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Kakkalide and irisolidone alleviate 2,4,6-trinitrobenzenesulfonic acid-induced colitis in mice by inhibiting lipopolysaccharide binding to toll-like receptor-4 and proteobacteria population

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ABSTRACT

The flower of *Pueraria lobata* (family Fabaceae) has been clinically used in traditional Chinese medicine to counteract symptoms associated with drinking alcohol and liver injury and to alleviate inflammatory diseases. Its major constituent kakkalide is metabolized to irisolidone by gut microbiota. This research study was undertaken to understand the anti-colitis mechanism of kakkalide and irisolidone in vitro and in vivo. Kakkalide and its metabolite irisolidone inhibited lipopolysaccharide (LPS)-stimulated NF-κB activation and TNF-α expression in macrophages. They also inhibited LPS-induced phosphorylation of IRAK1 and TAK1 and activation of NF-κB by inhibiting the binding of Alexa Fluor 488-conjugated LPS in vitro. Orally administered irisolidone or kakkalide alleviated colon shortening and myeloperoxidase activity in mice with 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis. Their treatments also protected epithelial cell disruption and infiltration of $CD11b^+/CD11c^+$ cells in the colon. Furthermore, they suppressed TNBS-induced expression of M1 macrophage markers TNF-α, CD80, CD86, and Arg2 expression while the expression of M2 macrophage markers Arg1, CD163, CD206, and IL-10 was induced. They also suppressed the fecal Proteobacteria population. Overall, the anti-colitic effects of irisolidone were superior to those of kakkalide. Kakkalide and its metabolite irisolidone inhibited inflammation in vitro and in vivo by inhibiting LPS binding to toll-like receptor 4 and gut proteobacteria population.

1. Introduction

Inflammation can be classified as either acute or chronic inflammation [1]. Acute inflammation is a normal and helpful response to injury and infection. Chronic inflammation, which persistently and excessively secretes inflammatory mediators in the immune cells such as tumor necrosis factor (TNF)-α, causes the severe damage to the body, resulting in the occurrence of diverse inflammatory diseases, such as inflammatory bowel disease (IBD) and rheumatoid arthritis [2,3]. IBD, the chronically relapsing disorder, occurs most frequently in the terminal ileum and colon where many intestinal microbes reside but does not progress significantly in germ-free animals [4,5]. The disruption of gut microbiota and excessive production of gut microbiota endotoxin(s) can cause IBD, which accelerates the excessive production of gut microbiota endotoxins [6,7]. These endotoxins such as lipopolysaccharide (LPS) induce the expression of proinflammatory cytokines such as TNF-α, interleukin (IL)-1β, and IL-6, via a toll-like receptor (TLR)4-linked nuclear transcription factor κB (NF-κB) pathway in immune cells, leading to the activation of inflammatory responses [7,8]. NF-κB is an essential transcription factor in the immune cells to stimulate immune responses but its excessive and persistent activation can cause inflammatory diseases [8,9]. Chronic exposure to excessive LPS activates TLR4-mediated NF-κB signaling pathway, leading to the expression of proinflammatory cytokines such as TNF-α [9,10]. Therefore, to regulate chronic inflammatory diseases, the studies on traditional Chinese medicines and their constituents regulating TNF-α expression or NF-κB activation has been consistently performed [10,11].

The flower of *Pueraria lobata* (family Fabaceae), which contains kakkalide as a main constituent [12,13], is frequently used in the traditional Chinese medicine to counteract symptoms associated with drinking alcohol and liver injury and to alleviate inflammatory diseases [12–14]. Oral administration of kakkalide, a hydrophilic glycoside, is

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metabolized to irisolidone through kakkalidone by gut microbiota [13,15]. Kakkalide and its metabolite irisolidone exhibit estrogenic, anti-oxidant, ant-inflammatory, anti-*Helicobacter pylori*, hepatoprotective, and neurodegenerative effects [16–21]. Irisolidone protects against ethanol-induced lethality and gastric injury by regulating CXCL-4 or IL-8 expression in immune cells [22]. Furthermore, irisolidone suppresses LPS-stimulated NF-κB activation in peritoneal macrophages and microglial cells as well as carrageenan-induced inflammation in the air-pouch of mice [18,21,22]. However, the anti-inflammatory mechanism of kakkalide and irisolidone remains elusive.

Therefore, in the present study, to understand the anti-inflammatory mechanism of irisolidone and its parental compound kakkalide, we examined their anti-inflammatory mechanism in LPS-stimulated macrophages and mice with 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis.

2. Materials and methods

2.1. Materials

LPS purified from *Escherichia coli* O111:B4, TNBS, and RPMI 1640 were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Antibodies for interleukin (IL)-1 receptor-associated kinase 1 (IRAK1), p-IRAK1, transforming growth factor β-activated kinase 1 (TAK1), p-TAK1, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα), p-IκBα, p65, p-p65, and β-actin were purchased from Cell Signaling Technology (Beverly, MA). Enzyme-linked immunosorbent assay (ELISA) kits for cytokines were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Radioimmunoprecipitation assay buffer (RIPA) lysis buffer, phosphatase inhibitor cocktail, and protease inhibitor cocktail were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Alexa 488-conjuaged LPS was purchased from Invitrogen (Waltham, MA, U.S.A.).

2.2. Isolation of kakkalide and irisolidone

Kakkalide and irisolidone were isolated from the flower of *Pueraria lobata* according to the previously reported methods [13,22] and their purities were analyzed by thin layer chromatography and high-performance liquid chromatography. The endotoxin content was assayed according to the method of Jang et al. [23].

Kakkalide (purity, > 93%) pale yellowish needles; mp 251–253 °C; FAB-MS: 609 $[M + H]^+$; < 0.01 ng of endotoxin/mg.

Irisolidone (purity, > 95%) pale yellowish amorphous powder; mp 189–190 °C; FAB-MS: 315 $[M + H]^+$; < 0.01 ng of endotoxin/mg.

2.3. Animals

Male C57BL/6 mice (19–21 g, 6 weeks old) were purchased from Orient Bio (Seongnam-shi, Korea). Mice (four mice per cage) were housed in a wire cage under controlled condition (12-h light and 12-h dark cycle; temperature, 20–22 °C; and humidity, 50 ± 10%) and fed with standard laboratory chow and water ad libitum. Mice were acclimated for > 7 days before the experiment. The experiments were approved by the Committee for the Care and Use of Laboratory Animals in the Kyung Hee University (KHUASP(SE)-17-113) and performed in accordance with the NIH and Kyung Hee University Guidelines for Laboratory Animals Care and Usage.

2.4. Isolation and culture of macrophages

Mice were intraperitoneally injected with 4% sodium thioglycolate solution (2 mL) and sacrificed 4 days after the injection [13,24]. Their peritoneal cavities were collected by flushing with RPMI 1640, centrifuged at 300 ×*g* for 10 min, and washed with RPMI1640 twice. Collected cells were suspended in RPMI 1640 containing 10% FBS and 1% antibiotic-antimycotic (RFA), seeded in 6-well plate, incubated at 37 °C for 24 h, and washed with RFA. Attached cells (2×10^6 cells/well) were used as macrophages.

To measure anti-inflammatory effect of irisolidone and kakkalide, macrophages were treated with LPS (100 ng/mL) in the absence or presence of irisolidone or kakkalide (2 or 10 μM) for 90 min (for NF-κB signaling molecules) or 20 h (for TNF-α).

2.5. Confocal microscopy

Inhibitory effects of irisolidone and kakkalide on the binding of LPS to TLR4 of macrophages were assayed according to the method of Kang et al. [24]. Briefly, peritoneal macrophages (1×10^5 cell) were attached in small glass, treated with Alexa Fluor 488-conjugated LPS in the presence or absence irisolidone or kakkalide (10 μM) for 1 h, dyed with propidium iodide solution, and measured by a confocal microscope (Nanoscope Systems, Daejeon, Korea).

2.6. Preparation of TNBS-induced colitis

TNBS-induced colitis were prepared according to the method of Jang et al. [25]. Mice were randomly separated into seven groups: normal control group, which was treated with saline instead of TNBS and test agents, and six groups with TNBS-induced colitis, which were induced by the intrarectal injection of 2.5% (w/v) TNBS solution (100 μL, dissolved in 50% ethanol) into the colon of mice anesthetized with ether. Each group consisted of six mice. TNBS-induced colitic mice were orally gavaged with test agents (vehicle [1% tween 20 solution], 20 mg/kg and 50 mg/kg of kakkalide, 10 mg/kg and 20 mg/kg of irisolidone and 50 mg/kg of sulfasalazine) daily for 3 days 24 h after the TNBS injection. Mice were sacrificed by CO_2 asphyxiation, 18 h after the final administration of test agents. Colons were removed, opened longitudinally, gently washed with ice-cold saline, and stored at −80 °C until used in the experiment. The external severity of colitis was macroscopically scored (0 to 5: 0, no ulcer and no inflammation; 1, no ulceration and local hyperemia; 2, ulceration with hyperemia; 3, ulceration and inflammation at one site only; 4, two or more sites of ulceration and inflammation; 5, ulceration extending > 2 cm).

Immunostaining analysis of colon slices was performed according to the method of Jang et al. [25]. Macrophages and dendritic cells were visualized by staining with anti-CD11b and anti-CD11c antibodies, and measured by a confocal microscope (LSM510, Carl Zeiss, Oberkochen, Germany).

2.7. Assay of myeloperoxidase activity

Colon tissues were homogenized with cold RIPA lysis buffer and centrifuged at 10,000 ×*g* for 10 min [24]. The supernatant was used as a crude enzyme solution. An aliquot of the supernatant was added in the reaction mixture containing 0.03% hydrogen peroxide and 1.6 mM tetramethylbenzidine and measured the absorbance at 650 nm time over 5 min. Activity was defined as the quantity degrading 1 μmol/mL of peroxide.

2.8. ELISA and Immunoblotting

For ELISA, colons were homogenized in cold RIPA lysis buffer containing 1% phosphatase inhibitor cocktail and 1% protease inhibitor cocktail and centrifuged at 15,000 ×*g* and 4 °C for 10 min [22]. The cytokines were measured using ELISA kit (Ebioscience, Atlanta, GA, USA).

For the immunoblot analysis, the supernatant were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membrane [25]. The membrane was treated with blocking buffer, probed with antibodies, and washed with phosphate buffered saline with 0.1% Tween 20, treated with

horseradish peroxidase-conjugated secondary antibodies, and then visualized with an enhanced chemiluminescence detection kit.

2.9. Immunofluorescence assay

The sections were washed with PBS, blocked with normal serum, incubated with CD11b (1:200, Abcam) and CD11c (1:200, Abcam) antibodies overnight, and treated with the secondary antibodies for 2 h [24]. Secondary antibodies conjugated with Alexa Fluor 488 (1:1000, Invitrogen) or Alexa Fluor 594 (1:500, Abcam) were then treated to visualize. Nuclei were stained with 4′,6-diamidino-2-phenylindole, dilactate (DAPI, Sigma). Immunostained samples were scanned with a confocal laser microscope.

2.10. Quantitative real time - polymerase chain reaction (qPCR)

Real time PCRs for TNF-α, IL-10, arginase-1 (Arg1), Arg2, CD80, CD86, CD163, CD206, and glyceraldehyde 3-phophate dehydrogenase (GAPDH) were performed on the Rotor-Gene Q® using DNA polymerase and SYBR Green I (a reaction volume, 20 μL), as previously reported [26]. Gene expression levels were calculated relative to β-actin, using Microsoft Excel. Primers are shown in Table 1.

2.11. Statistical analysis

Data were expressed as the mean ± standard deviation, using one-way ANOVA followed by the student-Newman-Keuls test for multiple comparisons ($P < 0.05$).

3. Results

3.1. Kakkalide and irisolidone inhibited the NF-κB activation and TNF-α expression in LPS-stimulated macrophages

Irisolidone, a metabolite of kakkalide, inhibits NF-κB activation in LPS-stimulated macrophages [18]. Therefore, to confirm its anti-inflammatory effect, we examined the effects of irisolidone and kakkalide on the NF-κB activation and TNF-α expression in LPS-activated macrophages. They significantly inhibited LPS-induced NF-κB activation and TNF-α expression, while NF-κB activation and TNF-α expression were not affected by irisolidone or kakkalide treatment (Fig. 1A–D and Supplement Fig. S1). Irisolidone inhibited them more potently than kakkalide. However, they did not affect NF-κB activation and TNF-α expression in macrophages.

3.2. Irisolidone and kakkalide inhibited the binding of LPS to TLR4 on macrophages

In order to confirm the effects of kakkalide and irisolidone on the NF-κB activation in LPS-stimulated macrophages, we examined the translocation of p65 into the nucleus, assessed by a confocal microscope (Fig. 2A). Stimulation with LPS significantly increased p65 translocation into the nucleus. However, treatment with irisolidone or kakkalide (10 μM) significantly inhibited the translocation of NF-κB: Irisolidone inhibited p65 translocation more potently than kakkalide. Irisolidone and kakkalide showed no cytotoxic effects against macrophages under the tested condition (data not shown).

Thereafter, we examined the effects of irisolidone and kakkalide on the expression of NF-κB signaling molecules in LPS-stimulated macrophages (Fig. 2B and Supplement Fig. S2). They inhibited LPS-induced IRAK1, TAK1, IκBα, and p65 (NF-κB) phosphorylation. Overall, irisolidone inhibited their phosphorylation more potently than kakkalide. However, they did not affect TLR4 expression.

Next, we examined whether kakkalide and irisolidone could regulate the binding of Alexa Fluor 488-conjugated LPS to TLR4 on macrophages, assessed by a flow cytometer (Fig. 3A). Alexa Fluor 488-conjugated LPS treatment significantly increased the population of shifted macrophages. However, treatment with irisolidone or kakkalide significantly reduced the population of macrophages shifted by treatment with Alexa Fluor 488-conjugated LPS. To confirm whether kakkalide and irisolidone regulated the binding of LPS on the TLR4 of macrophages, we examined whether they could inhibit the binding of Alexa Fluor 488-conjugated LPS on macrophages, assessed by a confocal microscope. Treatment with kakkalide or irisolidone inhibited the LPS binding on the macrophages.

3.3. Irisolidone and kakkalide alleviated TNBS-induced colitis in mice

To understand whether irisolidone and kakkalide could alleviate inflammation in vivo, we examined the effects of orally administered irisolidone and kakkalide on TNBS-induced colitis in mice. TNBS treatment severely caused colitis: it thickened, shortened, and inflamed colons (Fig. 4A–D). Oral administration of irisolidone or kakkalide alleviated TNBS-induced bodyweight loss, macroscopic score, and colon shortening. They also significantly suppressed TNBS-induced myeloperoxidase activity in the colon: of them, irisolidone at a dose of 20 mg/kg inhibited it the most potently. Their treatments also protected epithelial cell disruption and infiltration of $CD11b^+/CD11c^+$ cells in the colon (Fig. 4E). The anti-colitic effect of irisolidone at a dose of 20 mg/kg was comparable with that of sulfasalazine at a dose of 50 mg/kg.

Next, we investigated the effects of irisolidone and kakkalide on the expression of TLR-4 linked NF-κB signal molecules in the colon of TNBS-treated mice (Fig. 5A). TNBS treatment significantly activated TLR4-linked NF-κB signaling pathway in the colon. However, oral administration of irisolidone and kakkalide significantly inhibited the IRAK1, TAK1, IκBα, and p65 (NF-κB) phosphorylation and TLR4 expression in the colon. Furthermore, their treatments suppressed TNBS-induced TNF-α expression and increased TNBS-suppressed IL-10 expression, assessed by ELISA (Fig. 5B). We also examined the effects of irisolidone and kakkalide on the expression of macrophage polarization markers in mice with TNBS-induced colitis (Fig. 5C). Treatment with TNBS induced the expression of M1 macrophage polarization markers

Table 1
Primers for qPCR.

	Sequence	
	Forward	Reverse
TNF-α	5′-CTGTAGCCCACGTCGTAG-3′	5′-TTGAGATCCATCCCGTTG-3′
IL-10	5′-GCTCTTACTGACTGGCATGAG-3′	5′-GCGAGCTCTAGGAGCATGTG-3′
Arginase 1	5′-CCAGAAGAATGGAAGAGTCAGTGT-3′	5′-GCAGATATGCAGGGAGTCACC-3′
Arginase 2	5′-ATATGGTCCAGCTGCCATTCGAGA-3′	5′-TAACCACTTCAGCCAGTTCCTGGT-3′
CD80	5′-GGCAAGGCAGCAATACCTTA-3′	5′-CTCTTTGTGCTGCTGATTCG-3′
CD86	5′-TCTCCACGGAAACAGCATCT-3′	5′-CTTACGGAAGCACCCATGAT-3′
CD163	5′-TCCACACGTCCAGAACAGTC-3′	5′-CCTTGGAAACAGAGACAGGC-3′
CD206	5′-TTGGACGGATAGATGGAGGG-3′	5′-CCAGGCAGTTGAGGAGGTTC-3′
β-Actin	5′-TGTCCACCTTCCAGCAGATGT-3′	5′-AGCTCAGTAACAGTCCGCCTAGA-3′

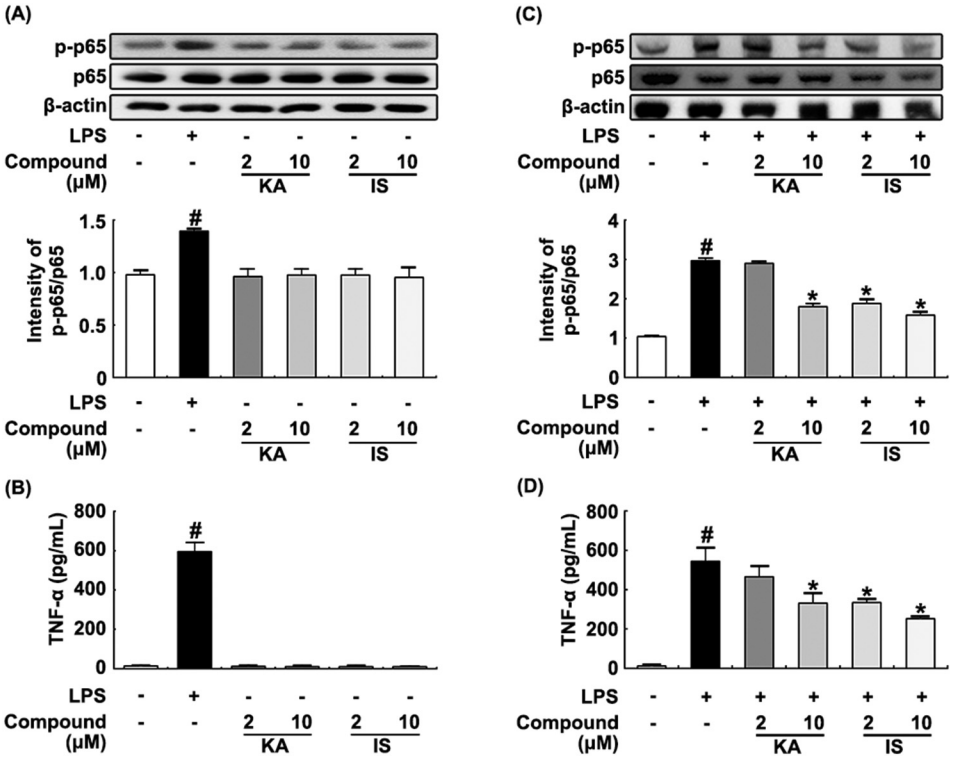

Fig. 1. Effect of kakkalide and irisolidone on the expression of inflammatory markers and the activation of NF-κB activation in LPS-stimulated macrophages. (A) Effect on the activation of NF-κB. (B) Effect on TNF-α expression. Peritoneal macrophages (2×10^5 cells) were incubated with LPS in the absence or presence of kakkalide (KA) or irisolidone (IS) (2 and 10 μM) for 90 min (for NF-κB) or 20 h (for TNF-α). All data are shown as the mean ± SD (n = 4). $^{\#}p < 0.05$ vs. group treated without LPS and test agents. $^{*}p < 0.05$ vs. group treated with LPS alone.

such as TNF-α, CD80, CD86, and Arg2 compared to normal control mice while the expression of M2 macrophage polarization markers such as Arg1, CD163, CD206, and IL-10 was suppressed. Treatment with irisolidone or kakkalide inhibited TNF-α, CD80, CD86, and Arg2 expression and induced Arg1, CD163, CD206, and IL-10 expression. The inhibitory effect of irisolidone (20 mg/kg) against TNBS-induced M1 macrophage polarization was more potent than that of kakkalide (50 mg/kg). Its potency was comparable to that of sulfasalazine (50 mg/kg). Next, we examined the effects of kakkalide and irisolidine on the gut microbiota of mice treated with TNBS (Fig. 5D). TNBS treatment significantly increased the γ-Proteobacteria population at the phylum level while decreasing the Bacteroidetes, Firmicutes, and Actinobacteria populations. Treatment with kakkalide or irisolidone significantly restored TNBS-disrupted γ-Proteobacteria, Actinobacteria, and Bacteroidetes populations.

4. Discussion

Gut bacterial LPS stimulates TLR4-mediated transcription factor NF-κB signaling pathway in gastrointestinal inflammation [8]. TLR4 recognizes pathogen-associated molecular patterns such as LPS and activates TAK1 via the activation of interleukin-1 receptor-associated

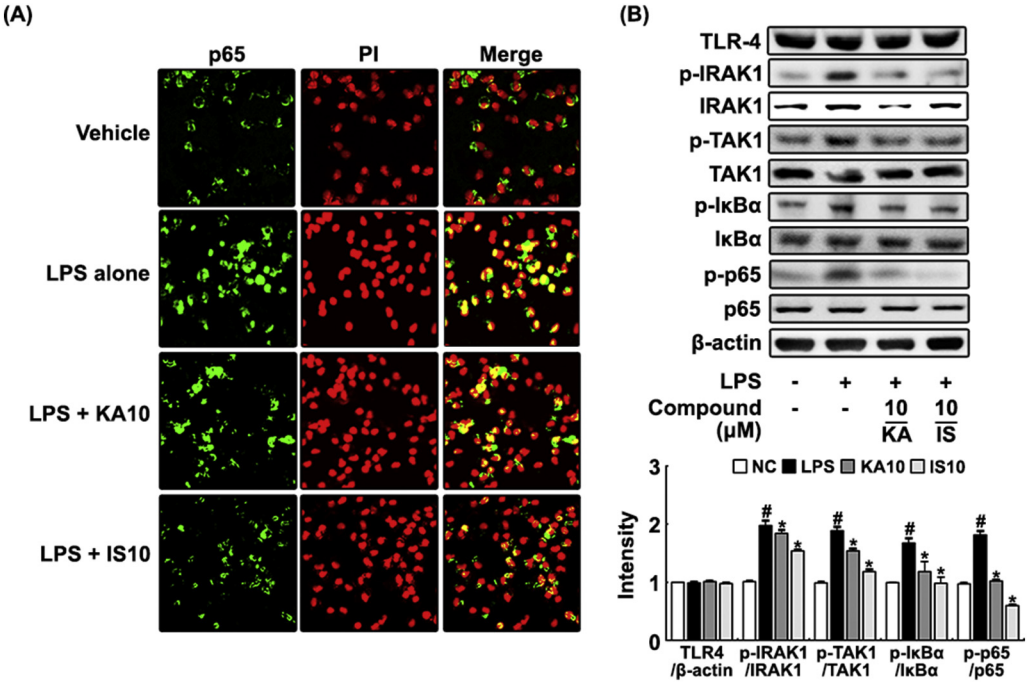

Fig. 2. Anti-inflammatory effect of kakkalide and irisolidone in LPS-stimulated peritoneal macrophages. (A) Effect on the translocation of NF-κB in the nuclei using a confocal microscope. (B) Effect on TLR4-linked NF-κB signaling pathway. The peritoneal macrophages were isolated from mice and incubated with or without LPS (100 ng/mL) in the absence or presence of kakkalide (KA) or irisolidone (IS) (10 μM) for either 90 min.

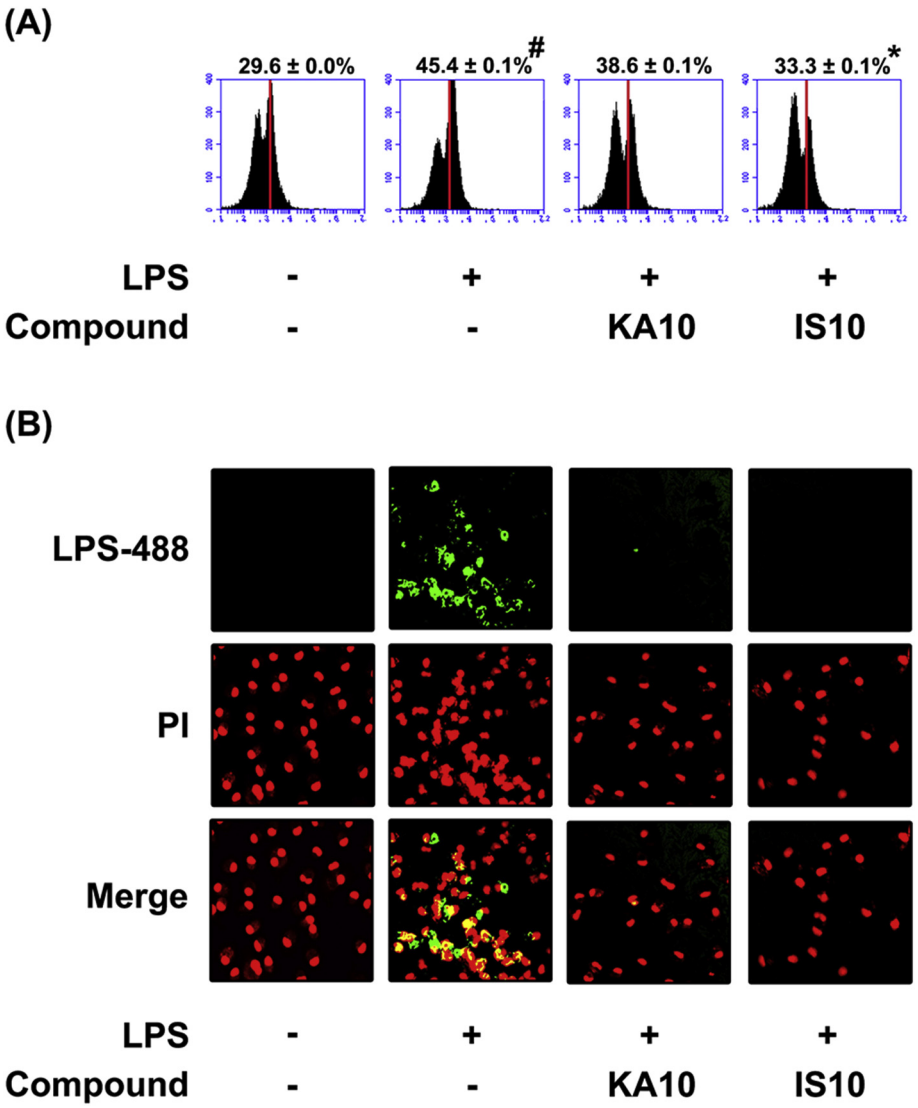

Fig. 3. Effect of kakkalide and irisolidone on the binding of LPS on the TLR4 of macrophages. Peritoneal macrophages were incubated with Alexa 488 conjugated LPS binding in the absence or presence of kakkalide (KA) or irisolidone (IS) (10 μM) for 30 min and measured by a flow cytometer (A) and a confocal microscope (B). All values are mean ± SD (n = 6). $^{\#}p < 0.05$ vs. group treated without LPS and test agents. $^{*}p < 0.05$ vs. group treated with LPS alone.

kinases (IRAKs). The activated TAK1 phosphorylates IKKs, which phosphorylate IκBα, leading to its ubiquitination and subsequent degradation by the proteasome [7,8]. The IκBα degradation accelerates NF-κB to translocate to the nucleus and bind to specific promoter sequences. The NF-κB activation in mucosal macrophages induces the biosynthesis and secretion of proinflammatory cytokines TNF-α and IL-1β and suppresses IL-10 expression [27,28]. The inhibition of TNF-α expression through the regulation of NF-κB activation by traditional Chinese medicines and phytochemical such as Ginseng radix, ginsenoside Rb1, Compound K and resveratrol alleviate IBD [29–31]. Ginseng radix and ginsenoside compound K inhibited NF-κB activation and TNF-α expression in murine macrophages by inhibiting IRAK1 activation, resulting in the alleviation of TNBS-induced colitis [30]. Resveratrol inhibits dextran sulfate sodium-induced colitis in mice by inhibiting the activation of NF-κB, signal transducer and activator of transcription-3, and extracellular signal-regulated kinase [31].

In the present study, we found that kakkalide and irisolidone, which is a metabolite of kakkalide by gut microbiota [13,15], inhibited TNBS-induced colitis: they suppressed myeloperoxidase activity, colon shortening, and TLR4 and TNF-α expression as well as polarization of M1 into M2 macrophages. They increased TNBS-suppressed expression of IL-10 and M2 macrophage markers, which stimulates the differentiation of T cells to regulatory helper T cells [32,33]. These results suggest that oral administration of irisolidone and kakkalide can restore the TNBS-induced disturbance of Th1, Th17, and Treg cell balance: they can inhibit the differentiation into Th1 and Th17 by inducing the differentiation into Treg. Thus, they can inhibit the activated innate immune cells such as macrophages and restore the disturbed adaptive immune cells such as Th1 and Treg cells, leading to the alleviation of colitis. Furthermore, kakkalide and irisolidone inhibited TNF-α expression and NF-κB activation in LPS-stimulated macrophages but did not inhibit TLR4 expression. Oral administration of kakkalide and irisolidone also inhibited TNBS-induced colitis in mice. They inhibited the phosphorylation of IRAK1, TAK1, IκBα and activation of NF-κB in LPS-stimulated macrophages while the TLR4 expression was not influenced. However, they suppressed TNBS-induced TLR4 expression in mice. Furthermore, they inhibited the binding of LPS to TLR4 on macrophages. These results suggest that irisolidone and kakkalide may inhibit the NF-κB activation by inhibiting the binding of LPS to TLR4 on immune. Furthermore, the successive inhibition of the NF-κB signaling pathway by kakkalide and irisolidone can inhibit TLR4 expression in vivo, resulting in the attenuation of colitis. Phytochemicals traditional

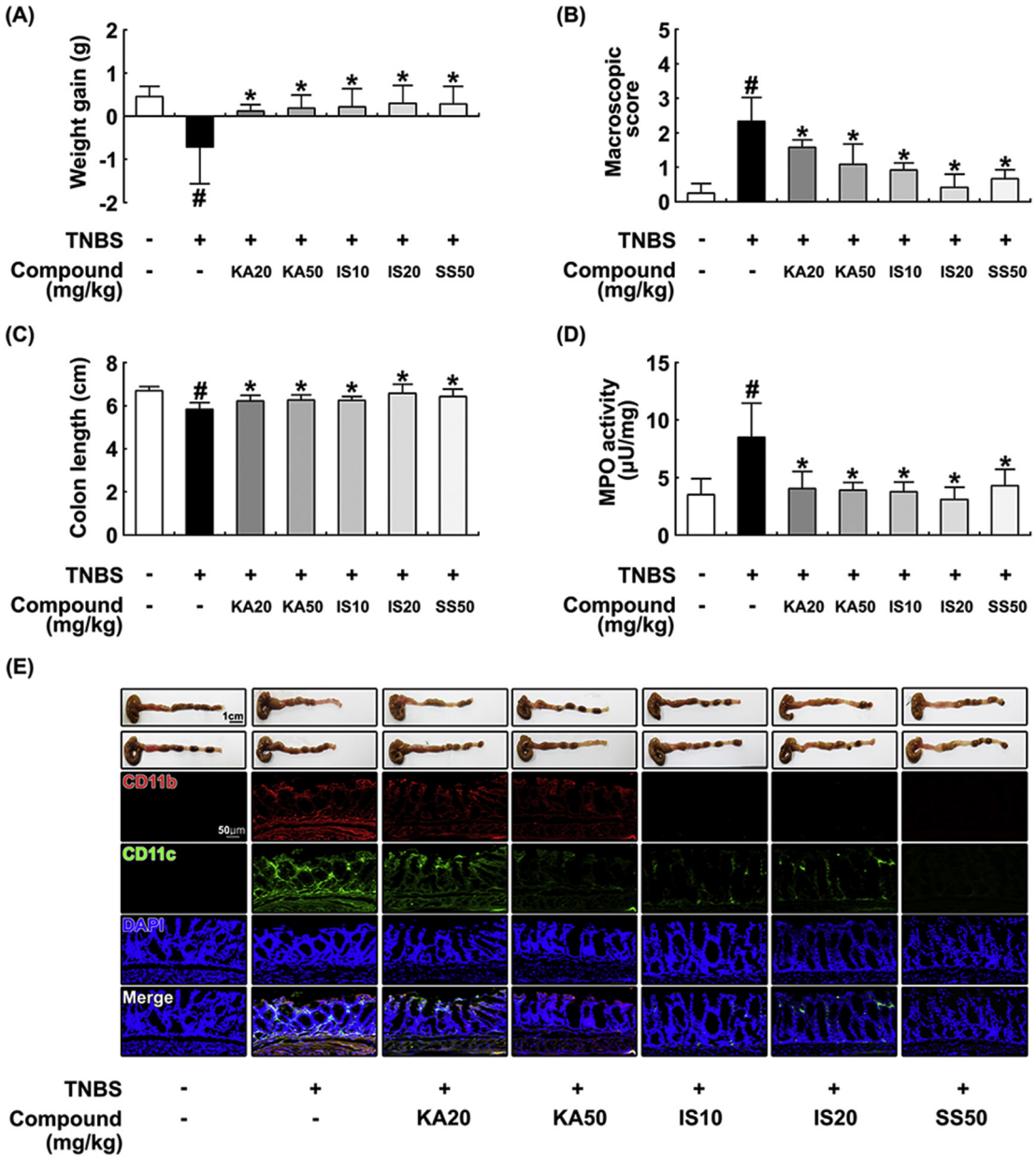

Fig. 4. Effects of kakkalide and irisolidone on body weight (A), macroscopic disease (B), colon length (C), myeloperoxidase (MPO) activity (D), and immunohistological examination (E) in mice with TNBS-induced colitis. TNBS, except in the normal control group, was administered intrarectally to mice, which were then orally administered test agents (KA20, 20 mg/kg of kakkalide; KA50, 50 mg/kg of kakkalide; IS10, 10 mg/kg of irisolidone; IS20, 20 mg/kg of irisolidone; SS50, 50 mg/kg of sulfasalazine for 3 days). The colon tissues were stained with fluorescent anti-CD11b and CD11c antibodies. All data are shown as the mean ± SD (n = 6). $^{\#}p < 0.05$ vs. normal control group treated with vehicle instead of test agents and TNBS. $^{*}p < 0.05$ vs. TNBS group treated with vehicle instead of test agents.

Chinese medicines are generally orally administered in humans and animals [34,35]. Orally administered hydrophilic phytochemicals contact with gut microbiota due to the difficult absorption and are transformed into hydrophobic compounds, which are easily absorbed from the intestine to the blood compared to parental hydrophilic components [34,35]. Orally administered kakkalide is metabolized to irisolidone via kakkalidone by gut microbiota [13]. Of these, a main metabolite is irisolidone, which is absorbed into the blood [13]. Therefore, if kakkalide or PF is orally administered, it is metabolized to irisolidone, which should express its pharmacological activities. Therefore, the anticolitic effects of kakkalide in vivo may be increased dependently on the metabolism into irisolidone. Interestingly, treatment with kakkalide or irisolidone significantly suppressed the fecal Proteobacteria population, which significantly produces gut inflammation-inducing endotoxins, and induced the Actinobacteria and Bacteroidetes populations. These results suggest that the anti-colitic effects of kakkalide and irisolidone may be elevated by modifying gut microbiota composition.

Based on these findings, irisolidone may alleviate gut inflammatory diseases including IBD by inhibiting the macrophage activation through the inhibition of LPS binding to TLR4 and the modification of gut microbiota and the anti-inflammatory effect of kakkalide may be elevated dependently on its transformation to irisolidone.

Declaration of Competing Interest

The authors declare no conflict of interest in this paper.

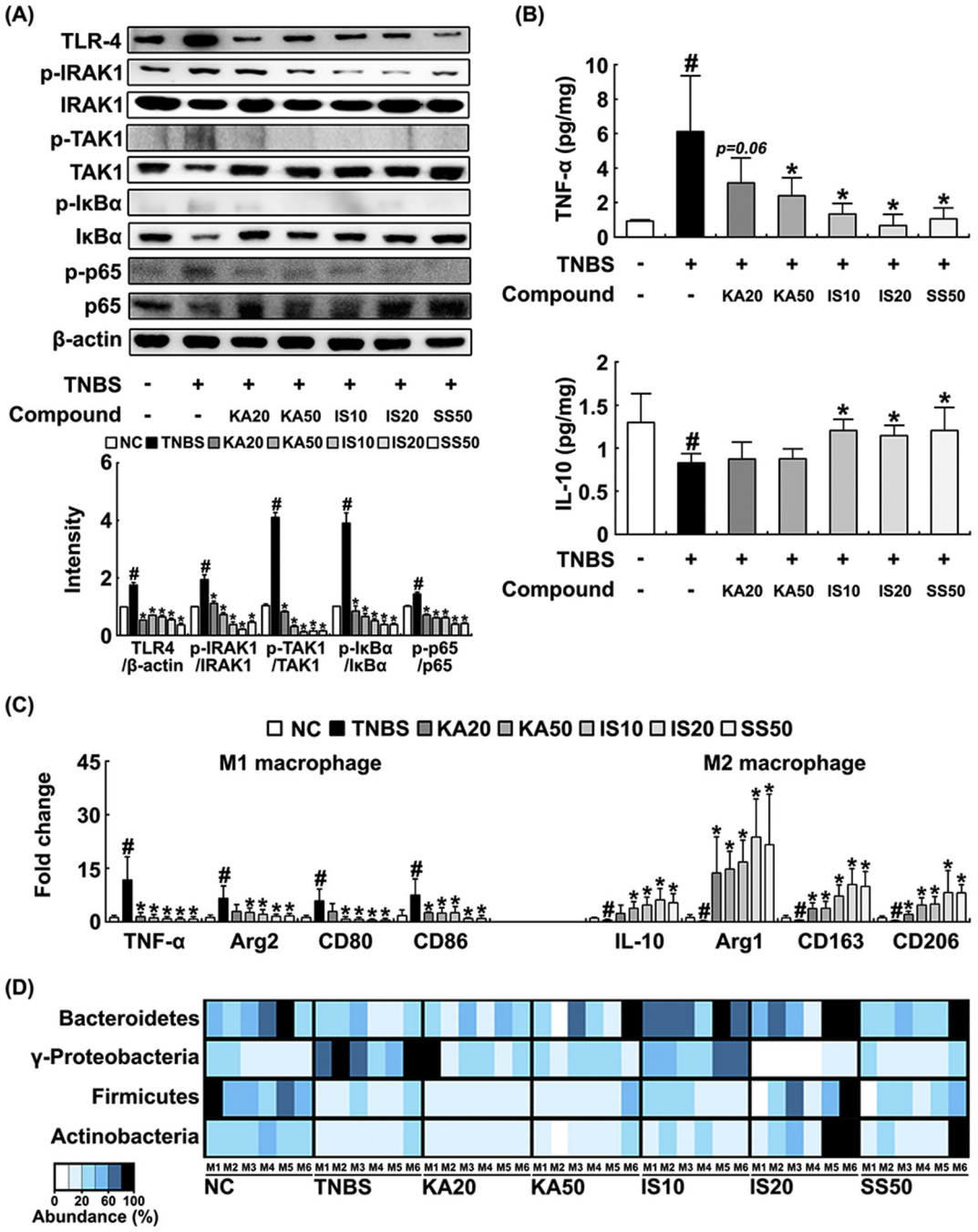

Fig. 5. Effects of kakkalide and irisolidone on inflammatory marker expression and gut bacterial populations in mice with TNBS-induced colitis. NF-κB signaling molecule expression (A) was measured by immunoblotting. TNF-α and IL-10 expression (B) was measured by ELISA. M1/M2 macrophage marker expression (C) and gut bacterial populations (D) were measured by qPCR. TNBS, except in the normal control (NC) group, was administered intrarectally to mice, which were then orally administered saline, test agents (KA20, 20 mg/kg of kakkalide; KA50, 50 mg/kg of kakkalide; IS10, 10 mg/kg of irisolidone; IS20, 20 mg/kg of irisolidone; SS50, 50 mg/kg of sulfasalazine for 3 days). $^{\#}p < 0.05$ vs. normal control group treated with vehicle instead of test agents and TNBS. $^{*}p < 0.05$ vs. TNBS group treated with vehicle instead of test agents.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.05.008.

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