



Bergapten enhances mitophagy to regulate intestinal barrier and Th17/Treg balance in mice with Crohn's disease-like colitis via PPAR γ /NF- κ B signaling pathway

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Abstract

This study aimed to investigate whether bergapten (BG), a furanocoumarin phytohormone, holds promise for Crohn's disease (CD)-like colitis treatment and to preliminarily explore its potential mechanisms. 2,4,6-Trinitrobenzenesulfonic acid (TNBS)-treated mice were applied to establish an *in vivo* research model, and BG was administered with different concentrations. The status of mice in each group was evaluated by disease activity index (DAI), and the severity was evaluated by pathological sections. The intestinal barrier was assessed by measuring *in vivo* intestinal permeability, peripheral blood intestinal fatty acid-binding protein (I-FABP) levels, epithelial resistance values, and tight junction protein levels. Markers were then used to assess Th17/Treg levels, mitophagy, and the peroxisome proliferator-activated receptor (PPAR) γ /nuclear factor kappa B (NF- κ B) signaling pathway. BG significantly reduced colon tissue damage in a concentration-dependent manner. DAI scores showed that the loose feces, occult blood, and weight loss of mice in the BG treatment were significantly reduced, and pathological section results revealed reduced inflammatory infiltration and fibrosis. Reduced serum FITC-dextran and I-FABP and increased levels of epithelial resistance and tight junction proteins support that the intestinal barrier was protected upon BG. The proportion of Th17 in mesenteric lymph nodes increased while Treg decreased in the model group. BG treatment effectively reduced the conversion of Treg to Th17. Additionally, BG was found to enhance mitophagy and activate the PPAR γ /NF- κ B signaling. BG demonstrates promising effects in ameliorating intestinal barrier damage and Th17/Treg imbalance in a murine model of CD-like colitis, while also promoting intracellular mitophagy. The PPAR γ /NF- κ B signaling pathway may serve as a key mediator of BG's regulatory mechanisms.

Keywords Inflammatory bowel disease · Crohn's disease · T cell · PPAR · Intestinal barrier

Introduction

Crohn's disease (CD) is a chronic, relapsing condition that primarily affects the intestines. Historically, CD was predominantly observed in Europe, but over the past decade, its incidence has been steadily rising in Asia (Ng et al. 2017). Due to its tendency for lifelong relapse, patients require long-term drug therapy to maintain intestinal inflammation

at a manageable level (Roda et al. 2020). Current treatments for CD predominantly include glucocorticoids, immunosuppressants, and intestinal mucosal protective agents. However, these medications often exhibit varying degrees of drug resistance and adverse reactions. Despite the widespread adoption of biological agents such as tumor necrosis factor- α (TNF- α) monoclonal antibodies, the surgical intervention rate for CD has not significantly decreased (Cockburn et al. 2023). Therefore, there is an urgent need for the development of new drugs with low toxicity, minimal adverse reactions, and precise efficacy (Noor et al. 2020). Although the exact pathogenesis of CD remains unclear, dysfunction of the intestinal barrier due to defects in the structure and function of intestinal epithelial cells is considered a crucial factor in initiating or perpetuating chronic intestinal inflammation (Kaminsky et al. 2021).

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Bergapten (BG), primarily derived from bergamot essential oil, is a furanocoumarin phytohormone found in various herbs and fruits, exhibiting anti-inflammatory properties. Studies have also demonstrated significant antioxidant and anti-cancer effects of BG (Quetglas-Llabrés et al. 2022). For instance, in mouse models of *Escherichia coli*-induced sepsis and *Citrobacter*-induced intestinal inflammation, BG inhibited the activation of the NOD-like receptor thermal protein domain-associated protein 3 (NLRP3) inflammasome and cellular pyroptosis by maintaining mitochondrial homeostasis, thereby significantly ameliorating tissue inflammation and damage (Luo et al. 2023). Additionally, in an isolated rabbit jejunum model, BG exhibited concentration-dependent relaxation effects on both spontaneous and potassium ion-induced contractions, indicating its potential as an anti-ulcer and anti-diarrheal agent with relative safety demonstrated in acute toxicity tests (Aslam et al. 2022). These findings underscore the potential of BG in the management of gut-related diseases through the modulation of mitochondrial function.

Furthermore, recent studies suggest that the development of CD may largely depend on the balance between Th17 cells and regulatory T cells (Tregs) (Gomez-Bris et al. 2023; Saez et al. 2021). Th17 cells primarily secrete pro-inflammatory factors such as interleukin (IL)-17, inducing neutrophil migration to the site of inflammation (Akhter et al. 2023). Single-cell analysis revealed a reduction in Tregs within tissues at sites of intestinal inflammation in severe CD patients, potentially exacerbating inflammation (Jaeger et al. 2021). BG has been shown to mitigate allergic rhinitis in mice exposed to PM2.5 by balancing Treg/Th17 expression (Jiang et al. 2023), suggesting its potential to modulate the course of CD by restoring Treg/Th17 balance, an area currently lacking in BG research in CD. Using the Super-PRED site, BG was found to target peroxisome proliferator-activated receptor (PPAR) γ , and previous studies have demonstrated that stimulating PPAR- γ expression alleviates inflammatory bowel disease (IBD) (Venkataraman et al. 2022).

Therefore, we hypothesize that BG may attenuate intestinal barrier damage to mitigate CD severity by regulating the PPAR γ signaling pathway to promote mitophagy and balance Th17/Treg. This study utilized 2,4,6-trinitrobenzenesulfonic acid (TNBS)-treated mice to establish an *in vivo* research model to investigate whether BG holds promise for CD treatment and to preliminarily explore its potential mechanisms.

Methods and materials

Animals

Male C57BL/6 mice (6–8 weeks, ~20 g) were purchased from Cavens-Biogle (Soochow, Jiangsu, China). All mice

were housed in an SPF environment under controlled temperature (~22 °C) and humidity (~55%) with natural light cycles, with *ad libitum* access to food and water. All experimental procedures followed the National Institute of Health guidelines and were approved by the Animal Ethics Committee.

Grouping and treatment

TNBS-treated mice established preclinical models that mimic clinical CD as previously reported (Katsandegwaza et al. 2022). Mice were randomly divided into five groups (6 mice per group): Sham (saline), TNBS (model), and BG treatment (TNBS + 3, 10, and 30 mg/kg BG) groups. Briefly, after fasting overnight and anesthetizing with 2% isoflurane, saline or TNBS (50 mg/kg) dissolved in 0.5 ml 45% ethanol was carefully dripped into the colon through a flexible catheter. The mice remained inverted for 1 min to ensure contact with the intestinal mucosa. The experiment was repeated weekly for 4 weeks. BG treatment was started at the beginning of TNBS induction, and BG (dissolved in 0.5 ml of sterile water) was administered via intragastric infusion once a day for 4 weeks. The sham and model groups received sterile water via the same route. The disease activity index (DAI) based on fecal consistency, occult blood, and weight loss was scored according to a previous study (Yan et al. 2018).

Sample collection

Before the end of the handling procedure, mice were deprived of food and water for 4 h and given FITC-dextran (600 mg/kg) by gavage. Cardiac puncture and blood collection were performed under anesthesia, followed by euthanasia by cervical dislocation. FITC-dextran levels in serum were measured using a spectrophotometer (Molecular Devices, Shanghai, China). Intestinal tissue was collected, the length of the colon was measured, and then the samples were evenly divided to prepare lysates and tissue wax blocks for subsequent analysis.

Hematoxylin and eosin (H&E) staining

Colons flushed with ice-cold PBS were fixed in 4% paraformaldehyde and embedded in paraffin to prepare sections for histological evaluation. Sections cut into 4 μ m thickness were treated with xylene and decreasing concentrations of alcohol. The sections were routinely stained with hematoxylin–eosin (HC0513, Fantawild Biotechnology, Beijing). Images were captured under a microscope (Olympus, Tokyo, Japan).

Masson staining

This assay was performed using a Masson staining kit (BL1538A, Biosharp, Hefei, China) according to the manufacturer's instructions. Dewaxed and hydrated tissue sections were obtained as described above. Following the staining with Weigert's iron hematoxylin for 5 min, samples were differentiated with acidic ethanol for 10 s, treated with the aniline blue solution for 1 min, and ponceau red magenta dyeing solution for 5 min. Subsequently, sections were washed with the phosphomolybdic acid solution for 1 min and counterstained with the aniline blue dyeing solution for 1 min. The results were observed after dehydration under a light microscope (Olympus, Tokyo, Japan).

ELISA

The blood was allowed to stand at 4 °C for 30 min, and the serum was separated by centrifugation at 1000 × g for 10 min. Serum intestinal fatty acid-binding protein (I-FABP) was determined using an ELISA kit (NBP2-82,214; Bio-Techne China) according to the manufacturer's instructions. Absorbance was measured at 450 nm using the microplate reader (Molecular Devices, Shanghai, China).

Transepithelial electrical resistance (TEER) assay

Clean colon segments were placed in Krebs buffer (pH=7.3) and cut lengthwise into flat plates. The tissue was sandwiched between two film squares and placed into the Ussing chamber system (AD Instruments, Shanghai, China). Glucose Krebs buffer (10 mM; 1.5 mL) was added to one side as an energy source, and mannitol Krebs buffer (10 mM; 1.5 mL) was added to the other side to maintain osmotic pressure balance. The voltage and current were adjusted through the system, and the data at 37 °C was recorded with the built-in acquisition software after balancing (Shao et al. 2023). TEER was calculated based on Ohm's law (resistance = voltage / current) / the exposed issue area (given as Ω/cm^2) (Thomson et al. 2019).

Immunofluorescence (IF) assay

The mesenteric lymph node sections were dewaxed, permeabilized in 0.1% Triton X-100, and antigen retrieved. After serum blocking, the sections were incubated with primary antibodies against IL-17A or forkhead box protein P3 (FOXP3) and CD4 (Proteintech, Wuhan, China) overnight at 4 °C, followed by FITC-labeled and FRITC labeled secondary antibodies. For mitophagy, antibodies against LC3B and MitoTracker were used and slides were counterstained with DAPI. Results were examined with a fluorescence microscope (Olympus, Tokyo, Japan).

Reverse transcription-quantitative (RT-q) PCR

This assay was performed to determine the levels of T cell differentiation markers. Total RNA was gathered using TRIzol® reagent (Thermo Fisher Scientific) and subsequently reverse transcribed into cDNA using a universal reverse transcription kit (Qiagen). The mRNA expression levels were measured using QuantiTect SYBR Green PCR kit (Qiagen) on a real-time PCR system (Agilent China). Beta-actin was used as the internal reference and the relative mRNA expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method.

Western blot analysis

Total proteins were extracted from colon segments using RIPA lysis buffer, determined using NanoDrop equipment, separated via SDS-PAGE on a gel, and subsequently transferred onto PVDF membranes. Following blocking with 5% skimmed milk for 1.5 h, the membranes were incubated with primary antibodies (Proteintech) at 4 °C overnight. Following the membranes being washed with TBS-0.01% Tween-20 (TBST), they were incubated with a goat anti-rabbit secondary antibody (Abcam) for 2 h. Blots were visualized using an ECL kit, and the gray values were analyzed using ImageJ software (version 1.8).

Statistical analysis

Data are presented as the mean ± standard deviation, and statistical analysis was performed using GraphPad Prism (version 8.0). Comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ indicates a statistically significant difference.

Results

BG reduces TNBS-induced CD-like colitis

The average weight of mice in the TNBS-induced group was significantly lower than that in the sham group ($P < 0.001$), and the weight of the BG-treated group was restored in a concentration-dependent manner. There was a significant difference between the body weight of mice in the highest dose treatment group and the model group ($P < 0.001$; Fig. 1A). The mouse DAI score is calculated by adding three observations from 0 to 4 and dividing by 3. The results showed that the DAI of the TNBS-induced group was close to 4, and the values in the BG-treated groups decreased gradually with concentration (Fig. 1B). Additionally, the length of the colon was recorded for assessment of visual intestinal damage. The colon in the TNBS-induced group was significantly shortened ($P < 0.001$), and BG contributed

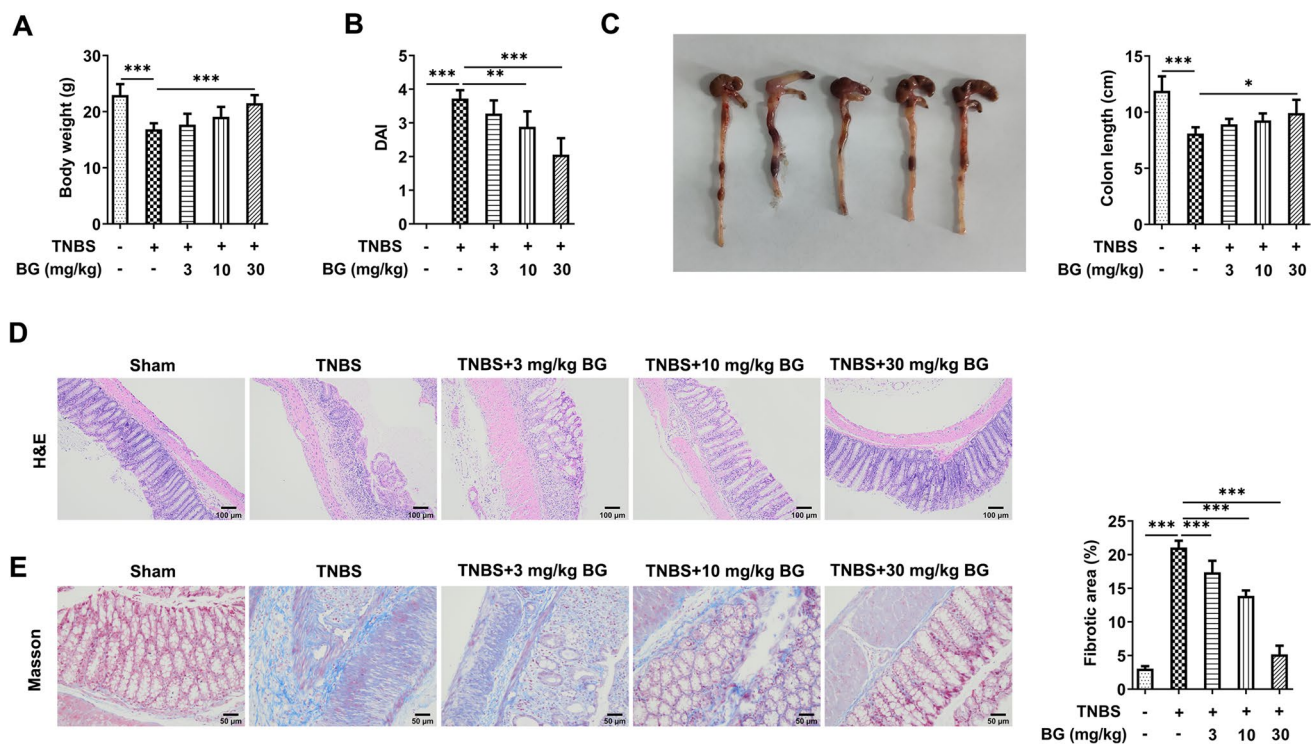


Fig. 1 BG reduces TNBS-induced CD-like colitis. **A** The average weight of mice. **B** DAI score based on fecal consistency, occult blood, and weight loss. **C** The length of the colon was recorded for

assessment of visual intestinal damage. **D** H&E staining exhibited the mucosal layer structure. **E** Masson staining displayed blue-labeled collagen fiber deposition. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

to the maintenance of colon length ($P < 0.05$; Fig. 1C). H&E staining exhibited that compared with the sham group, the mucosal layer structure in the model group was destroyed, inflammatory cells infiltrated, and the villus structure was disordered. The villi in the high-dose BG treatment group tended to be neat, and a clear normal mucosal layer could be observed (Fig. 1D). Masson staining displayed blue-labeled collagen fiber deposition in the model group, indicating the presence of fibrosis. In the BG treatment group, the blue visible part was significantly reduced and was very small in the high-dose group ($P < 0.001$; Fig. 1E).

BG reduces TNBS-induced intestinal barrier damage

The level of FITC-dextran in the serum of mice in the TNBS induction group was significantly increased ($P < 0.001$), compared with that in the sham group. Compared with the model group, the FITC-dextran level in the BG treatment group showed a concentration-dependent decrease (Fig. 2A). In addition, the serum level of I-FABP, a marker of intestinal injury, was significantly increased in the model group ($P < 0.001$), and BG treatment could alleviate the release of I-FABP into the blood circulation ($P < 0.01$; Fig. 2B). The decrease in TEER also indicated that the barrier in the model group was damaged, and BG

could play a role in maintaining the barrier ($P < 0.001$; Fig. 2C). For further validation, the levels of tight junction proteins were measured. The levels of ZO-1 and Claudin-1 protein decreased significantly in the model group, while Claudin-2 increased ($P < 0.001$). BG was conducive to the maintenance of high-level ZO-1 and Claudin-1 protein levels ($P < 0.05$; Fig. 2D). This suggested that BG antagonized the disruption of the integrity and stability of intercellular tight junction structures caused by TNBS.

BG maintains Th17/Treg balance

In IF analysis, IL-17A and FOXP3 were used as markers for Th17 and Treg, respectively. The results revealed that Th17 increased and Treg decreased in the mesenteric lymph nodes of the model group. BG treatment effectively alleviated the increase of Th17 cells and the decline of Treg, indicating that it might inhibit the conversion of Treg to Th17 (Fig. 3A, B). In the model group, the levels of IL-17, IFN- γ , ROR γ t, and T-bet elevated, and the levels of IL-10, IL-5, Foxp3, and GATA3 decreased ($P < 0.001$). BG treatment alleviated the fluctuations in the levels of these cytokines or transcription factors (Fig. 3C).

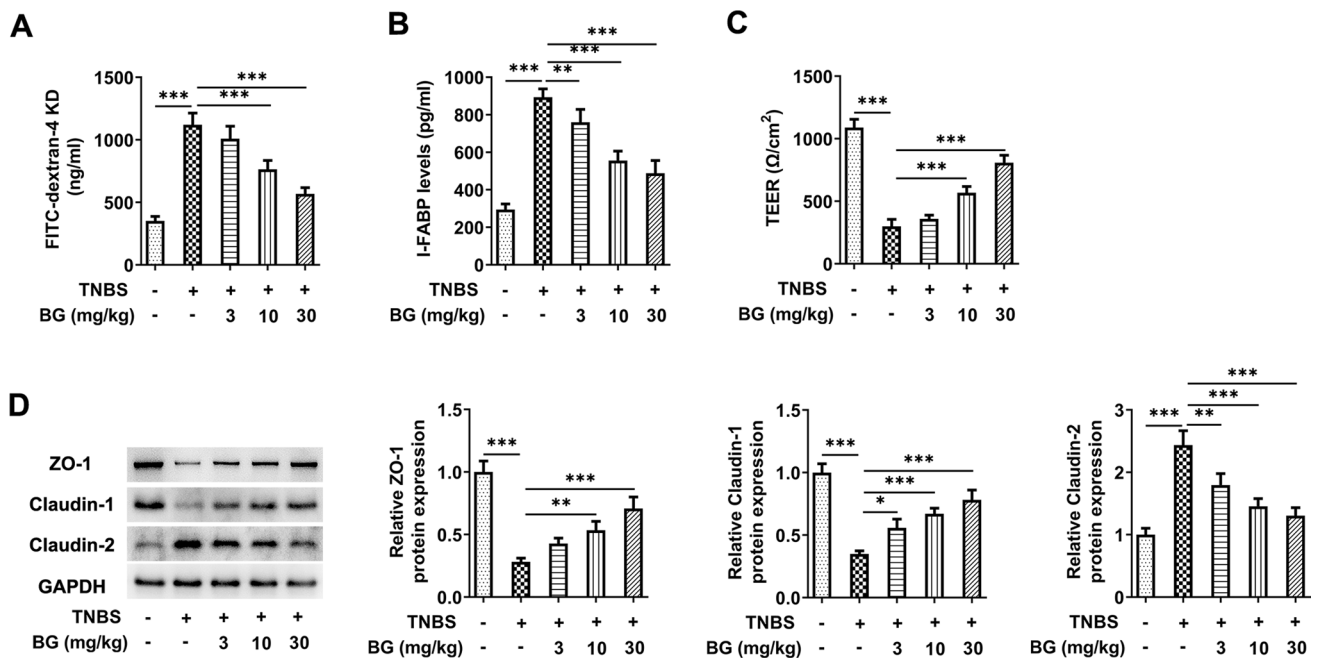


Fig. 2 BG reduces TNBS-induced intestinal barrier damage. **A** The level of FITC-dextran in the serum of mice. **B** The serum level of I-FABP. **C** TEER indicated the barrier. **D** The levels of tight junction proteins were measured using western blotting. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

BG regulates mitophagy and the PPARγ/ nuclear factor kappa B (NF-κB) signaling pathway

According to IF analysis, Mito-Tracker and LC3B levels were found to be decreased in the model group, while their levels increased upon BG treatment, suggesting that BG might contribute to autophagy (Fig. 4A). By detecting mitophagy-related proteins, BG was found to promote the expression of Beclin-1, PINK1, and mito-Parkin, increase the LC3II/LC3I ratio, and reduce p62 and cyto-Parkin (*P* < 0.001). This proved that mitophagy was enhanced in the BG-treated groups (Fig. 4B). In addition, TNBS reduced PPARγ levels in tissues, accompanied by enhanced NFκB p65 phosphorylation (*P* < 0.001), indicating that the NFκB inflammatory channel was activated. BG activated PPARγ and inhibited NF-κB activation in a concentration-dependent manner, reflecting its function in regulating this pathway (Fig. 4C).

Discussion

IBD constitutes a chronic inflammatory condition characterized by intermittent exacerbations, leading to complications and often necessitating repeated surgeries. It encompasses two major subtypes: CD and ulcerative colitis (UC) (Cheng et al. 2021). In CD, inflammation affects all layers of the intestine, whereas UC primarily involves the mucosal layer. TNBS is widely applied to establish CD models (Flacs

et al. 2020; Zuo et al. 2023). Its induction mechanism is that it enters the intestinal mucosa damaged by ethanol and combines with macromolecular substances to form whole antigens and induce immune responses. TNBS induces chronic transmural colitis with severe diarrhea, mimicking the histological features of human CD (Neurath et al. 1995). This study, based on a mouse model, demonstrated that BG significantly attenuated colon tissue damage, particularly notable in CD, in a concentration-dependent manner. Assessment using DAI scores revealed significant reductions in loose feces, occult blood, and weight loss among mice in the BG treatment group. Pathological analysis indicated decreased inflammatory infiltration, restoration of crypt structure towards normalcy, and reduced fibrosis. Given that anti-inflammatory therapies often prove ineffective in patients with fibrosis in IBD (Wang et al. 2022), the observed reduction in fibrosis suggests a potentially novel mechanism of action for BG. It is speculated that beyond inflammatory stimuli, BG may target independent cellular or molecular factors contributing to fibrosis. Furthermore, the impact of BG on the intestinal barrier integrity was assessed. I-FABP, a sensitive marker for early identification of intestinal ischemia and injury (Straarup et al. 2023), exhibited a significant decrease in serum levels following BG treatment. Additionally, increased levels of TEER and tight junction proteins support the notion of BG-mediated repair or protection of the intestinal barrier.

Th17 cells, a subset of CD4⁺ T cells, play a crucial role in IBD pathogenesis. It has been confirmed that cytokines

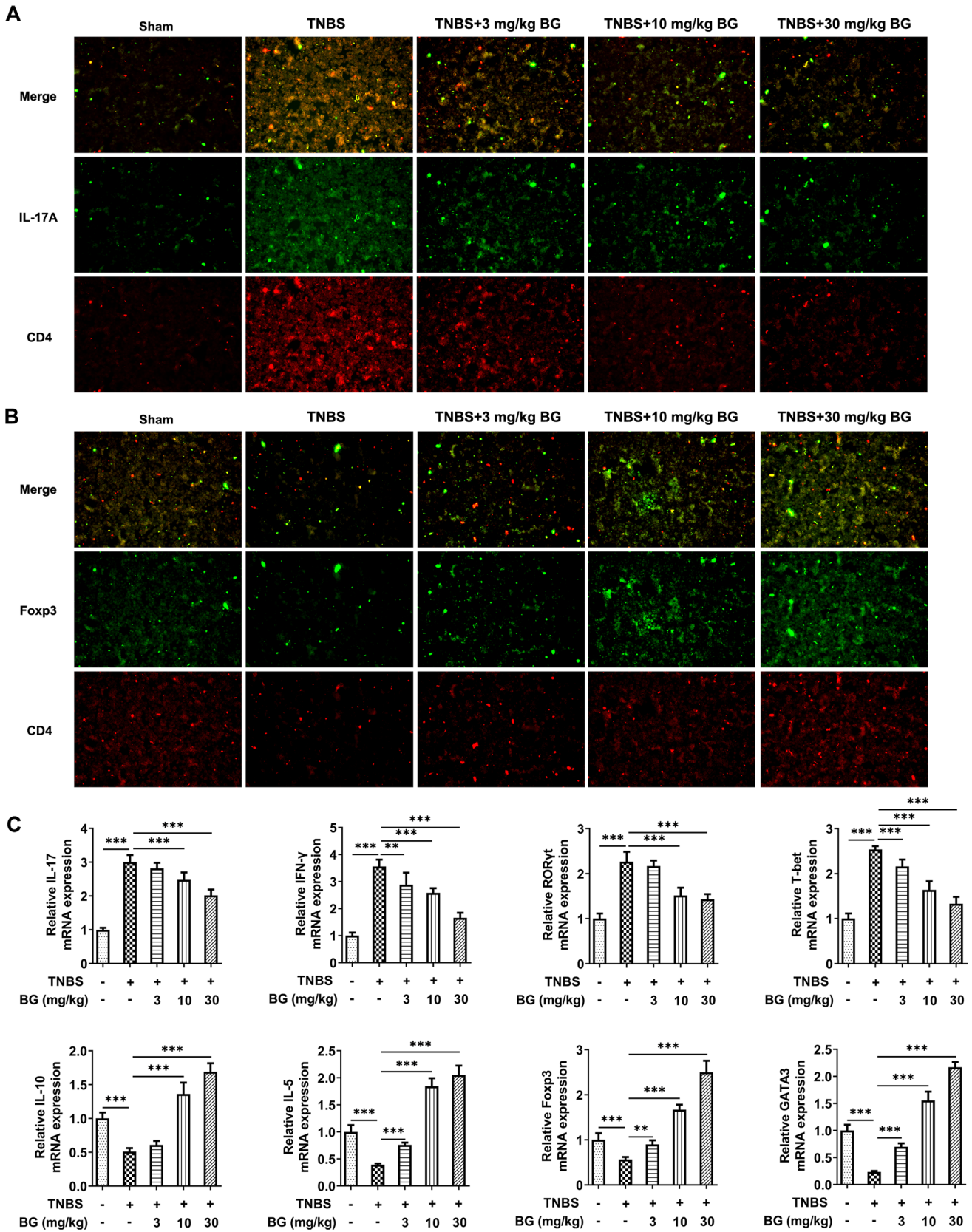


Fig. 3 BG maintains Th17/Treg balance. **A, B** IL-17A and FOXP3 were used as markers for Th17 and Treg in IF analysis. **C** RT-qPCR determined the levels of T cell differentiation markers. $**P < 0.01$, $***P < 0.001$

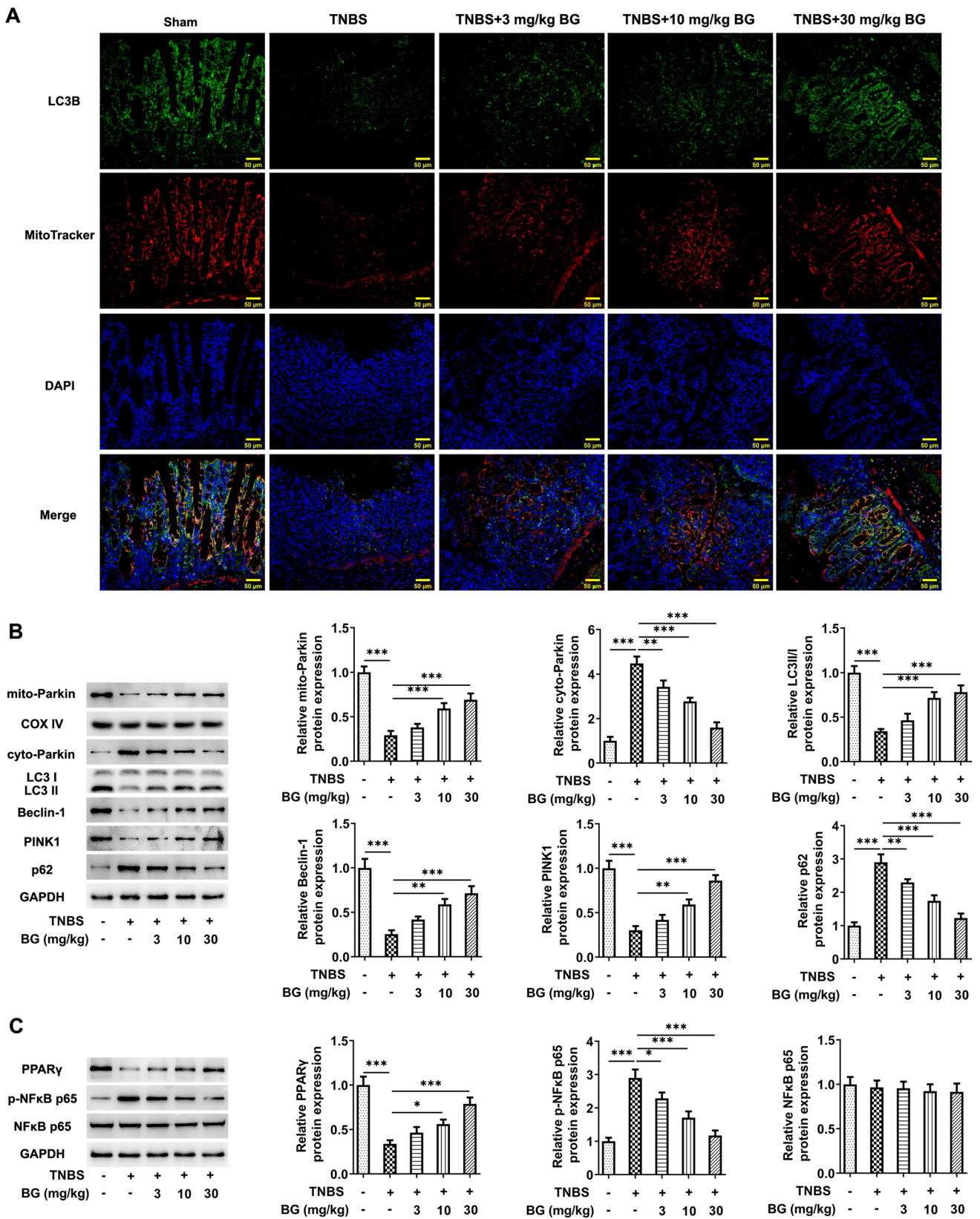


Fig. 4 BG regulates mitophagy and the PPAR γ /NF- κ B signaling pathway. **A** Mito-Tracker and LC3B levels were determined using IF to indicate autophagy. **B** Mitophagy-related proteins were determined

using western blotting. **C** PPAR γ level and NF κ B p65 phosphorylation were determined using western blotting. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

and transcription factors, such as IL-6 and ROR γ t, are required for the early differentiation of Th17 cells (Kumar et al. 2021). This study revealed a significant increase in Th17 cells alongside a decrease in Tregs in mesenteric lymph nodes of the model group. BG treatment effectively mitigated this imbalance by reducing the conversion of Tregs to Th17 cells. Elevated local and serum IL-17 levels in CD patients underscored the significance of IL-17⁺ T cells in disease severity (Pedersen et al. 2022). BG treatment increased anti-inflammatory factors such as IL-10, IL-5, Foxp3, and GATA3 in the colon, indicative of enhanced anti-inflammatory responses. Notably, GATA3 expression in Tregs residing in barrier sites, including the intestine (Choi et al. 2023), highlights its potential role in BG-mediated immunomodulation.

Given BG has previously been demonstrated to have a protective effect on mitochondrial function as aforementioned, markers of mitophagy were evaluated in colon tissue. BG was found to enhance mitophagy, suggesting a potential mechanism by which BG regulates immune responses. Increasing evidence suggests that the elimination of dysfunctional mitochondria is a powerful means used by autophagy to control the immune system, and that the mitophagy process may limit inflammatory cytokine secretion (Xu et al. 2020). Moreover, the regulation of BG on the PPAR γ /NF- κ B signaling pathway was elucidated. Activation of PPAR γ has been associated with mitigating experimental CD through regulation of mitochondrial function (Yao et al. 2017), while inhibition of NF- κ B has been linked to reduced inflammation (Huang et al. 2020). Activating PPAR γ has also been found to have positive effects in other disease models. For example, regulating PPAR γ -dependent mitophagy enhances brain glucose metabolism and improves cognitive impairment in APP/PS1 mice (Li et al. 2022), and inhibiting NF- κ B reduces brain ischemia–reperfusion injury in rats (Yao et al. 2022). Therefore, PPAR γ is considered a strong candidate target.

In summary, BG demonstrates promising effects in ameliorating intestinal barrier damage and Th17/Treg imbalance in a murine model of CD-like colitis, while also promoting intracellular mitophagy. The PPAR γ /NF- κ B signaling pathway may serve as a key mediator of BG's regulatory mechanisms. These findings provide a valuable experimental foundation for the potential therapeutic use of BG in CD treatment. Nevertheless, in addition to the colon, the ileum is also the primary segment affected by CD (Alrubia et al. 2022; Narula et al. 2022). Therefore, the impact of Bergapten on ileal lesions deserves future investigation, and the lack of research on other intestinal segments is a limitation of the present study.

Authors contributions All authors contributed to the study conception and design. B.Z., H.C., and G.L. contributed to material preparation, data collection, and analysis. L.X. contributed to the draft and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. This study was approved by the Ethics Review Committee of Zhangjiagang Hospital affiliated with Soochow University.

Competing interests The authors declare no competing interests.

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