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Cannabinoids Receptor-2 (CB2) agonist ameliorates colitis in IL-10^{-/-} mice by attenuating the activation of T cells and promoting their apoptosis

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Abstract

Inflammatory bowel disease (IBD) is a chronic intestinal inflammation caused by hyperactivated effector immune cells that produce pro-inflammatory cytokines. Recent studies have shown that the cannabinoid system may play a critical role in mediating protection against intestinal inflammation. However, the effect of cannabinoid receptors induction after chronic colitis progression has not been investigated. Here, we investigate the effect of cannabinoid receptor-2 (CB2) agonist, JWH-133, after chronic colitis in IL-10^{-/-} mice. JWH-133 effectively attenuated the overall clinical score, reversed colitis-associated pathogenesis and decrease in body weight in IL-10^{-/-} mice. After JWH-133 treatment, the percentage of CD4⁺ T cells, neutrophils, mast cells, natural killer (NK1.1) cells, and activated T cells in the LP of colitis mice declined after JWH-133 treatment in the intestinal lamina propria (LP) and mesenteric lymph nodes (MLN). JWH-133 was also effective in ameliorating dextran sodium sulphate (DSS)-induced colitis. In this model, JWH-133 reduced the number and percentage of macrophages and IFN- γ expressing cells that were induced during colitis progression. Treatment with aminoalkylindole 6-iodopiravadolone (AM630), a CB2 receptor antagonist, reversed the colitis protection provided by JWH-133 treatment. Also, activated T cells were found to undergo apoptosis following JWH-133 treatment both *in-vivo* and *in-vitro*. These findings suggest that JWH-133 mediates its effect through CB2 receptors, and ameliorates chronic colitis by inducing apoptosis in activated T cells, reducing the numbers of activated T cells, suppressing induction of mast cells, NK cells, and neutrophils at

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sites of inflammation in the LP. These results support the idea that the CB2 receptor agonists may serve as a therapeutic modality against IBD.

Keywords

Cannabinoid-2 receptors; JWH 133; Colitis; inflammatory bowel disease

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are chronic intestinal inflammatory conditions that are collectively known as Inflammatory Bowel Disease (IBD). IBD affects colon pathologies in over a million people in the United States alone and represents an important and wide spread health problem in modern society (Mayer and Collins, 2002; Loftus, 2004). The symptoms of IBD include abdominal pain, diarrhea and poor ability to digest food and in half of the severe cases require surgery to remove the affected bowel segment. IBD is also associated with infiltration in the intestinal lamina propria (LP) by immune cells including macrophages, neutrophils and lymphocytes that over-express NF- κ B- regulated target genes, such as the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 (Podolsky, 2002). The occurrence of an enteric infection, trauma or inflammation has been suggested to relate to the initiation of these diseases (Mayer and Collins, 2002). Despite recent therapeutic advances and improved understanding on the underlying pathologies, patients with IBD are often resistant to treatment, justifying the continued search for new therapeutic approaches.

In IBD, it has been shown that neutrophil activation, migration, and degranulation are important effector mechanisms for intestinal damage (Verspaget *et al.*, 1988). It has been reported that a correlation between activated neutrophils and pathological changes in affected colonic mucosa in UC patients exists (Kayo *et al.*, 2006). There are reports showing that depletion of neutrophils decreases the severity of various experimental forms of colitis (Palmen *et al.*, 1995; Natsui *et al.*, 1997; Kuhl *et al.*, 2007). The role of mast cells in colitis development has been less documented. However, there are several reports, which indicate that mast cells are also involved in the development of clinical symptoms of IBD (Rijnierse *et al.*, 2006). It has been shown that mast cells are increased in human CD patients (Knutson *et al.*, 1990).

The major active constituents of the plant *Cannabis Sativa* have been shown to possess an anti-inflammatory activity (Zurier, 2003). Most of the biological action of these cannabinoids are mediated by the cannabinoid receptors-1 (CB1) and -2 (CB2), both coupled to G proteins (Howlett *et al.*, 2002). CB1 is located pre-dominantly on the neurons (Herkenham *et al.*, 1991) and CB2, primarily on the immune cells (Munro *et al.*, 1993). The role of both CB receptors in colitis is getting increased recognition in recent years. For example, CB1 receptor-deficient mice were shown to be more sensitive to colitis induction by dextran sodium sulphate (DSS) than wild-type mice, and a CB1 antagonist worsened the colitis that was induced by 2,4-dinitrobenzene sulfonic acid (DNBS) (Massa *et al.*, 2004). These results suggested that the endogenous cannabinoid system may regulate

inflammation-associated colitis and that activation of CB receptors may help in the amelioration of colitis. In the past, studies have also been carried out, using primarily CB1 agonists, which demonstrated their ability to protect from various induced forms of experimental colitis (Massa *et al.*, 2004; Kimball *et al.*, 2006; Storr *et al.*, 2008; Engel *et al.*, 2010). Furthermore, some studies focusing on the both CB1 and CB2 receptor agonists also showed protection against experimental colitis (Storr *et al.*, 2009). In contrast, using SAB378, a peripherally restricted CB1/CB2 receptor agonist, a recent study, indicated that this compound did not affect the progression of experimental colitis (Cluny *et al.*, 2010). It is noteworthy that the administration of CB1 receptor agonists as a therapeutic strategy is limited due to adverse psychotropic side effects. Instead, targeting CB2 receptors may constitute a novel approach because such a treatment would be restricted primarily to the immune cells, and would be devoid of psychotropic activity. Moreover, previous studies from our laboratory demonstrated that CB2 agonists can exhibit potent anti-inflammatory activity *in-vivo* (Lombard *et al.*, 2007).

IBD is a major burden to both patients and society and novel therapeutic options are warranted. The side effects associated with current available treatments could result in adverse reactions or poor responses by the patients, thereby limiting their clinical use (Mouser and Hyams, 1999). Taken this into consideration, the cannabinoid system has emerged as a potential therapeutic target for IBD in recent years. In the current study, we demonstrate that use of the CB2 selective agonist, JWH-133, reduces the chronic form of colitis by inducing apoptosis in activated T cells and inhibiting the infiltration of inflammatory cells in the colon.

Methods

Animals

Female IL-10^{-/-} mice on C57BL/6 background and C57BL/6 wild-type mice aged 8~10 weeks were purchased from Jackson Laboratories (Bar Harbor, ME). The animals were housed and maintained in micro-isolator cages under conventional housing conditions at the University of South Carolina School of Medicine animal facility. Care and use of animals was overseen and approved by the Animal Resource Facility (ARF). The ARF is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care-International (AAALAC). Experimental groups consisted of six mice each and the studies were repeated three times.

Spontaneous chronic colitis development in IL-10^{-/-} mice

Under conventional housing conditions, IL-10^{-/-} mice develop spontaneous colitis at 12 weeks of age. The histological feature of colitis presented by IL-10^{-/-} mice shows similarities with IBD patients. Based on published study from our laboratory, we determined that the onset of chronic colitis occurred when serum amyloid A (SAA) and IL-6 levels reached 300 µg/ml and 5.0 ng/ml (Singh *et al.*, 2003), respectively, with at least a 15-17 % decrease from the original body weight of mice at an age of 18 weeks. Following the onset of spontaneous chronic colitis, after 18 weeks, mice received either 100µl of vehicle or 1, 2.5, 5 mg/kg body weight dose of JWH 133, suspended in 70% alcohol and further diluted

with distilled water, intraperitoneally (i.p) every second day until the week 25. In addition, ten week-old mice termed “pre-colitis”, were also used as a control for comparison with mice that had developed chronic colitis. The body weight of each mouse was monitored once a week. JWH-133 a purified compound with the linear formula $C_{22}H_{32}O$ was obtained from Sigma Chemical Co (St. Louis, MO). The certificate of origin indicated that it was originally purified and found to be greater than 99% pure by both gas chromatography and thin-layer chromatography. Pilot studies demonstrated that the 2.5mg/kg body weight dose was optimal in reducing colitis in IL-10^{-/-} mice and therefore this dose was used throughout the study. At the end of the experiment on week 27, mice were euthanized. The blood was collected for serum separation and colon samples were washed with phosphate-buffered saline (PBS), cut longitudinally, formalin fixed, and embedded in paraffin. The spleen, MLN and LP lymphocytes were isolated for flow cytometry staining.

Induction of DSS mediated acute colitis

To chemically induce experimental colitis, 8 to 10 week old BL/6 mice received either water or water containing 3% DSS (MP Biomedical, LLC Ohio) (*ad libitum*) for seven days. This was followed by water alone for next seven days. The body weight of mice was recorded every day from day 0 at the start of both JWH-133 agonist and antagonist (AM630) treatment. AM630 select CB2 receptor antagonist was purchased from Tocris Biosciences, (Ellisville, Missouri). Mice received either 100µl of both JWH-133 agonist (10 or 20 mg/kg body weight) or antagonist (AM630) at 20 mg/kg body weight or vehicle, every day till day 14 at the experimental end-point. We used a higher dose of agonist because DSS-induced colitis is an acute model unlike the colitis seen in IL-10^{-/-} mice wherein we treated the mice for 7 weeks. At the end of the experiment, spleen, MLN and LP lymphocytes were collected for flow cytometry analysis of macrophages (CD11b⁺) and IFN-γ⁺ expressing cells. Furthermore, the colon samples were washed with phosphate-buffered saline, cut longitudinally, formalin fixed, and paraffin embedded for histological analysis.

In-vitro proliferation assays

Lymphocytes from mesenteric lymph nodes (MLN ~ 75% T cells) were cultured either alone, or co-cultured *in-vitro* with (0, 5 µM or 50 µM/ml dose of JWH-133), stimulated with anti-CD3 (5µg/ml) and CD28 (1µg/ml) (BD PharMingen) Abs at 37°C in 5% CO₂ for three days. Proliferation was measured by 5-Bromo-2'-deoxy uridine (BRDU) absorption and detection (Roche Diagnostics; Dusseldorf, Germany). In brief, after 72 hour of culture, 10 µl of BrdU labeling solution (10 µM/ml final concentration per well) was added and incubated for 18 hour at 37°C with 5 % CO₂. The cells were then fixed and incubated with 100 µl of nuclease in each well for 30 minute at 37°C. Next, cells were pulsed with BrdU labeling solution and incorporation was detected by ELISA assay (Roche Molecular Biochemical).

In-vitro apoptosis assays

Lymphocytes from MLN (~75% T cells) were cultured either with the vehicle, or with JWH (0, 1, 5, 10, 20 and 50 µM) in the presence or absence of 20 or 50µM concentration of AM630, stimulated with anti-CD3 (5µg/ml) and CD28 (1µg/ml) (BD PharMingen) Abs at 37°C in 5% CO₂. After 3 days of culture, cells were washed and T cell apoptosis was

measured as per company protocol by Vybrant apoptosis assay kit #2 (Molecular Probes™ Eugene, OR). The washed T cells were resuspended with 100 µl/ml of annexin binding buffer and add 5µl of Alexa Fluor® 488 annexin V and 1µl of the 100 µg/ml of PI working solution. The cells were incubated for 15 minutes at room temperature. After the incubation period, we added 400 µL annexin-binding buffer, mix gently and keep the samples on ice and analyze the stained cells by flow cytometry (FC 500, Beckman Coulter Fort Collins, CO).

In situ apoptosis assays in the colon

To determine the role of JWH-133 on *in-vivo* apoptosis on T cells, colon from vehicle or JWH-133 treated mice were fixed, paraffin blocks were prepared, and microtome sections generated. The *in situ* tunnel assays was performed using company protocol on the tissue section using a tunnel assay kit from (Roche Diagnostics Germany). The sections were examined for apoptosis in cellular infiltrates under fluorescence microscope. The percentage and intensity of apoptosis staining in colon sections were enumerated in a blinded fashion using a method similar to that previously described (Cui *et al.*, 2010). In brief, for each tissue section, the percentage of tunnel positive cells was scored on a scale of 0 to 4 for the percentage of tissue stained: 0 (0% positive cells), 1-3 (1-7%), 4-7 (8% to 18%), 7-10 (19% to 30%), or 11-12 (> 30%). The scores obtained in three experiments were depicted resulting in an apoptosis score value ranging from 0 to 12.

JWH-133 induces co-stimulatory molecule expression

MLN cells were isolated from normal C57 BL/6 mice. These cells (1×10^6 /ml) were cultured for two days in complete medium containing 0, 5, or 50 µM/ml of JWH-133. A similar dose of Anti-CD3 and CD28 Abs were used to activate the T cells as described above. After incubation for two days, the cells were stained with FITC conjugated CD3, B220 and PE conjugated rat anti-mouse CD80, CD86, and 41BB and respective isotype controls, (PharMingen) for 30 minutes with shaking. The cells were then washed with FACS buffer (PBS with 1% BSA), fixed in 2% paraformaldehyde in PBS, and analyzed by (FACS aria II) flow cytometry (Becton Dickinson, San Diego, CA).

Cell isolation

Spleens and MLN from individual mice were mechanically dissociated. RBCs were lysed with lysis buffer (Sigma St. Louis, MO). Single cell suspensions of spleen and MLN were passed through a sterile wire screen (Sigma St. Louis, MO). Cell suspensions were washed twice in RPMI 1640 (Sigma St. Louis, MO) and stored in media containing 10% FBS on ice until used within two hours. The small intestine was cut into 1-cm strips and stirred in PBS containing 1mM EDTA at 37°C for 30 minutes. The cells from intestinal LP were isolated as described previously (Singh *et al.*, 2003). In brief, the LP was isolated by digesting intestinal tissue with collagenase type IV (Sigma St. Louis, MO) in RPMI 1640 (collagenase solution) for 45 minutes at 37°C with moderate stirring. After each 45 minutes interval, the released cells were centrifuged, stored in complete medium and mucosal pieces were replaced with fresh collagenase solution at least two times. LP cells were further purified

using a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient collecting at the 40–75% interface.

Lymphocytes were maintained in complete medium, which consisted of RPMI 1640 supplemented with 10 ml/L of nonessential amino acids (Mediatech, Washington, DC), 1 mM sodium pyruvate (Sigma), 10 mM HEPES (Mediatech), 100 U/ml penicillin, 100 µg/ml streptomycin, 40 µg/ml gentamycin (Elkins-Sinn, Inc., Cherry Hill, NJ), 50 µM mercaptoethanol (Sigma) and 10 % FCS (Atlanta Biologicals).

Flow cytometry analysis

Cells from the spleen, MLN, and LP were freshly isolated as described above for each experimental group as described earlier (Singh *et al.*, 2010). For cell surface antigen staining, cells were pre-blocked for Fc receptors (Becton Dickinson) for 15 minutes at 4°C. The cells were then washed with FACS staining buffer (PBS with 1% BSA) followed by staining with Abs. The cells were stained with Fluorescein Isothiocyanate (FITC) conjugated anti-CD4 (H129.19), CD11b (M1/70), Ly 6G (neutrophils), NK1.1, IFN-γ (XMG-1.2, e bioscience, San Diego, CA), phycoerythrin (PE) conjugated anti-CD8 (LY-2 53-6.7), anti-CD69 (H1.2F3), CD3 (145-2C11), allophycocyanin (APC) conjugated rat anti-mouse CD117, (BD-PharMingen, San Diego, CA) Abs for 30 minutes with occasional shaking at 4°C. The cells were washed two times with FACS staining buffer thoroughly, resuspended in 500 µl of FACS buffer and analyzed by flow cytometry (FC 500, Beckman Coulter Fort Collins, CO).

Histology

Colons were fixed in 10% buffer neutral formalin and embedded in paraffin. Fixed tissues were sectioned at 6 µm and stained with hematoxylin and eosin. Two independent pathologists blinded to the study design gave an inflammation score to samples taking into account the number of lesions as well as severity of the disease. Each colon segment was scored from 0 to 12 on the following well establish criteria (Singh *et al.*, 2003). In brief, grade 0 represented no change from normal tissue; grade 1, 1 or few multifocal mononuclear cell infiltrates in the LP; grade 2, intestinal lesion involved with several multifocal cellular infiltrates in LP; grade 3, lesions involved moderate inflammation and epithelial hyperplasia; grade 4, inflammation involved most of the colon sections. The summation of scores per mouse provided a total colonic disease score. Disease scores ranged from 0 to 12, with grade 4 lesions in ascending, transverse, and descending colon segments. Summation of these disease scores provides a total colonic disease score with graded lesions in proximal, middle, and distal colon segments.

Quantifying inflammation

The histological slides were examined and scored by two individuals separately in a blinded fashion, as described previously (Cui *et al.*, 2010). Briefly, inflammation was graded by extent (focal, multifocal, diffuse or extensive areas) and depth/penetration of inflammation (lamina propria, into submucosa, into subserosa), then given a numerical value of 0 – 12, where 0, is none observed, and 12 is severe inflammation and/or ulceration/erosion. *Statistics.* The data were expressed as the mean ± SEM and compared using a two-tailed

paired Student's *t*-test or an unpaired Mann Whitney U test. The results were analyzed using the Statview II statistical program (Abacus Concepts, Inc., Berkeley, CA) and Microsoft Excel (Microsoft, Seattle, WA). Single-factor variance ANOVA analyses were used to evaluate changes in groups. Results were considered statistically significant if *p* values were < 0.05 between the control and experimental groups.

Results

JWH-133 reduces weight loss typically associated with colitis

We first examined the effect of JWH-133, a CB2 agonist, on body weight loss in normal C57BL/6 and IL-10^{-/-} mice. In addition, throughout this study, we also compared pre-colitis IL-10^{-/-} mice with post-chronic colitis IL-10^{-/-} mice treated with either vehicle or JWH-133. As shown in Fig 1A, vehicle-treated IL-10^{-/-} mice developed spontaneous colitis as shown by significant weight loss, diarrhea, and ruffled fur. Associated with the development of colitis was a ~12% to 17% reduction in initial body weight. Body weights in the vehicle-treated group continued to decline throughout the study (Fig. 1A). In contrast, IL-10^{-/-} mice treated with 2.5 mg/kg JWH-133 showed significant improvements in the body weight. We found that 2.5 mg/kg was the optimum dose because 1 mg/kg was not effective while treatment with 5 mg/kg JWH showed improvements in body weights, although this dose was not as effective as 2.5 mg/kg (Fig. 1A). The body weight in normal C57BL/6 mice did not change much after JWH-133 treatment. Together, these findings suggested that 2.5 mg/kg of JWH-133 was the optimum dose that was able to protect IL-10^{-/-} mice from colitis-induced weight loss.

Next, we quantified the protective effects of JWH-133 treatment on colitis severity. We did this by analyzing colonic sections from pre-colitis mice and JWH-133 versus vehicle treated IL-10^{-/-} mice. The mean histological scores of IL-10^{-/-} mice with colitis that received vehicle were significantly higher than those mice treated with 2.5 mg/kg of JWH-133 (Fig. 1B). No significant protection was noticed in 1 and or 5 mg/kg dose of JWH treatment (Fig. 1 B). Increase in leukocyte infiltrate mainly consisted of lymphocytes and distortion of glandular architecture in the colon was noticed during colitis (Fig. 1C). Mice that received JWH-133 showed a significant reduction in the colon inflammation, reduced leukocyte infiltration, and a partially restored glandular and goblet cell architecture as compared to control mice. Pathologic changes included leukocyte infiltrates in the LP of the colon of vehicle treated IL-10^{-/-} mice, with the number of these infiltrates being reduced after 2.5 mg/kg of JWH-133 treatment (Fig. 1C). Because 2.5 mg/kg of JWH-133 was the optimum dose in this chronic colitis model, we used this dose throughout this study.

JWH-133 modulation of co-stimulatory molecule expression by lymphocytes. LP lymphocytes and immature dendritic cells are induced to rapid maturation and CD86 expression after entering in the MLN (Huang *et al.*, 2000). To further establish the JWH-133-mediated effects on the regulation of co-stimulatory molecules, we next assessed the potential of JWH-133 to modulate the expression of CD80, CD86 (gated on B220 cells), and 4-1BB (gated on T cells) after CD3 and CD28 antigen stimulation in MLN lymphocytes. JWH-133 significantly increased the expression of CD80, and CD86 by B220 cells during JWH-133 stimulation in a concentration-dependent fashion (Table 1). In

contrast, JWH-133 also significantly down-regulated the expression of 4-1BB, a T cell activation marker. Taken together, these findings suggested that JWH-133 might mediate differential activity, which could result in distinct effects on the expression of T- and B-cell costimulatory molecules.

JWH-133 Modulates of T helper lymphocytes

It is well known that colitis may be the result of massive cellular infiltrates and/or an overall autoimmune dysregulation/imbalance in T cells. It has been shown that CD4⁺ Th1 cells mainly mediate the chronic inflammation in the colons of IL-10^{-/-} mice (Berg *et al.*, 1996). Next, we delineated the effect of JWH-133 on T cells (Fig 2). The leukocytes that infiltrated the spleen, MLN and LP were characterized by flow cytometry analysis. After chronic colitis development, JWH-133 treatment significantly increased the percentage and absolute numbers of CD4⁺ T cells in the spleen when compared to the vehicle-treated control mice. In contrast, in the MLN and LP, the CD4⁺ T cell percentage and numbers decreased in JWH-treated groups as compared to vehicle treated group (Fig 2). We did not noticed, any major changes in the absolute numbers of CD8⁺ T cells in any groups except in the MLN of JWH-133 treated group. Together, these findings suggested that JWH-133 treatment considerably reduces the percentage and absolute numbers of CD4⁺ T helper lymphocytes in the MLN and LP (inductive and effector site) while increasing both the percentage and the number of CD4⁺ T cells in the spleen (systemic).

JWH-133 inhibits neutrophils infiltration during colitis

It has been reported that circulating and activated neutrophils, a major source of inflammatory cytokines, are elevated in UC patients and neutrophils are among the first cell types to arrive at a site of inflammation. Further, it has been hypothesized in recent reports that neutrophils are involved in many experimental models of colitis (Breider *et al.*, 1997; Natsui *et al.*, 1997). Based on this information, we next examined the changes in percentage and numbers of mucosal and systemic neutrophils after JWH-133 treatment after the chronic colitis. To this end, we used LY6G⁺ marker that is primarily associated with neutrophils. We noticed a significant increase of the neutrophils proportion in the spleen, MLN and LP of vehicle treated post-colitis mice as compared to pre-colitis mice (Fig 3). JWH-133 significantly reduced the percentage and numbers of neutrophils in spleen, MLN and LP compared with vehicle-treated group (Fig. 3). Taken together, these results indicate that the increased neutrophils seen during chronic colitis were significantly reduced both at systemic and mucosal sites, after JWH-133 treatment.

JWH-133 decreases activated T cells associated with colitis

To determine whether JWH-133 has any effect on activated T cells in this colitis model, we enumerated the percentage of CD4⁺CD69⁺ expressing T cells from spleen, MLN and LP by flow cytometry. A significant decline in the percentage and numbers of CD4⁺CD69⁺T cells were noticed in the spleens, MLN and LP of IL-10^{-/-} mice that received JWH-133 as compared to vehicle-treated mice (Fig. 4). Taken together, these data indicated that JWH-133 reduces activated T cell populations from the spleen, MLN and LP.

Effect of JWH-133 treatment on NK 1.1 and mast cells during colitis

NK cells have been shown to be involved in the differentiation of naïve CD4⁺ T cells into Th1 cells (Romagnani, 1992). We therefore examined the effect of JWH-133 on NK 1.1 cells in chronic colitis model. The splenic NK 1.1 cell number as well as percentage did not change after JWH-133 treatment (Fig 5). However, we noticed that JWH-133 reduced the number and percentage of LP NK1.1 cells that were increased during this disease as compared with pre-colitis mice (Fig 5 A & B).

We also examined the changes in mast cell percentage and numbers after JWH-133 treatment. Mast cells were significantly increased in the spleen, and LP of control IL-10^{-/-} mice as compared with pre-colitis mice (Fig 5 C & D). However, this population reduced significantly after JWH-133 treatment both in the spleen and LP.

JWH-133 induces apoptosis in T cells in the colon and reduces T cell proliferation

Next, we tested whether JWH-133 induces apoptosis *in-vitro* and *in-vivo* at sites of inflammation after chronic colitis. JWH-133 induced higher levels of apoptosis in activated T cell in vitro when compared with vehicle controls, in a dose-dependent manner (Fig. 6A). Also, addition of CB2 antagonist, AM630, reversed the T cell apoptosis induced by JWH-133 thereby suggesting that JWH-133 was acting through CB2 receptors (Fig. 6A). Correspondingly, IL-10^{-/-} mice that received JWH-133 after onset of chronic colitis showed significant increase in apoptosis in the colon when compared to vehicle-treated mice (Fig. 6 B) as well as IL-10^{-/-} mice with pre-colitis. The mean apoptosis scores of IL-10^{-/-} mice with colitis that received vehicle were significantly lower than those mice treated with JWH-133 (Fig. 6 C). Taken together, these data indicated that JWH-133 induces apoptosis mainly in T cells thereby reducing its populations from the LP, while AM630 reversed the induction of apoptosis mediated by JWH-133. Together, these data indicated that JWH-133 decreased T cells from colon and that this may result from induction of apoptosis in T cells. We also examined whether, JWH-133 reduces proliferation of T cells. To this end, we used lymphocytes from MLN (~75% T cells) from C57BL/6 mice cultured with various doses of JWH-133 after anti-CD3 and CD28 Ab stimulation. We found that JWH-133 caused a significant decrease in T cell proliferation as compared to activated control T cells (Fig. 6D).

JWH-133 is effective against DSS-induced colitis and its effects are reversed by CB2 antagonist

To determine if the effects of JWH-133 can also be expanded to other models of colitis, we studies DSS-mediated colitis. Also, we determined if the protective effect of JWH-133 was mediated through CB2 receptors by using the CB2 antagonist (AM630). To this end, C57BL/6 mice received 3% DSS alone in drinking water. The treatment groups received a combination of DSS in drinking water and JWH-133 (10 or 20 mg/kg body weight). We used a higher dose of JWH-133 because of the acute nature of colitis induced. In addition, some groups received JWH and 20 mg/kg body weight of antagonist (AM-630). DSS administration caused acute colitis in these mice, as shown by weight loss (Fig. 7A) and occasional bleeding. Mice treated with 10 mg/kg body weight of JWH-133 showed significant improvement in body weight, however, complete reversal of body weight was seen only at a dose of 20 mg/kg body weight (Fig. 7A). In contrast, mice that received JWH

+AM-630 showed increased inflammation and decreased body weight when compared to those that received JWH-133 alone. These results indicated that JWH-133 can also protect mice against DSS-induced acute form of colitis, and that it mediates these effect in vivo through CB2 receptors. We confirmed these results also by mean inflammation scores as well as histopathological analysis (Fig 7 B and C respectively). Taken together, these data clearly indicated that JWH-133 protects mice from both spontaneous and DSS-induced colitis, through the activation of CB2 receptors.

Next, we examined the changes in percentage of macrophages and IFN- γ expressing cells in DSS-induced colitis after treatment with JWH-133 alone JWH-133+ AM630. Macrophage percentage (CD11b⁺) was significantly increased in MLN and LP during DSS-induced colitis as compared to control mice (Fig. 8A). JWH-treatment decreased the percentage of macrophages from MLN and LP when compared to DSS-induced mice without treatment (Fig. 8A). We found very modest changes in the percentage of macrophages in the spleen. In contrast, the AM630 treatment reversed the JWH-133-mediated effects in macrophages. We noticed a similar trend in the changes in the percentage and expression of IFN- γ ⁺ expressing cells in the spleen and LP after JWH-133 and AM630 treatment (Fig 8B). We did not notice any changes in MLN IFN- γ expression in any group (data not shown).

Discussion

Inflammation activates several cellular pathways in the intestinal tract, leading to a pathological state. IBD is a major burden for society since conventional therapies are neither uniformly effective nor without side effects, novel therapeutic options are always warranted. There are reports indicating that the both CB1 and CB2 cannabinoid receptors as well as their endogenous ligands can serve as potential targets for the treatment of IBD (Massa *et al.*, 2004; Kimball *et al.*, 2006; Storr *et al.*, 2008; Storr *et al.*, 2009). It has also been reported that cannabinoids relieve symptoms in patients with CD (Grinspoon and Bakalar, 1995). Previous immunologic and therapeutic evidence suggests that animal models mimicking colitis that are relevant to human IBD and the pathologic process are similar (Neurath *et al.*, 2000).

We therefore identified the functional effect of CB2 receptor-selective cannabinoids to abrogate spontaneous mouse model of chronic colitis, using IL-10^{-/-} mice that has pathological similarities with human IBD patients (Berg *et al.*, 1996). In the current study, we demonstrated that JWH-133, a CB2 selective agonist, reversed colitis in IL-10^{-/-} mice. JWH-133 treatment reversed the body weight loss and corrected the inflammation score and disease severity. Our *in-vitro* results indicated that JWH-133 reduced T cell proliferation, induced apoptosis, and reduced 4-1BB expressions on activated T cells. The increased numbers of mucosal and systemic mast cells during chronic colitis was also mitigated by JWH-133 treatment. Taken together, these results suggest that JWH-133 abrogates colitis in IL-10^{-/-} mice by suppressing the activation of Th cells by promoting their apoptosis and thereby potentially reducing other inflammatory cells seen at sites inflammation in the colon.

Several recent studies have indicated a role for exogenous and endogenous cannabinoids in suppressing experimental models of colitis. Delta-9 tetrahydrocannabinol (THC) and cannabidiol alone and in combination were found to suppress inflammation of the 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced acute colitis in rats (Jamontt *et al.*, 2010). AM630, a CB2 agonist, was shown to protect against experimental TNBS-induced colitis in mice. In this study, colitis was associated with increased expression of CB2 receptor mRNA (Storr *et al.*, 2009). In contrast, human studies indicated that CB2 receptor expression increased in mild and moderate pancolitis patients, whereas, during quiescent pancolitis, CB1 and CB2 expression dropped (Marquez *et al.*, 2009). The endocannabinoid system may also exert inhibitory effects on inflammatory responses in the colon. For example, mice lacking the CB1-, CB2-receptor or both receptors, showed aggravation of inflammation in the TNBS-induced model of colitis (Engel *et al.*, 2010). Furthermore, blocking endocannabinoid degradation or cellular reuptake in experimental TNBS-induced colitis in mice was shown to inhibit inflammation and colon length (Storr *et al.*, 2008). Additionally, human studies indicated that CB1 1359 G/A (p.Thr453Thr, rs1049353) single nucleotide polymorphism (SNP) modulated UC susceptibility and the CD phenotype (Storr *et al.*, 2010). Also, increased levels of endocannabinoid, anandamide, have been reported from biopsies of human UC patients and these levels were increased in the sub-mucosal layer, but not in the mucosal layer of the inflamed colon (D'Argenio *et al.*, 2006). Unlike the TNBS-induced experimental models of colitis in which the effect of cannabinoids has been studied previously, there are no reports, thus far, on the effect of cannabinoids treatment on chronic spontaneous model of colitis. Of the many spontaneous models of chronic colitis that have been developed involving gene-targeting the IL- IL-10^{-/-} mouse represents a model that has been extensively studied. This model mimics pathology similar to that seen in human IBD. The advantage of using the IL-10^{-/-} model is that it is a well-established Th1-mediated model of colitis (Ostanin *et al.*, 2009). Thus, our studies demonstrate for the first time that CB2 receptor selective agonist during the spontaneous development of colitis can improve the body weight, prevent the progression of the disease and ameliorate colitis. The increase in body weight in normal BL/6 mice that received JWH-133 alone might be due to increase in food consumption. To this end there is report that CB receptor antagonist AM4113 regulates the body weight by induction of initial transient reduction of food intake which results in long term reduction of body weight gain (Cluny *et al.*, 2011). Another report also showed that CB2 antagonist reduces the body weight (Ignatowska-Jankowska *et al.*, 2011). This may be one of the mechanisms through which, JWH-133 may increase body weight in our colitis models as well. However, it should be noted that we used increase in body weight as only one of the indicators of effectiveness of treatment against colitis and that we tested several additional inflammatory markers which clearly indicated suppression of inflammation in the colon mediated by JWH-133.

Several IBD models of colitis indicate that CD4⁺ T cells play a major role in the induction of IBD and that much of the intestinal damage of this disease is a result of T cell-mediated injury (Elson *et al.*, 1996). This is consistent with the current study wherein we found that the percentage and number of CD4⁺ T cells in the LP increased significantly during colitis progression. Moreover, JWH-133 treatment caused marked decrease in these cells in the LP. We have shown previously that CD4⁺ T cells were a source of IFN- γ in the LP during

severe colitis and the numbers of IFN- γ ⁺ as well as TNF- α ⁺ expressing CD4⁺ T cells were elevated in the LP during severe colitis (Singh *et al.*, 2008). It has been shown that IFN- γ play critical role during induction and progression of colitis (Neurath *et al.*, 1995). In the present study, we demonstrated that IFN- γ expression was decreased following JWH-133 treatment, which was reversed after AM630 treatment. The current study also indicated that JWH-133 *in-vitro* induced the expression of CD80 and CD86 on B cells. In general, this indicated that JWH-133 was effectively modulating expression of co-stimulatory molecules that might be necessary for the activation of antigen presenting cells (APCs) and consequent activation of T cells. However, such activated T cells may be killed by the JWH-133 by inducing apoptosis. 4-1BB, a member of the TNFR super-family, is a costimulatory receptor primarily expressed on activated T cells. *In vitro* studies have shown that 4-1BB signaling augments T cell proliferation and cytokine production (Sun *et al.*, 2002). Thus, administration of an agonistic anti-4-1BB mAb was shown to reduce the incidence and severity of autoimmune diseases such as experimental autoimmune encephalomyelitis (Sun *et al.*, 2002). In the current study, we found that JWH-133 decreased 4-1BB expressions on stimulated T cells. In addition to 4-1BB, we also noted a decrease *in vivo* in the percentage and numbers of colitis-induced CD69⁺ cells, with CD69 being an early activation marker. Together, these observations suggest that activation of CB2 by JWH-133 may also suppress colitis by down-regulating the T cell activation process.

The recruitment and activation of neutrophil are considered to play a causative role in mucosal injury during IBD. Disease activity in IBD is linked to an influx of neutrophil in the mucosa and subsequently in the intestinal lumen, resulting in the formation of crypt abscesses and granulomatous. It has been shown that monoclonal antibody-mediated depletion of neutrophil decreases several parameters of DSS-induced colitis (Natsui *et al.*, 1997), reduces the cellular infiltrates in rectal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis (Palmen *et al.*, 1995). This also exerts therapeutic effects in DSS-induced colitis by correcting weight loss and inflammation scores (Morohoshi *et al.*, 2006). Recently, it has been shown that Curcumin protects from IBD by mediating neutrophil motility (Larmonier *et al.*, 2010). JWH-133 treatment significantly diminished the neutrophil numbers and percentage as compared to vehicle treated mice. Our study also corroborates the finding in part that activation of CB2 receptor protects against cerebral ischemia mainly by inhibiting neutrophil recruitment (Murikinati *et al.*, 2010). The present study corroborates the above findings, and suggests that JWH-133 suppress both percentage and number of the neutrophil to attenuate chronic colitis.

It has been reported that macrophages are prominently seen in active IBD (Strober and James, 1986), but absent in normal mucosa. Studies also indicate that in DSS-induced colitis, macrophage percentage increased during progression of disease severity (Grose *et al.*, 2001; Ogawa *et al.*, 2004). In the present study, we also noted a similar increase in macrophages in MLN and LP of mice with colitis as compared to control group. JWH-133 treatment reversed the increased CD11b⁺ cells to normal levels, but AM630 reversed it back, indicating that effect of JWH-133 was mediated through activation of CB2 receptors.

In addition to the decrease in T cell activation, our studies also demonstrated that JWH-133 induces apoptosis in T cells. Activation of cannabinoid receptors was shown by us

previously to down-regulate Raf-1/mitogen-activated protein kinase/ERK kinase (MEK)/ERK/RSK pathway leading to translocation of Bad to mitochondria and consequent induction of apoptosis (Jia *et al.*, 2006). Also, we found that cannabinod receptor activation triggers apoptosis in antigen presenting cells such as the dendritic cells (Do *et al.*, 2004). Using CB2 selective agonist, the induction of apoptosis in immune cells and in colon cancer cell lines (Lombard *et al.*, 2007; Cianchi *et al.*, 2008) has been reported. Together, our study suggests that CB2 activation *in vivo* during colitis by JWH-133 may induce apoptosis in T cell mainly in the MLN and LP to suppress colitis.

Constant activation of the immune system occurs in the gastrointestinal tract that is exposed to exogenous agents entering the body by intestinal bacteria. Since mast cells are involved in the recognition of food allergens, bacteria and invading pathogens can therefore be found in a continuously activated state in the intestinal tract (Argenzio, 1997). Several lines of new evidence suggest that mast cells are involved in the pathogenesis of IBD (He, 2004). The increased mast cell number and its mediators can be found in the mucosa of IBD patients (Raithel *et al.*, 2001). Recently, it has been shown that mast cells are critically involved in the development of symptoms in the murine model of IBD (Rijnierse *et al.*, 2006). In the present study, we demonstrated that JWH-133 effectively suppressed the number and percentage of mast cells compared to control mice. Our present study supports the conclusions of a few reports that show CB2 receptor ligands significantly reduce the immunological release of histamine from guinea pig mast cells (Samson *et al.*, 2003) and endocannabinoids down regulate the activation of the RBL-2H3 mast cell line through CB2 receptor-mediated AKT phosphorylation (Vannacci *et al.*, 2002). Furthermore, recently it has been shown that CB2 ligands modulate mast cell activation by NO and PGE₂ release, acting in an autocrine manner to inhibit histamine release (Vannacci *et al.*, 2004). Taken together, our data suggest the potential role of mast cells in chronic models of colitis, and that CB2 agonists suppress the mast cells, which may contribute towards attenuation of colitis.

It has been well known that endogenous cannabinoid system protects against colonic inflammation. Previous study also indicated that mice that lack CB1 and CB2 receptors show increased susceptibility to TNBS-induced colitis (Engel *et al.*, 2010). In the present study, our data strongly suggested that CB2 receptor agonists protect mice from both spontaneous as well as DSS-induced colitis. Using CB2 receptor antagonist (AM630), we demonstrated both *in vitro* and *in vivo* that JWH-133 mediates these effects through activation of CB2 receptors.

In summary, the current study suggests that JWH-133 acts as an anti-inflammatory agent by targeting multiple cellular pathways during the development of spontaneous chronic colitis. While many mechanisms of CB2-receptor agonist-mediated immunosuppression of colitis have been identified, the current study, for the first time, has suggested that JWH-133 may also mediate anti-inflammatory properties through inhibition of activated T cells, and induction of apoptosis in T cells.

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Highlights

- JWH-133, a cannabinoid receptor-2 agonist ameliorated spontaneous and DSS induced colitis.
- JWH-133 suppressed inflammation and toxicity to colon by inducing apoptosis in activated T cells.
- JWH-133 decreased mast cells, macrophages, NK cells, IFN- γ^+ cells and neutrophils in the LPL.
- AM630 a cannabinoid receptor-2 antagonist reversed the protection afforded by JWH-133 against colitis.
- Cannabinoid receptor-2 may serve as a novel therapeutic target for Inflammatory Bowel Disease

Fig. 1. Change in body weight, histological characterization and inflammation score after JWH-133 treatment in C57BL/6 and IL-10^{-/-} mice

IL-10^{-/-} mice, on a C57BL/6 background, and normal C57BL/6 mice (Δ) received, 1, 2.5 and 5 mg/kg body weight of JWH-133, (□), (○), (Δ) or vehicle (●), every second day, starting at the beginning of week 18 when mice had lost about 15-17 % of their body weight and continuing until the week 25. The mice were sacrificed two weeks after the last injection at week 27. The body weight of the IL-10^{-/-} mice was recorded every week, and the change from initial body weight was expressed as a percentage change in body weight (Fig. 1A). Histological sections of colons from the pre-colitis and two groups of IL-10^{-/-} mice at the week 27 were presented (as described in Fig 1C). Vehicle treated mice (Fig. 1 C lower panel) showed significant lymphocyte infiltration and distortion of glands, while JWH-133-treated mice (Fig. 1 B upper panel) showed markedly decreased lymphocyte infiltration. Other pathologic changes included diffuse leukocyte infiltrates, distorted crypts, and thickening of the lamina propria in the area of distorted crypts in the colon. The histological inflammation scores of these two groups are presented in (Fig. 1 C). Asterisks (*) indicate statistically significant differences ($p < 0.01$) between vehicle and JWH-133 treated groups. Representative sections from three separate experiments (10 and 40X magnification) are shown, with each group containing six mice.

Fig. 2. Effect of JWH-133 on T cells during colitis

Splenic, MLN and LP lymphocytes were isolated from pre-colitis mice and two groups of IL-10^{-/-} mice (at 27 weeks) after the end-points of experiment as described in Fig 1 legend. The lymphocytes were stained for CD4⁺ and CD8⁺ T cells and analyzed using flow cytometry. The numbers in the bottom right quadrant indicate the total percentage of CD4⁺ T cells (Panel A); the upper left quadrant indicates the total percentage of CD8⁺ T (Panel A) cells. The absolute number (mean of 6 samples $\pm$ SEM) of CD4⁺ T cells from spleen, MLN and LP in each group was enumerated and depicted (Panel B). We did not notice any major changes in the CD8⁺ T cells in any experimental group. Asterisks (*) indicate statistically significant difference ($p < 0.01$) between vehicle vs JWH-133.

Fig. 3. JWH-133 reduces percentage and numbers neutrophil during colitis

Spleen, MLN and LP lymphocytes were isolated from pre-colitis IL-10^{-/-} mice and at the week 27 from IL-10^{-/-} mice that received 2.5 (mg/kg body weight) of JWH-133 and vehicle control. The lymphocytes were stained for CD3⁺ T and neutrophil (LY6G⁺) cells. The spleen, MLN or LP-derived CD3⁻ lymphocytes were characterized for neutrophil expression by flow cytometry. The numbers in the upper left quadrant indicate the total percentage of neutrophil. Data represent the total number of cells $\pm$ SEM from three independent experiments involving 6 mice per groups. Asterisks (*) indicate statistically significant differences ($p < 0.01$) between vehicle and JWH-133 treated groups.

Fig. 4. JWH-133 reduces the induction of activated T cells during colitis

Spleen, MLNs and LP lymphocytes were isolated from the pre-colitis and two groups of IL-10^{-/-} mice at the week 27 and stained for CD4⁺ and CD69⁺ T cells. Panel A shows a representative experiment indicating the percentages of various types of cells and panel B depicts the absolute numbers of these cells in various lymphoid organs. In panel B, the data represent the total number of cells $\pm$ SEM from three independent experiments. Asterisks (*) indicate statistically significant differences ($p < 0.01$) between vehicle and JWH-133 treated groups.

Fig. 5. JWH-133 treatment mediates mast and NK1.1 during colitis

Spleen and LP cells were isolated from IL-10^{-/-} mice before the onset of colitis (pre-colitis) and two groups of IL-10^{-/-} mice post-colitis at the week 27. The cells were stained for expression of CD3 and NK1.1 or CD117 (mast cell). Panel A and B show a representative experiment indicating the percentages and numbers of NK1.1 cells in various lymphoid organs. Panels C, and D represent the total number and percentage of mast cells $\pm$ SEM from three independent experiments. Asterisks (*) indicate statistically significant differences ($p < 0.01$) between vehicle and JWH-133 treated groups.

Fig. 6. JWH-133 treatment induces apoptosis both in-vitro and in-vivo in the colon and inhibits T cell proliferation

Lymphocytes obtained from MLN (~ 75% T cells) of normal C57BL/6 mice were cultured after anti-CD3/CD28 Abs activation in the presence of vehicle or JWH-133 (0, 1, 5, 10, 20 and 50 μ M). Some cultures received JWH+ 20 or 50 μ M of AM630. The apoptosis of T cells was measured by flow cytometry (A). *In situ* tunnel assay sections of colons from the pre-colitis and two groups of IL-10^{-/-} at the week 27 (as described in Fig 1) and normal C57BL/6 mice (8 weeks) were presented (B). Representative sections from three separate experiments (20X magnification) are shown. Vehicle treated normal as well as IL-10^{-/-} mice showed less apoptosis as compared to JWH-133-treated mice. The percentage of apoptosis scores for each group is presented (C). Panel D shows BrdU cell proliferation assay performed using lymphocytes obtained from MLN (~ 75% T cells) of normal C57BL/6 mice cultured with anti-CD3/CD28 Abs in the presence of vehicle or JWH-133 (0, 5 and 50 μ M) at a density of 5×10^6 cells/ml. The Proliferation of T cells were measured by BrdU incorporation. The panel D data presented are the mean OD₄₅₀ for proliferation $\pm$ SEM of quadruplicate cultures. The statistical significance between experimental and control group was assessed using Student's t tests. Data represent the mean of three independent experiments involving six mice per group. Asterisks (*) indicate statistically significant differences ($p < 0.01$) between groups.

Fig. 7. Change in body weight, colitis score and severity of DSS-induced colitis after JWH-133 and AM-630 treatment

BL/6 mice were given vehicle (Δ , control), JWH-133 alone (20 mg/kg body weight)($\square$), DSS alone (3%) in drinking water ($\bullet$, DSS), or a combination of DSS with 10 ($\blacktriangle$) or 20 ($\bullet$) mg/kg body weight dose of JWH-133 or a combination of JWH-133 (20 mg/kg) + 20 mg/kg dose of AM-630 ($\circ$) for fourteen days. DSS was replaced after seven days with a water cycle (*ad libitum*) for another seven days. The body weight of these mice was recorded daily. Changes in body weight were expressed as a percentage of the weight (panel A). Panel B represents the persistence or improvement of colitis inflammation scores. Histological sections of colons from the 4 groups of mice were presented (panel C). DSS induced mice showed significant lymphocyte infiltration and distortion of glands (arrow), while JWH-133 treated mice showed colon lumen having markedly decreased lymphocyte infiltration. The AM-630 treatment further increased leukocyte infiltrates, distorted crypts (arrow) in the colon. The statistical significance between values of each group was assessed by Student's t test. Data represents the mean of three experiment involving 6 mice per group.

Fig. 8. Effect of JWH-133 treatment on CD11b⁺ and IFN-γ⁺ expression during colitis
Colitis was induced using DSS and these mice were treated with JWH-133 and AM630 as described in Fig 7 legend. Cells from the spleen, MLN and LP were stained for CD11b⁺ (panel A) macrophages and for IFN-γ⁺ (panel B) cells using flow cytometry.

Table 1

Percent change in co-stimulatory molecule expression by JWH-133

Lymphocytes	Dose of JWH133 (μ M/ml)			
	<u>Unstimulated</u> Cells	Stimulated		
		0 μ M/ml	5 μ M/ml	50 μ M/ml
B220+ CD80	3.2% $\pm$ 0.6	5.3% $\pm$ 0.9	8.82 [*] $\pm$ 1.6	11.04% $\pm$ 0.9
B220+ CD86	2.1% $\pm$ 0.6	8.6% $\pm$ 1.4	12.3% [*] $\pm$ 1.2	19.2% [*] $\pm$ 3.1
<u>CD3+ 4-1BB</u>	2.4% $\pm$ 1.9	11.6% $\pm$ 2.3	4.1 [*] % $\pm$ 1.1	3.4% [*] $\pm$ 0.3

CD3+CD28-stimulated or unstimulated MLN cells from C57BL/6 mice were incubated with 0, 5, 50 μ M/ml of JWH-133 for 48 h and prepared for FACS analysis. The percentages increase or decrease of the costimulatory molecule expression was calculated as the percent of double positive of each population. Data represent mean $\pm$ SEM of three independent experiments.

* Statistically significant differences ($p < 0.01$) relative to culture without JWH.