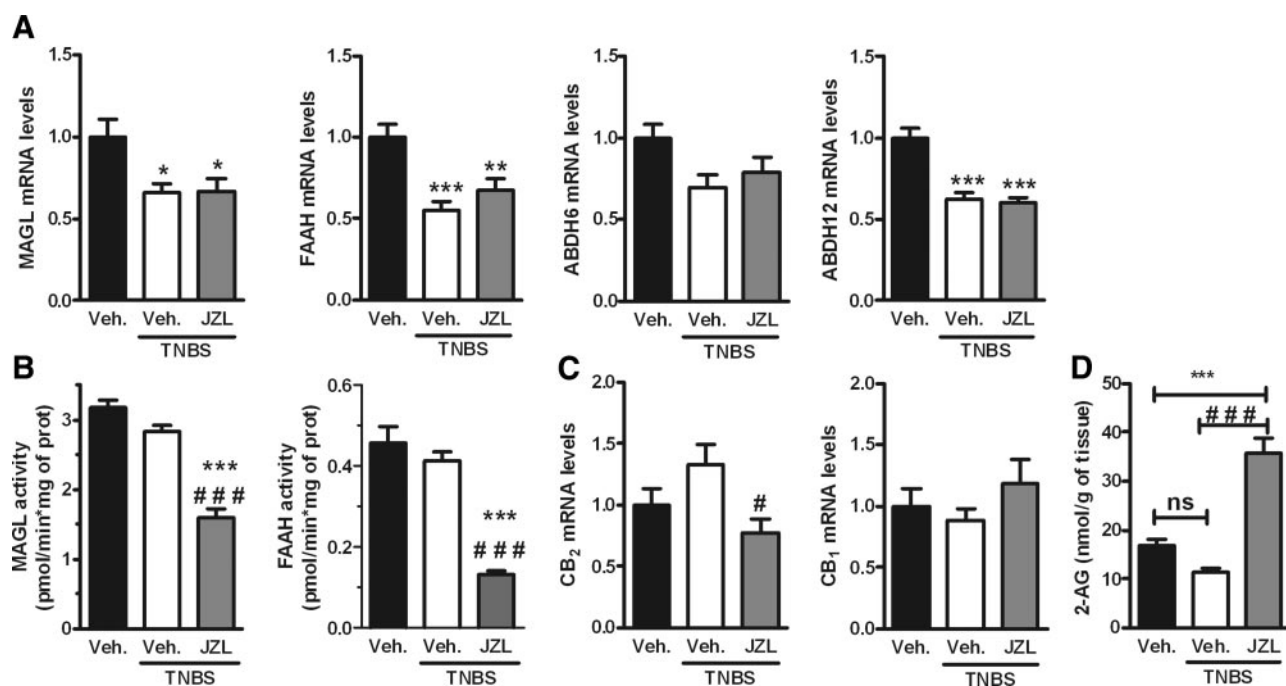


Figure 2. Effect of TNBS-induced colitis on the endocannabinoid system. A) Colon mRNA expression of the main enzymes responsible for endocannabinoid catabolism, *i.e.*, MAGL, FAAH, ABHD6, and ABHD12, normalized to RPL19 mRNA expression. B) MAGL and FAAH activities measured in supernatant and pellets, respectively, of mice colon homogenates using [3 H]2-oleoylglycerol (MAGL) and [3 H]anandamide (FAAH) are decreased after JZL184 administration. C) Colon mRNA expression of CB₁ and CB₂ cannabinoid receptors, normalized to RPL19 mRNA expression. D) 2-AG levels (expressed as nmol/g of fresh tissue) are increased in the colon of JZL184-treated mice (TNBS+JZL) compared with the levels found in control (Veh.) and inflamed mice (TNBS+Veh.). Values are means $\pm$ SE. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs.* control; # $P < 0.05$, ### $P < 0.001$ *vs.* TNBS.

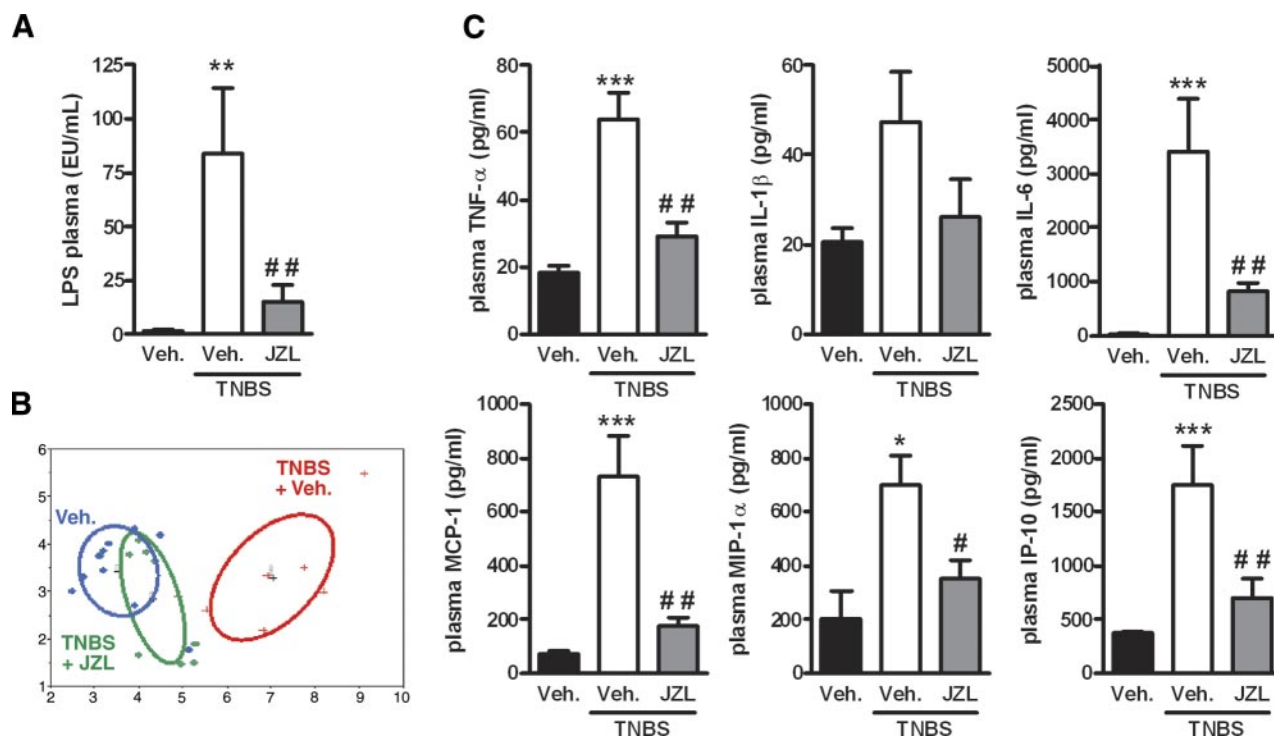


Figure 3. MAGL inhibition decreases colitis-induced endotoxemia and circulating inflammatory markers. **A)** MAGL inhibition (TNBS+JZL) quenches the increase in plasma LPS levels found in TNBS-induced colitis (TNBS+Veh.) compared with that in control mice (Veh.). **B)** Multiple correlation analysis of the colon histological score, colon weight/length ratio, and LPS plasma levels (EU/ml). **C)** Plasma cytokine (IL-1β, IL-6, and TNF-α) and chemokine (MCP-1, MIP-1α, and IP-10) levels (pg/ml) measured in JZL184-treated mice (TNBS+JZL) compared with the levels found in control (Veh.) and inflamed mice (TNBS+Veh.). Values are means ± SE. * $P < 0.05$, ** $P < 0.01$ vs. control; # $P < 0.05$, ## $P < 0.01$ vs. TNBS.

correlation analysis of the colon histological score, colon weight/length ratio, and LPS plasma levels was performed. The analysis shows that the TNBS group (TNBS+vehicle) clustered far from the control group (vehicle), whereas the treated group (TNBS+JZL) clustered together with the control group (vehicle), demonstrating the normalization of both colon structure and LPS blood levels upon MAGL inhibition (Fig. 3B). In addition to LPS, TNBS also increased plasma levels of several circulating inflammatory markers, which were normalized [IL-1β, TNF-α, and macrophage inflammatory protein 1 (MIP-1)] or strongly reduced [IL-6, MCP-1, and 10-kDa IFN-γ-induced protein (IP-10)] after MAGL inhibition (Fig. 3C). Of note, the plasma levels of these cytokines and chemokines are correlated with LPS plasma levels (e.g., plasma LPS vs. plasma TNF-α: $r=0.7$, $P=0.0008$; plasma LPS vs. plasma MCP-1: $r=0.74$, $P=0.0003$).

MAGL inhibition reduces colitis-related systemic and central inflammation

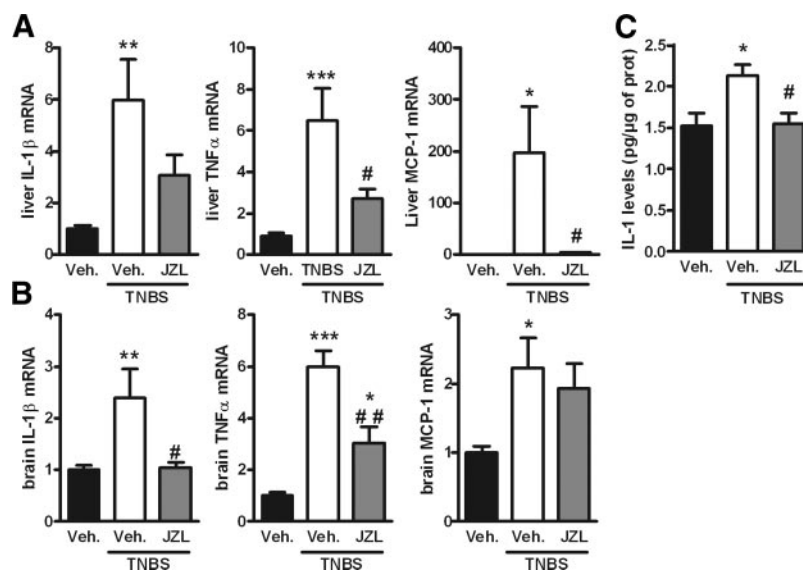
It is expected that endotoxemia will lead to peripheral tissue inflammation. Accordingly, liver mRNA expression of IL-1β, TNF-α, and MCP-1 was increased during colitis and reduced after JZL184 administration (Fig. 4A). This was further confirmed by quantification of IL-1β protein levels in the liver. Indeed, JZL184 administra-

tion counteracted the colitis-induced increase of IL-1β (Fig. 4C). These results clearly suggest a reduction in liver inflammation following MAGL inhibition and a subsequent increase in 2-AG levels. Because peripheral inflammation is often associated with inflammation of the central nervous system, we also measured mRNA expression of these cytokines in the brain (38). Here, TNBS-induced colitis resulted in a significant increase in brain cytokine levels, whereas MAGL inhibition significantly reduced mRNA expression of IL-1β and TNF-α (Fig. 4B). When the effects of MAGL inhibition at the colon, liver, and brain level were analyzed by performing a heatmap profile and dendrogram analysis, taking into account all the parameters measured here, it clearly appears that the MAGL inhibitor-treated mice (TNBS+JZL) formed a cluster apart from the vehicle-treated TNBS-induced colitis mice (TNBS+vehicle) (Fig. 5).

Beneficial effects of MAGL inhibition are mediated by both cannabinoid receptors

2-AG is a full agonist at both cannabinoid receptors (CB₁ and CB₂), and MAGL is expected to be the main enzyme responsible for its hydrolysis. Therefore, we sought to investigate whether the beneficial effect of MAGL inhibition, and consequent increase of 2-AG levels, was indeed mediated by one or both cannabinoid receptors. To this end, we used selective CB₁ and

Figure 4. MAGL inhibition reduces colitis-related systemic and central inflammation. *A, B*) Liver (*A*) and brain (*B*) cytokine (TNF- α and IL-1 β) and chemokine (MCP-1) mRNA expression analysis by RT-qPCR, normalized to RPL19 mRNA expression. *C*) IL-1 β protein expression in the liver, as measured by ELISA, is also decreased after MAGL inhibition. Values are means $\pm$ SE. * P < 0.05, ** P < 0.01 *vs.* control (Veh.); # P < 0.05, ## P < 0.01 *vs.* TNBS.



CB₂ cannabinoid receptor antagonists, SR141716A and AM630, respectively. In this second study, administration of JZL184 had the same beneficial effects on colitis as in the first study and effectively reduced macroscopic and histological alterations of the colon (**Fig. 6**). These effects were completely abrogated by both antagonists, suggesting an involvement of the two cannabinoid receptors (**Fig. 6A, B**). Moreover, MAGL inhibition decreased mucosal mRNA levels of the proinflammatory cytokines IL-6, TNF- α , and MCP-1, and its effects were partially reversed by each of the antagonists used (**Fig. 7**). We also quantified liver IL-1 β protein levels to assess peripheral tissue inflammation. JZL184 administration reduced IL-1 β expression compared with that of the colitis group, and this effect was also partially reversed by each of the two cannabinoid receptors antagonists (Supplemental Fig. S1).

DISCUSSION

Extensive research has been performed on animal models of IBD to unravel the pathophysiology of these diseases, given that such models allow the examination of inflammatory processes as well as the evaluation of new therapeutic pathways. Considerable evidence suggests that the animal models presently used to study IBD are relevant to human disease. Among these models, TNBS-induced colitis in mice is widely regarded as sharing numerous features with Crohn's disease (18). In addition, TNBS-induced colitis responds to current Crohn's disease treatments, including 5-aminosalicylic acid, corticosteroids, and anti-TNF antibodies, thus providing a useful model to test new biological hypotheses and therapeutic concepts (6, 8).

The endocannabinoid system is involved in protective mechanisms in the gastrointestinal tract during inflammation and, more particularly, colitis. Increased levels of anandamide, but not 2-AG, have been found in biopsy samples of patients with ulcerative colitis as well as in the colon of TNBS-treated rats and 2,4-dinitrobenzene sulfonic acid-treated mice (32). Moreover, studies with knockout mice suggest that both CB₁ and CB₂ cannabinoid receptors are required to mediate beneficial effects (12, 13, 20). Thus, we opted to target both receptors because it should be more efficient in protecting against colitis than a receptor-specific approach. Of note, a concomitant increase in anandamide and 2-AG levels is more likely to induce side effects than the sole increase in 2-AG (39). Therefore, there is a strong rationale for studying the effects of a selective increase of 2-AG levels in colitis.

Four enzymes, MAGL, FAAH, ABHD6, and ABHD12, have been reported to hydrolyze 2-AG (16, 40, 41). To date, most of the 2-AG hydrolysis is thought to be mediated by MAGL, which therefore represents the preferred target to increase endoge-

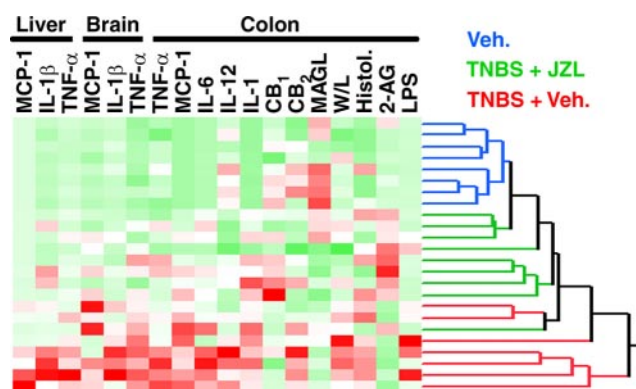


Figure 5. MAGL inhibition in mice with TNBS-induced colitis (TNBS+JZL) results in a cluster apart from that of mice with untreated TNBS-induced colitis (TNBS+Veh.). Heatmap profile and dendrogram analysis performed on the basis of colon (IL-12, IL-6, IL-1 β , TNF- α , and MCP-1) and liver and brain (IL-1 β , TNF- α , and MCP-1) inflammatory markers, colon integrity markers [weight/length ratio (W/L), histology (Histol.) score, and plasma LPS levels], CB₁, CB₂, and MAGL mRNA expression, and 2-AG colon levels. Veh., vehicle control.

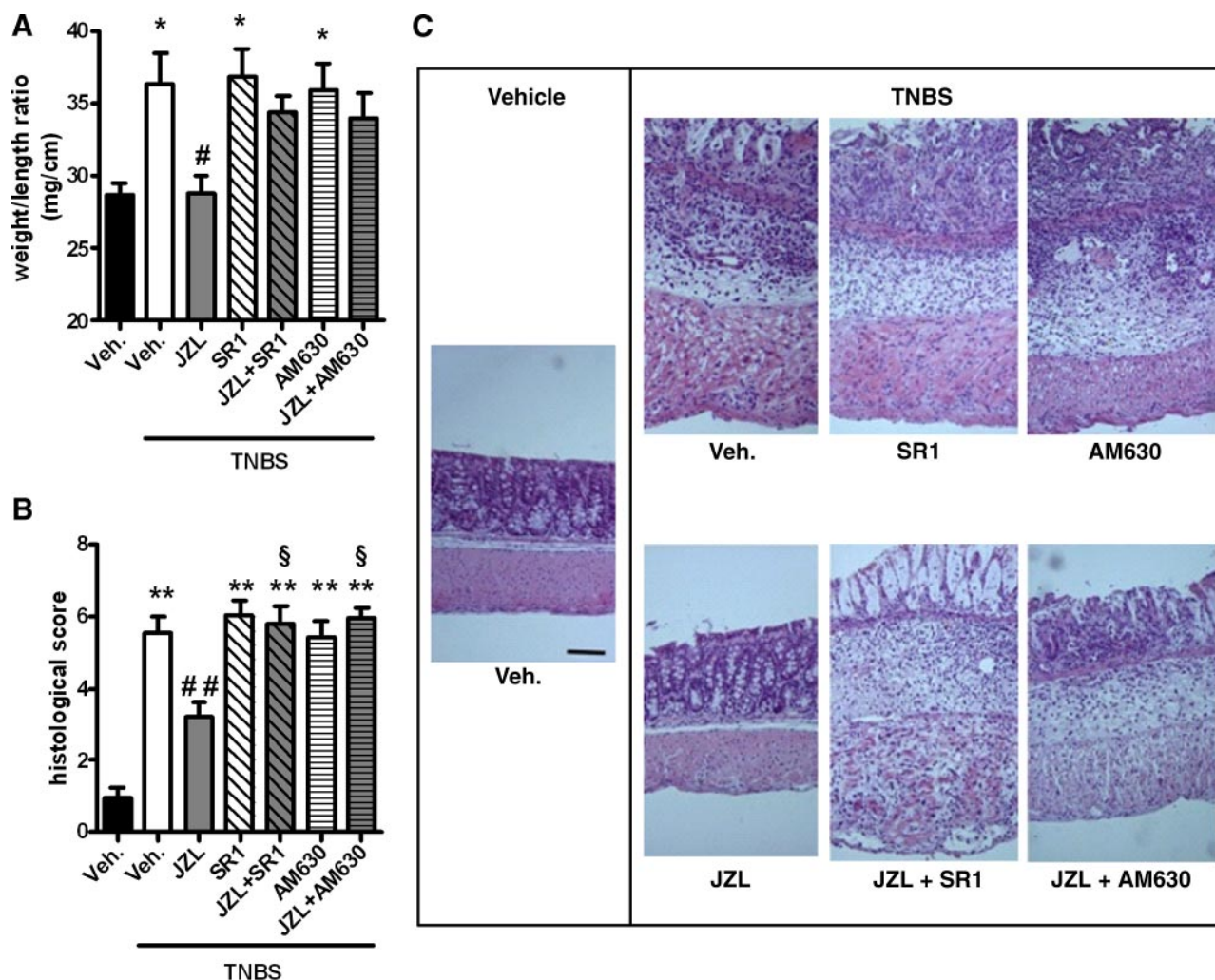


Figure 6. CB₁ and CB₂ cannabinoid receptor blockade prevents the beneficial effects of MAGL inhibition on colon morphology. Colonic inflammation was induced by intrarectal administration of TNBS to mice treated with vehicle (saline-ethanol-Tween 80 i.p., b.i.d.; Veh.) or JZL184 (16 mg/kg i.p., b.i.d.; TNBS+JZL) plus or minus CB₁ (SR141716A, 3 mg/kg i.p.; SR1+JZL) and CB₂ (AM630, 10 mg/kg i.p.; AM630+JZL) cannabinoid receptor antagonists. Two groups of mice with TNBS-induced colitis received the antagonists alone at the same regimen (SR1 and AM630 groups). Control mice (Veh.) received i.p. injections of vehicle (b.i.d.) and an intrarectal administration of 50% ethanol. **A**) Colon weight/length ratio. **B**) Histological score obtained by blind evaluation of 2 × 3 colon sections/mouse. **C**) Representative photomicrographs of hematoxylin and eosin-stained paraffin sections of colons. Scale bar = 100 μm. Values are means ± SE. **P* < 0.05, ***P* < 0.01 vs. control; #*P* < 0.05, ##*P* < 0.01 vs. TNBS; §*P* < 0.05 vs. TNBS+JZL.

nous 2-AG levels (16). To do so, we used the carbamate derivative JZL184 that was recently described as a potent and highly selective MAGL inhibitor, increasing 2-AG levels upon administration to mice (17, 42).

Previous studies have shown a reduction in FAAH expression and a parallel increase in anandamide levels in murine colitis (32), as well as increased levels of anandamide but not 2-AG in biopsy samples

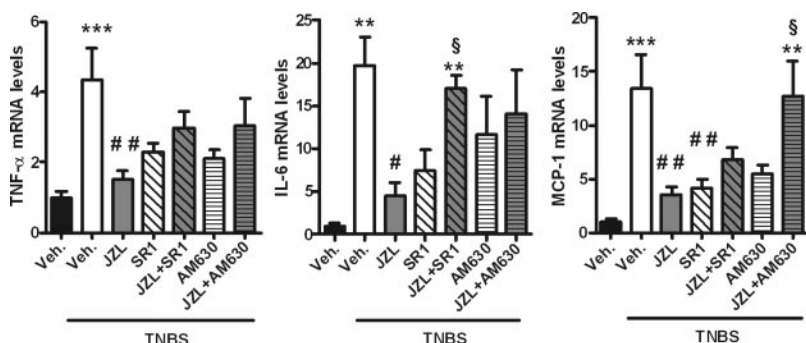


Figure 7. CB₁ and CB₂ cannabinoid receptor antagonism prevents the beneficial effects of MAGL inhibition on colon cytokines. Proinflammatory cytokine TNF-α and IL-6 and chemokine MCP-1 colonic mRNA expression analysis by RT-qPCR (data are normalized to RPL19 mRNA expression). Values are means ± SE. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. control; #*P* < 0.05, ##*P* < 0.01 vs. TNBS; §*P* < 0.05 vs. TNBS+JZL.

of patients with ulcerative colitis. These findings suggest that reduced endocannabinoid hydrolysis could be a homeostatic response to colon inflammation and points mostly to anandamide, rather than 2-AG, for the physiological control of colon inflammation. In our study, we show a reduction in MAGL expression in TNBS-induced colitis, but with no increase in 2-AG levels, which is consistent with the fact that MAGL activity was not altered by the colitis. However, the finding that the administration of the MAGL inhibitor JZL184 increases 2-AG levels in the colon (by reducing MAGL activity) and reduces colitis-induced colon alteration and inflammation confirms the relevance of MAGL and 2-AG as therapeutic targets in IBD. It is noteworthy that the administration of JZL184 also reduced colon FAAH activity measured in colon homogenates; however, this was not accompanied by an increase in colon anandamide levels. This result is consistent with studies showing that partial blockade of FAAH is insufficient to raise *N*-acylethanolamines levels *in vivo* (17, 43, 44). These findings show that *in vivo* MAGL inhibition by JZL184 selectively increases colon 2-AG levels without affecting those of anandamide, suggesting that the latter is not involved in the improvement of colitis observed here.

The reduction in macroscopic and histological scores after MAGL inhibition was accompanied by a reduction of the Th1-driven immunological response found in TNBS-induced colitis and Crohn's disease. In immunocompetent mice, TNBS-induced colitis evolves rapidly, weight loss and colon injury already appearing the first day, and is associated with transmural infiltrates of neutrophils and macrophages. This leads to an unbalanced Th1 T-cell response, driven by excessive secretion of IL-12 by activated macrophages. This Th1 response is characterized by further induction of the production of proinflammatory cytokines by macrophages, such as TNF- α , IL-1 β , and IL-6, which then serve as the immediate causes of inflammation (18, 19). In general, inflammatory processes are complex biological responses to harmful stimuli; however, during the initiation and course of inflammation, protective mechanisms are activated simultaneously and the balance between pro- and anti-inflammatory responses determines the duration of the process. IBD is the result of an aberrant inflammatory response leading to a pathological state (1, 3). The relevance of TNF- α or IL-12 in the physiopathology of IBD is well recognized because TNF- α blockade constitutes one of the major advances in IBD treatment, and administration of IL-12 antibodies to mice completely abolishes experimental colitis (2, 6). We found that increasing endogenous 2-AG levels results in a dramatic reduction of these mediators of TNBS-induced inflammation and thus seems to diminish the excessive inflammatory response characterizing IBD.

We also wanted to assess whether the reduction in TNBS-induced alteration of intestinal integrity would

translate into a decrease of the systemic inflammation present in human IBD, as well as IBD animal models. Indeed, IBD-induced colon alterations result in impaired gut barrier function and thus increased plasma LPS levels leading to peripheral tissue inflammation (*e.g.*, liver) (45). Accordingly, endotoxemia is found in a significant proportion of subjects with IBD and is correlated with disease activity (36, 37). Thus, endotoxemia reduction can be seen as an important therapeutic goal in colitis. In our model, the permeability of the intestinal mucosa was most probably increased, which led to elevated plasma LPS levels in the TNBS-induced colitis group. The beneficial effect of the increased 2-AG levels on macroscopic and microscopic alterations in the colon led to an improvement in the gut barrier function and thus to a drastic reduction in endotoxemia. Plasma LPS levels were also correlated with the levels of circulating inflammatory mediators, which substantiates the link between endotoxemia and disease activity. It is noteworthy that, just like MCP-1, the chemokines MIP-1 α and IP-10 have been found to be up-regulated in patients with IBD, and their levels correlated with disease activity. Knockout mice lacking receptors for such chemokines are resistant to IBD-like inflammation, and systemic inhibition of these chemokines has been shown to have a protective effect in experimental colitis (46–49). Accordingly, treatment with N-terminal modified antagonistic chemokines showed improvement of gut inflammation in some preclinical IBD models (46, 47). Here we show that increasing 2-AG levels translates into decreased levels of several proinflammatory mediators in the colonic mucosa and the plasma, thus suggesting that 2-AG metabolism could be an upstream target allowing the control of numerous proinflammatory cytokines.

Peripheral inflammation is often associated with CNS inflammation, which induces behavioral and pathological alterations such as sickness behavior, depression, or enhanced susceptibility to seizures (50). In the present study, we confirmed the existence of a link between gut inflammation and CNS inflammation (38), possibly after blood-brain barrier alteration, by showing that TNBS-induced colitis results in a significant increase in brain proinflammatory cytokine levels. Again, MAGL inhibition significantly reduced mRNA expression of IL-1 β and TNF- α .

The use of selective CB₁ and CB₂ antagonists clearly indicates that the effects of 2-AG on colitis are mediated by CB₁ and CB₂ cannabinoid receptors together. In fact, blocking either CB₁ or CB₂ receptors effectively abolished the beneficial effects of 2-AG on colon morphology, suggesting that simultaneous activation of both receptors is needed to maintain colon integrity. This observation is consistent with data from knockout mice, in which invalidation of CB₁ or CB₂ receptors resulted in an increased sensibility to TNBS-induced colitis. Indeed, in each knockout strain (CB₁^{-/-} or CB₂^{-/-}), the

remaining cannabinoid receptor activity was not sufficient to suppress colonic inflammation (9, 13). This finding was further confirmed by the double-knockout mice (CB₁^{-/-} and CB₂^{-/-}), in which the increased sensibility to TNBS colitis was similar to that of mice lacking only the CB₁ or CB₂ receptor (13). Note that, similarly to findings we obtained here, pharmacological blockade of cannabinoid receptors did not always result in increased susceptibility to colitis (21).

However, the effect of administration of antagonists on cytokine mRNA expression was slightly different, because blocking one receptor did not result in a complete reversal of the anti-inflammatory effect. Indeed each antagonist partially blocked MAGL inhibition-induced decrease of proinflammatory cytokines. For instance, coadministration of JZL184 with either antagonist blocked by ~50% the effect of JZL184 on TNF-α mRNA expression in the colon. This trend is visible for all the cytokines measured, in the colon or the liver, although with different percentages. Thus, the effect of JZL184 on IL-6 and MCP-1 mRNA expression was blocked 80 and 30%, respectively, by SR141716A coadministration, whereas AM630 coadministration resulted in 60 and 93% blockade, respectively. These results suggest that the effect of 2-AG on proinflammatory cytokine expression is mediated by both cannabinoid receptors and that they are each responsible for a fraction of the anti-inflammatory effect.

An interesting observation is the ability of the antagonists *per se* to modulate the colitis-induced cytokine responses. Although the antagonists effectively blocked the 2-AG-induced responses at cannabinoid receptors when coadministered with JZL184, they induced anti-inflammatory effects when administered alone. Such an effect was not reported in previous studies on colitis and cannabinoid receptor activation or inactivation, because most of these studies only focused on macroscopic and histological scoring or myeloperoxidase activity in assessing the severity of colitis and the effect of using cannabinoid receptors antagonists on agonist-induced responses in colitis (9, 20, 21). Of note, the anti-inflammatory effects of SR141716A and AM630 administered alone have been well documented in several experimental models and settings, such as macrophages in culture, adjuvant-induced arthritis in rats, type 2 diabetic rats or endotoxemic mice (51–54). The mechanisms underlying these effects are not fully elucidated, but may involve other targets in addition to the cannabinoid receptors. An alternative explanation is that the anti-inflammatory effect observed with SR141716A, for instance, might be mediated by alternative signaling of endocannabinoids through CB₂ receptors in the presence of functional CB₁ blockade and *vice versa* (55). Nevertheless, the two antagonists successfully blocked the anti-inflammatory responses of MAGL inhibition in colitis, thus confirming that CB₁ and CB₂ receptors play a primary role in suppression of inflammatory reactions in the gut.

Overall, this study provides additional evidence for the implication of the endocannabinoid system in IBD and points to an important role of 2-AG in reducing intestinal inflammation. We demonstrate here for the first time a protective role of increasing 2-AG levels, by blocking hydrolysis of 2-AG, on TNBS-induced colitis. This protective effect, mediated jointly by CB₁ and CB₂ cannabinoid receptors, is evident on the colonic inflammation itself as well as on the related systemic and central inflammation mediated by increased levels of circulating LPS.

In summary, the strategy explored here makes use of the autacoid action of endocannabinoids. Here we aimed to make use of the anti-inflammatory action of locally produced 2-AG by blocking its degradation. Moreover, 2-AG acts on both cannabinoid receptors, the activation of which was shown to reduce colitis. As shown here, MAGL inhibition and the consequent increase in 2-AG levels drastically reduce colitis and related central and peripheral inflammation, making this strategy a promising therapeutic approach in treating patients with IBD. **[F]**

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