

Ginsenoside Rg1 Ameliorated Colitis by Regulating the Homeostasis of M1/M2 Macrophage Polarization and Intestinal Flora

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Research Article

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Abstract

Background: Aberrant M1/M2 macrophage polarization and intestinal flora disruption are involved in the pathological processes associated with ulcerative colitis (UC). Ginsenoside Rg1 has good immunomodulatory and anti-inflammatory effects and is effective in treating UC of humans and animals. However, it is unclear how ginsenoside Rg1 regulate the homeostasis of M1/M2 macrophage polarization and intestinal flora.

Methods: BALB/c mice were randomly divided into 4 groups: Control, DSS, DSS+Rg1, DSS+Y27632 groups. In this study, experiment colitis was induced in BALB/c mice using sodium dextran sulfate (DSS). Mice of DSS+Rg1, DSS+Y27632 groups were treated respectively with ginsenoside Rg1 and Rock inhibitor Y27632 for 14 consecutive days. On day 21, all mice were sacrificed. Histopathological analysis of the colon tissues was performed by Hematoxylin Eosin staining. Cytokines (IL-6, IL-33, CCL-2, TNF- α , IL-4 and IL-10) were detected by Elisa. Flow cytometry was used to analyse macrophage activation and M1/M2 macrophage polarisation. Western blotting were applied to detect the levels of Macrophage polarization-associated protein (Arg-1, MIF-1, PIM-1, TLR2) and Nogo-B/RhoA signaling molecules (Rock1, RhoA and Nogo-B). The fecal microbial populations were analyzed using 16S gene sequencing.

Results: After ginsenoside Rg1 and Y27632 treatment, the changes of body weight, colon length, colonic weight index and colonic mucosal injury of colitis mice were effectively improved, accompanied by less ulcer formation and inflammatory cell infiltration, lower levels of pro-inflammatory cytokines (IL-6, IL-33, CCL-2, TNF- α) and higher anti-inflammatory cytokines (IL-4 and IL-10). Importantly, the percentage of CD11b⁺F4/80⁺, CD11b⁺F4/80⁺Tim-1⁺, CD11b⁺F4/80⁺TLR4⁺, and CD11b⁺F4/80⁺iNOS⁺ cells and the expression levels of MIF-1 and PIM-1 proteins were down-regulated significantly after ginsenoside Rg1 and Y27632 treatment, and CD11b⁺F4/80⁺CD206⁺ and CD11b⁺F4/80⁺CD163⁺ cells and Arg-1 up-regulated significantly. Intestinal flora composition were effectively improved after administration of ginsenoside Rg1. The Nogo-B/RchoA signaling pathway were obviously inhibited after ginsenoside Rg1 and Y27632 treatment, and the levels of Rock1, RhoA and Nogo-B proteins were significantly reduced.

Conclusions: Ginsenoside Rg1 has the protective effect on UC by inhibiting macrophage activation, restoring the balance of M1/M2 macrophage polarization, and improving intestinal flora composition, associated with inhibition of the Nogo-B/RhoA signaling pathway.

Background

Inflammatory bowel disease (IBD) is a chronic, non-specific inflammatory disease affecting the gastrointestinal tract and can be classified as Crohn's disease (CD) and ulcerative colitis (UC). The incidence of ulcerative colitis has been increasing globally over the last half century [1]. The clinical manifestations of UC are mostly diarrhoea, mucus, pus and blood stools, and abdominal pain. UC has become one of the top 10 intractable diseases in the world due to its tendency to recur and difficulty in complete cure [2]. It is now widely accepted that the development of UC is closely related to genetic

susceptibility, environmental factors, immune abnormality and intestinal flora disturbances [3]. In recent years, abnormal M1/M2 phenotype switching [4] and imbalanced intestinal flora [5] have been implicated in the onset and development of IBD. Currently, 5-aminosalicylates, steroids and immunosuppressive drugs are widely used for the treatment and maintenance of UC [6]. However, there are many resistance problems with these drugs, including major adverse effects and poor therapeutic response. Therefore, there is an urgent need to develop new safe and reliable drugs for UC treatment.

Ginseng has been used in Chinese medicine for over 2000 years as a tonic. Ginsenoside Rg1, the major active ingredient extract from the herb *Panax ginseng*, has a wide range of pharmacological activities, including anti-inflammation [7], anti-apoptosis, anti-oxidant [8], anti-tumour and neuroprotection [9]. Recently, the anti-inflammatory and immunomodulatory effects of ginsenoside Rg1 have attracted extensive attention. Ginsenoside Rg1 and its metabolites Rh1 or 20(s)-protopanaxatriol inhibited 2,4,6-Trinitrobenzenesulfonic acid (TNBS)-induced colitis by suppressing NF- κ B activation and MPO, IL-1 β , IL-17 and TNF- α expression, and regulating Th17/Treg balance [7]. In DSS-induced acute colitis model, ginsenoside Rg1 alleviated colonic inflammation by inducing NLRP12 expression and inhibiting IL-1 β and TNF- α [10]. However, ulcerative colitis is dominated by chronic spontaneous intestinal inflammation. Therefore, it is more reasonable to construct a model of chronic colitis is to go into the anti-inflammatory mechanisms of ginsenoside Rg1. In addition, the immunomodulatory effect of ginsenoside Rg1 is closely related to M1/M2 macrophage polarization. Ginsenoside Rg1 has been shown to treat a variety of diseases by regulating M1/M2 macrophage polarization, including acute lung injury [11], cardiovascular and cerebral-vascular diseases [12], autoimmune encephalomyelitis [13] etc. In vitro, ginsenoside Rg1 increased autophagic activity and anti-apoptosis in Raw264.7 macrophages [12]. However, whether ginsenoside Rg1 can act on macrophages in colitis has not been reported.

Accumulating evidence indicates that gut microbiota can promote or inhibit colonic inflammation [14]. Recently, research hotspots have shown that improving the composition of the intestinal flora is an emerging strategy to alleviate intestinal inflammation [15]. The addition of Arg to the feed prevents the development of colitis, accompanied by an improvement of the intestinal flora composition, with higher abundance of *Bacteroidetes* and lower *Verrucomicrobia* [16]. Hyaluronic acid-bilirubin nanomedicine (HABN) modulates the intestinal microbiota, increases overall diversity and the abundance of *Akkermansia muciniphila* and *Clostridium XIVa* to effectively restore intestinal injury [17]. However, the action mechanism of ginsenoside Rg1 in regulating intestinal flora composition and gut health is currently poorly understood. Here, the effects of ginsenoside Rg1 on mice with DSS-induced colitis and its action mechanism on macrophage activation, M1/M2 macrophage polarization and intestinal flora were investigated.

Materials And Methods

Drugs

Ginsenoside Rg1 (batch number: 16080053, purity: 99.91%) was purchased from Chengdu Purifa Technology Development Co., Ltd. (Chengdu, China). Dextran sodium sulfate (DSS; molecular weight: 36-50 kDa; batch number: 160110) was purchased from MP Biomedicals (California, USA). Y27632 (batch number: 129830-38-2) was purchased from APE&BIO (Texas, USA).

Mice

Male specific pathogen-free (SPF) BALB/c mice (8 - 9 weeks, 22 ± 2 g) were purchased from Hunan Slack Jingda Experimental Animal Co., Ltd. (Changsha, China) (Animal Certificate Number: SCXK (Xiang) 2019-0004). Reproduce freely with standard diet and tap water according to the Institutional Animal Care and Use Committee at the animal facility of Traditional Chinese Medicine (Nanchang, China). This protocol (license number: JZ2019-235) was approved by the Institutional Animal Care and Use Committee (IACUC) of Jiangxi University of Traditional Chinese Medicine.

DSS-induced colitis and treatment

All mice were divided randomly into 4 groups (10 mice per group): Control, DSS, DSS+Rg1 and DSS+Rg1 group. Male BALB/c mice of the DSS, DSS+Rg1, DSS+Y27632 groups received 3% (wet/vol) DSS in drinking water for 7 days, then normal water for 7 days, followed by administration of 2% (wet/vol) DSS for 7 days. Mice of the Control group received normal drinking water. At the beginning of the 8th day, mice in the DSS+Rg1 group were orally administrated with 200 mg/kg/day ginsenoside Rg1, mice in the DSS+Y27632 group were orally administrated with 10 mg/kg/day Y27632, and mice in the DSS and Control groups were treated with the equal volume of normal saline. Throughout the study, all mice were weighed once daily (09:00), and monitored daily for diarrhea, hematochezia, hunched posture and hair loss.

Macroscopic evaluation

Mice were anesthetized deeply with 20% pentobarbital sodium, the abdominal cavity was quickly opened, the colon tissue was directly separated, the length and weight of the colon were measured, and the colon index = colon weight/mouse weight \times 100%.

Pathological histology analysis

The proximal colon of mouse was fixed in 4% polyformaldehyde solution for 24 hours. The tissue was dehydrated with gradient ethanol, transparent with xylene, embedded in paraffin, and finally cut into 4 μ m-thickness slices. The sections were stained with hematoxylin and eosin (Solarbio, Beijing, China), and images were collected under the optical microscope (Lecia, Wetzlar, Germany) for pathological analysis. The pathological injure of the colon were blindly assessed by two different pathologists, including inflammatory cell infiltration and tissue damage [18]. The scoring of inflammatory cell infiltration was evaluated as 0 (rare inflammatory cells in the lamina propria) to 3 (transmural extension of the infiltration of inflammatory cells), and tissue damage was evaluated ranging from 0 (no mucosal damage) to 3 (extensive mucosal damage and extension through deeper structures of the bowel wall).

Enzyme-linked immunosorbent assay (Elisa)

The colon tissue (100 mg) was collected and lysised by RIPA (radio immunoprecipitation assay) solution at a ratio of 1:10, incubated at 4 °C for 1 hour, homogenized by ultrasonic homogenizer for 20 min, centrifuged at 4 °C for 15 min. Total protein in each sample was quantified by a total protein detection kit (Aidlab Biotechnologies Co., Ltd., Beijing, China), and then diluted in 1× PBS to a final concentration of 3000 ng/mL. And then the supernatant was obtained and detected. The concentrations of IL-6, IL-33, CCL-2, TNF- α , IL-4 and IL-10 were measured by commercial Elisa kits (Invitrogen, Calif., USA) according to the manufacturer's protocol, and then the optical density (OD) values at 450 nm was detected using a microplate reader (Thermo, Varioskan, MA, United States). Then, each cytokine was quantified basally based on a standard curve established using an Elisa kit.

Flow cytometry

Peripheral blood was collected in anticoagulated tubes, and mononuclear cells were isolated for further analysis. These cell samples were resuspended in RPMI 1640 and incubated in 5% CO₂ at 37 °C for 3 hours. All samples were detected on a FACS calibur analyser (Becton-Dickinson, Mountain View, CA). Then, these cells were incubated with an Fcy receptor-blocking mAb (CD16/32; BioLegend, San Diego, CA, USA) for 15 minutes at 4 °C. Subsequently, for surface antigen detection, the cells were shielded from light and labeled with with PE-conjugated anti-CD163 (1:100), PE-Cyanine7-conjugated anti-CD206 (Invitrogen, Calif., 1: 100), Alexa Fluor 488-conjugated anti-iNOS (Invitrogen, Calif., USA, 1: 200), PE/Cyanine7-conjugated anti-CD284 (Biolegend, Calif., USA, 1: 100), Alexa Fluor® 647-conjugated anti-F4/80 (BD Biosciences, Franklin Lakes, USA, 1: 100), PE-conjugated anti TIM-1 (BD Biosciences, Franklin Lakes, USA, 1: 100) and PerCP-Cy™5.5-conjugated anti-CD11b (BD Biosciences, Franklin Lakes, NJ, USA, 1: 100) antibodies. All data were analyzed using FlowJo 7.6.1 software (TreeStar, Ashland, OR, USA), and the inactive cells were excluded by gating.

Western blotting

The normalized supernatants (5 μ g/ μ L) of colonic tissues were prepared as described in 2.6. An equivalent amount of protein in each sample was fractionated onto sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane with a Bio-Rad Western blot apparatus. The PVDF membranes were blocked with 5% fat-free milk then incubated overnight with the following primary antibodies at 4°C. The primary antibodies were Nogo B (Abcam, ab47085, 1:2500), MIF (Abcam, ab187064, 1:2500), Pim-1 (CST 3247s, 1:1000), TLR2 antibody (Abcam, ab16894, 1:500), Rock1 (Abcam, ab45171, 1:1000), RhoA (Abcam, ab187027, 1:7500). These membranes were treated with the corresponding secondary antibody Goat-anti-Rabbit IgG (HRP) (Abcam, ab205718, 1:5000), Goat-anti-mouse IgG (HRP) (Abcam, ab205719, 1:10000) for 1-2 h at room temperature. Subsequently, these membranes were visualized with ECL western blot substrate. The specific protein bands were scanned with a UVP Chen Studio (Analytik Jena, Germany) and quantified using Image-Pro Plus 6.0 software (Media Cybernetic, MD, United States).

Microbial diversity analysis

The stool samples were collected on day 21 and immediately stored at -80°C for bacterial DNA extraction. The microbial diversity analysis was entrusted to Majorbio Bio-Pharm Technology (Shanghai, China). Total bacterial DNA was extracted from fecal samples according to QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) instructions. The V3-V4 region of the bacterial 16S rRNA gene was amplified with primers: 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). PCR products were purified using the AxyPrep DNA gel extraction kit (Axygen, Union City, USA) and quantified by Qubit (Invitrogen, USA). Then, the qualified libraries were sequenced using Illumina miseq platform. Operational taxonomic units (OTUs) were clustered with a 97% similarity cut-off using UPARSE (version 7.1). The taxonomy of each 16S rRNA gene sequence was assigned by the ribosomal database project (RDP) Classifier algorithm (<http://rdp.cme.msu.edu/>) against the SILVA (SSU123) 16S rRNA database using a confidence threshold of 70% [19]. Alpha diversity analysis (Mothur, version v.1.30.1) was used to evaluate the Chao1 abundance and the Shannon index. Principal coordinates analysis (PCoA) was performed using Mothur, and statistical analysis was performed based on the values of PC1 [20]. Linear discriminant analysis (LDA) coupled with effect size (LEfSe) measurements (based on non-parametric factorial Kruskal–Wallis sum-rank test and the Wilcoxon rank-sum test) was used to identify taxa that were significantly different (biomarkers) between groups, with $P < 0.05$ and an LDA score threshold of 4 [19]. Microbial difference analysis, correlation analysis, and co-occurrence network analysis were performed using I-sanger (Majorbio Bio-Pharm Technology Co. Ltd.; www.i-sanger.com). [21]

Statistical analysis

Data were expressed as the mean \pm SEM (Standard error of mean). Statistical analyses were carried out using GraphPad Prism 7.0 software (San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by the Tukey test for multiple comparisons were performed to determine significance. All p -values less than 0.05 were considered to be statistically significant.

Results

Ginsenoside Rg1 ameliorated DSS-induced colitis

In this study, the rate of body weight change (Fig. 1A) in colitis mice decreased significantly from day 4 to day 21, and mouse weight (Fig. 1B) and colon length (Fig. 1D, E) decreased significantly, and the colon weight index increased significantly (Fig. 1F) at the end of the experiment. At the same time, the mucosal tissue structure of colitis mice was found to be disorganized under light microscopy, with epithelial detachment, ulcer formation and massive inflammatory cell infiltration (Fig. 1G), and their pathological damage scores were significantly higher than that of the Control group (Fig. 1H). After treatment with ginsenoside Rg1 and Y27632, the rate of weight change (Fig. 1A) in mice increased significantly from day 16 to day 21, mouse weight (Fig. 1B) and colon length (Fig. 1D, E) increased significantly, and colon weight (Fig. 1C) and colon weight index (Fig. 1E) decreased significantly at the end of the experiment;

meanwhile, the pathological damage to the colon of mice in the DSS + Rg1 and DSS + Y27632 groups was effectively inhibited, less ulcer formation and inflammatory cell infiltration was occasionally observed under light microscopy (Fig. 1G), and its pathological damage scores were significantly lower than that of the DSS group (Fig. 1D). The above study showed that ginsenoside Rg1 and Y27632 was effective in alleviating DSS-induced experimental colitis.

Ginsenoside Rg1 regulated inflammatory cytokines in colon tissue

Cytokines are key pathophysiological factors that govern the initiation, progression and resolution of IBD inflammation [22], including pro-inflammatory cytokines IL-6, IL-33, CCL-2 and TNF- α , and anti-inflammatory cytokines IL-4 and IL-10. Therefore, in this study, the expression levels of these cytokines were detected in colonic tissue by Elisa assay. In the present study, the pro-inflammatory cytokines IL-6 (Fig. 2A), IL-33 (Fig. 2B), CCL-2 (Fig. 2C) and TNF- α (Fig. 2D) were significantly upregulated, and the anti-inflammatory cytokines, IL-4 (Fig. 2E) and IL-10 (Fig. 2F) were significantly down-regulated. After treatment with ginsenoside Rg1 in colitis mice, the pro-inflammatory cytokines IL-6 (Fig. 2A), IL-33 (Fig. 2B), CCL-2 (Fig. 2C) and TNF- α (Fig. 2D) were significantly down-regulated, and the anti-inflammatory cytokines, IL-4 (Fig. 2E) and IL-10 (Fig. 2F) were significantly up-regulated in colonic tissues. The trend of inflammatory cytokine changes in colitis mice treated with Y27632 was basically the same as that in the DSS + Rg1 group, but not including IL-10. These studies suggest that ginsenoside Rg1 and Y27632 can effectively regulate the expression of inflammatory cytokines in colonic tissues of colitis mice.

Ginsenoside Rg1 inhibited macrophage activation

Hyperactivation macrophages were closely linked to the onset and recurrence of mucosal inflammation in UC. Increasing evidence suggests that targeted regulation of macrophage activation is an emerging strategy for UC treatment [23]. In this study, the percentage of CD11b⁺F4/80⁺ (Fig. 3B1), CD11b⁺F4/80⁺Tim-1⁺ (Fig. 3C), and CD11b⁺F4/80⁺TLR4⁺ (Fig. 3D) macrophages were significantly increased in the peripheral blood of DSS-induced colitis mice. After treatment with ginsenoside Rg1 and Y27632, the percentage of CD11b⁺F4/80⁺ (Fig. 3B1), CD11b⁺F4/80⁺Tim-1⁺ (Fig. 3C), and CD11b⁺F4/80⁺TLR4⁺ (Fig. 3D) cells was significantly reduced. The above results indicate that ginsenoside Rg1 and Y27632 can effectively inhibit the activation of macrophages in colitis mice.

Ginsenoside Rg1 regulated the polarization balance of M1/M2 macrophage

Colonic mucosal injury caused by aberrant M1/M2 macrophage polarization contributes to IBD pathogenesis [24]. In this study, the percentage of M1 macrophage CD11b⁺F4/80⁺iNOS⁺ (Fig. 4C) cells of DSS-induced colitis mice were significantly increased, and M2 macrophages CD11b⁺F4/80⁺CD206⁺ (Fig. 4D) and CD11b⁺F4/80⁺CD163⁺ cells (Fig. 4E) were significantly reduced. After treatment with ginsenoside Rg1 and Y27632 in colitis mice, the percentage of M1 macrophages CD11b⁺F4/80⁺iNOS⁺

(Fig. 4C) was significantly decreased, and M2 macrophages CD11b⁺F4/80⁺CD206⁺ (Fig. 4D) and CD11b⁺F4/80⁺CD163⁺ cells (Fig. 4E) were significantly increased.

In this study, the level of Arg1 protein down-regulated significantly, whereas MIF-1 and PIM-1 proteins were significantly increased in the colon tissues of colitis mice (Fig. 5). After treatment with ginsenoside Rg1 and Y27632, the protein level of Arg1 increased significantly, whereas MIF-1 and PIM-1 proteins were significantly decreased in colon tissues of colitis mice (Fig. 5). These studies suggest that ginsenoside Rg1 and Y27632 can effectively regulate the balance of M1/M2 macrophage polarisation.

Ginsenoside Rg1 improved intestinal flora composition

The fecal microbial populations of mice in the Control, DSS, DSS + Rg1 and DSS + Y27632 groups were analyzed using 16S gene sequencing. Distances were calculated for each sample group at the OTU level, and the rank of distances (Bray_Curtis) was smaller for the Control, DSS, DSS + Rg1, and DSS + Y27632 groups relative to the Between group (Fig. 6A), with between-group differences greater than within-group differences. Sequencing coverage was high for all four groups and sequencing results found that most of the diversity was captured in all samples (Fig. 6B). 378 OTUs overlapped between the groups: 433 OTUs in both the Control and DSS groups; 436 OTUs in the DSS and DSS + Rg1 groups; and 433 OTUs in the DSS and DSS + Y27632 groups (Fig. 6C). Compared to percent of community abundance genus level of the Control group, *Lachnospiraceae* of the DSS group was significantly down-regulated and *Staphylococcus*, *Bacteroides* and *Ruminococcaceae_UCG_014* were significantly up-regulated (Fig. 6D). After ginsenoside Rg1 treatment, the percent of community abundance genus level of *Lachnospiraceae* were upregulated and *Staphylococcus*, *Bacteroides* and *Ruminococcaceae_UCG_014* were downregulated (Fig. 6D). A comparative analysis of the samples showed that stress = 0.059, which allows us to assume that the order of the samples in this experiment was quite good (Fig. 6E). The 20 samples in this experiment had different community compositions, but there was some similarity within the groups. The DSS and DSS + Rg1 groups had separate gut microbiota from the Control group, while the distance between the DSS + Rg1 group and the Control group was smaller than the distance between the DSS group and the Control group (Fig. 6E). To determine the role of Rg1 in regulating the intestinal flora of colitis mice, differential analysis among these four groups at the species level (Fig. 6F) were analysed. A one-way ANOVA was performed on data from the top seven bacterial rankings of microbiota abundance in the samples to detect species differences in microbiota community abundance between groups, including *Staphylococcus*, *Lachnospiraceae_NK4A136_group*, *unclassified_f_Lachnospiraceae*, *norank_f_Lachnospiraceae*, *Bacteroides*, *Prevotellaceae_UCG-001* and *Eubacterium_fissicatena* (Fig. 6F). The above results showed that Ginsenoside Rg1 improved gut microbiota composition in DSS-induced colitis mice.

Ginsenoside Rg1 regulated the crosstalk between M1/M2 macrophage and intestinal flora

The above results suggest that ginsenoside Rg1 is effective in regulating M1/M2 macrophage polarization and gut microbiota composition in colitis mice. However, the correlation or consistency

between whether ginsenoside Rg1 regulates macrophages and intestinal flora has not been elucidated. Here, redundancy analysis/canonical correlation analysis (RDA/CCA) was used to analyze their correlation. The correlation analysis between macrophages and intestinal flora at the genus level revealed that the DSS group was closer to TLR2, while the Control and Rg1 groups were closer to CD206 and CD163 (Fig. 7A). TLR4 is known to be involved in M1 macrophage differentiation, while CD206 and CD163 are involved in M2 macrophage differentiation. TLR2 was found to play a key role in intestinal flora composition, with the DSS group having the smallest spacing to TLR2 and the Control group having the largest spacing to TLR2 (Fig. 7B); and TLR2 protein expression was significantly elevated in colonic tissues of colitis mice and down-regulated significantly after ginsenoside Rg1 treatment (Fig. 7C). The results revealed that ginsenoside Rg1 effectively regulated the crosstalk between M1/M2 macrophage and intestinal flora in DSS-induced colitis.

Ginsenoside Rg1 regulated Nogo-B/RhoA signal pathway

The ATP-competitive ROCK-I and ROCK-II inhibitor Y27632 is effective in alleviating the development of UC [25]. Therefore, we further investigated whether ginsenoside Rg1 could play a critical role in the treatment of ulcerative colitis by regulating the Rock signaling pathway. In this study, the levels of Rock1 (Fig. 8A, B) and RhoA (Fig. 8A, C) proteins were significantly increased in the colonic tissues of colitis mice. After treatment with ginsenoside Rg1 and Y27632, the levels of Rock1 (Fig. 8A, B), RhoA (Fig. 8A, C) and Nogo-B (Fig. 8A, D) proteins in colonic tissues of colitis mice decreased significantly. These results suggest that ginsenoside Rg1 inhibit the activation of Nogo-B/RhoA signaling pathway in colitis mice.

Discussion

DSS-induced colitis model can mimic the pathogenesis of human colitis [26] and is commonly used in the study of colitis pathogenesis and development of new drugs [27]. In the present study, the colitis model was successfully replicated with lower body weight, shorter colonic length, larger colonic weight index, more ulcer formation and massive inflammatory cell infiltration in the colonic mucosa, and higher pathological damage scores. Ginsenoside Rg1, one of the main components of *Panax ginseng*, has potent anti-inflammatory properties [10]. In this study, the changes of body weight, colon length, colonic weight index and colonic mucosal injury of colitis mice were effectively improved after treatment of ginsenoside Rg1. Our study demonstrated that ginsenoside Rg1 was also efficacious in DSS-induced chronic colitis.

Macrophages are core effector cells of the innate immune system. Macrophages are functionally plastic and differentiate into pro-inflammatory (M1-like) or anti-inflammatory (M2-like) phenotypes in response to different stimuli in the local microenvironment [28]. M1 macrophages secrete pro-inflammatory cytokines like TNF- α and IFN- γ , causing mucosal damage and exacerbates IBD [24]. In contrast, M2 macrophages promote tissue repair and inflammation to alleviate IBD symptoms [29] by highly express Arg1, YM-1 and cytokines including IL-10 [30]. More M1 macrophages and fewer M2 macrophages were founded in peripheral blood of UC patients [31]. In DSS-induced experimental colitis, M1/M2 macrophage

polarisation imbalance was observed in the spleen and mesenteric lymph nodes [32]. Macrophages expressed Tim-1 [33] and TLR4 upon activation, M1 macrophages expressed iNOS [34], and M2 macrophages expressed CD206 and CD163 [35]. In this study, macrophage hyperactivation and M1/M2 macrophage polarization imbalance in DSS-induced colitis mice; CD11b⁺F4/80⁺Tim-1⁺ and CD11b⁺F4/80⁺TLR4⁺ and M1 macrophage CD11b⁺F4/80⁺iNOS⁺ were significantly increased, whereas M2 macrophages CD11b⁺F4/80⁺CD163⁺ and CD11b⁺F4/80⁺CD206⁺ were significantly reduced. The changes of CD11b⁺F4/80⁺Tim-1⁺, CD11b⁺F4/80⁺TLR4⁺, CD11b⁺F4/80⁺iNOS⁺, CD11b⁺F4/80⁺CD163⁺ and CD11b⁺F4/80⁺CD206⁺ macrophages were effectively reversed of after ginsenoside Rg1 treatment. Arg1 activation promotes the polarization of M2 macrophages [36]. PIM-1 induces M1 macrophages and enhances their effector functions [37]. An important pro-inflammatory factor, MIF (macrophage migration inhibitory factor) is a member of a family of bioactive proteins that can be released by a variety of cells, with monocytes/macrophages being the main source of MIF [38]. In this study, Arg1 protein inhibition and activation of MIF-1 and PIM-1 protein in colitis mice were reversed by Rg1. It suggested that ginsenoside Rg1 inhibited macrophage activation and regulated M1/M2 polarization balance to effectively alleviate UC,

Dysbiosis of the intestinal flora is a typical feature of DSS-induced colitis [23]. There are differences in the composition of gut microbiota between IBD patients and healthy individuals, such as microbial diversity and the relative abundance of specific bacterial taxa [39]. Alpha diversity analysis reveals intestinal flora imbalance in IBD patients with higher abundance of harmful bacteria such as *E. coli*, *Shigella* and *gamma-amoeba*, and lower abundance of beneficial bacteria *Lactobacillus*, *Bifidobacterium* and *Phytophthora* [40, 41]. In colitis model, the composition and relative abundance of intestinal flora present significant changes, with lower abundance of *Bacteroidales_S24-7_group_unidentified*, *Bacteroides Prevotellaceae_UCG-001*, *Lactobacillus* and *Alistipes*, and higher abundance of *Streptococcus* and *Shigella* [23, 42]. Not entirely consistent with these studies, we found that the relative abundance of *Lachnospiraceae_NK4A136_group*, *unclassified_f_Lachnospiraceae* and *norank_f_Lachnospiraceae* were significantly decreased, and that of *Staphylococcus*, *Bacteroides*, *Prevotellaceae_UCG-001* and *Eubacterium_fissicatena_group* were significantly increased. Recent hotspots suggest that correcting and restoring flora diversity and abundance plays a key role in resistance to intestinal inflammation, such as Baitouweng decoction [43], nitrate [42], Ping weisan [44], *Saccharomyces boulardii* [45]. In this study, ginsenoside Rg1 reversed the changes of relative abundance of *Staphylococcus*, *Bacteroides*, *Eubacterium_fissicatena_group*, *Lachnospiraceae_NK4A136_group* and *norank_f_Lachnospiraceae*. Combined with the previous efficacy evaluation, ginsenoside Rg1 effectively alleviated DSS-induced experimental colitis by improving intestinal flora composition.

Nogo-B is a member of the reticulohistone family of proteins, mainly located in the endoplasmic reticulum (ER), and is involved in a variety of cell biological processes. Nogo-B is required for macrophage homing, and Nogo-B deficiency impairs macrophage migration [46]. In vivo experiments, Nogo-B is involved in inflammatory responses and macrophage infiltration and macrophage-mediated tissue remodelling were found in Nogo-B^{-/-} mice [47]. In vitro experiments, LPS stimulated macrophage

activation and Nogo-B protein was downregulated in a time- and dose-dependent manner [48]. In addition, Nogo-B is required for the immune response and its over-expression induces the expression of the pro-inflammatory cytokines CCL-1, TNF- α , IL-1 β and TGF- β , and enhances the migratory activity of macrophages and recruits macrophages to move chemotactically towards local inflammation sites [48]. Impaired RhoA function leads to over-expression of CD4⁺ T cell integrin α 4 β 7 and colonic localization, inflammatory cytokine storm and the development of colitis [49]. The Rho kinase (ROCK) inhibitor Y27632 reduced intestinal permeability and inhibited the Rock-MLC and Rock-NF- κ B pathways, effectively attenuating TNBS-induced colitis in mice [50]. In our study, both of ginsenoside Rg1 and Y27632 inhibited the expression of Rock 1, RhoA, and Nogo-B proteins in colonic tissues of colitis mice. Importantly, the trends of Nogo-signaling in regulating pro-inflammatory cytokines and macrophages of colitis mice were largely consistent with ginsenosides Rg1. These findings suggest that the key target of ginsenoside Rg1 in regulating macrophage polarization in colitis mice is closely related to Nogo-B signaling, laying the foundation for future studies.

The interaction between TLR and intestinal flora can maintain immune system homeostasis [51], and the underlying mechanisms may be closely related to macrophage homeostasis. The disruption of intestinal flora leads to the activation of immune cells by TLR [52], which further leads to inflammation. In TLR2 gene knockout mice, expression of M1 macrophages was reduced and expression of M2 macrophages was increased [53]. Compared to wild-type mice, levels of IL-6 and TNF- α were significantly reduced in M1 macrophage culture supernatants from TLR2^{-/-} mice, while levels of IL-10 were significantly increased in M2 macrophage culture supernatants [53]. Inhibition of TLR2 expression reduces the secretion of inflammatory factors by macrophages for the treatment of osteoarthritis [54]. Interestingly, we found that changes in macrophage activation or polarization proteins TLR2, TLR4, CD206, and CD163 were closely associated with intestinal flora composition. It implies that macrophage polarization during the course of colitis affects the structure of the intestinal flora. The correlation among colitis, macrophages, and intestinal flora needs to be further investigated, which is the focus of the next study.

Conclusions

Ginsenoside Rg1 has the protective effect on UC by inhibiting macrophage activation, restoring the balance of M1/M2 macrophage polarization, and improving intestinal flora composition, associated with inhibition of the Nogo-B/RhoA signaling pathway.

Abbreviations

UC: Ulcerative colitis; IBD: Inflammatory bowel disease; CD: Crohn's disease; DSS: Dextran sodium sulfate; TNBS: 2,4,6-Trinitrobenzenesulfonic acid; HAN: Hyaluronic acid-bilirubin nanomedicine; OD: Optical density; PVDF: Polyvinylidene fluoride; Elisa: Enzyme-linked immunosorbent assay; RDP: Ribosomal database project; PCoA: Principal coordinates analysis; LDA: Linear discriminant analysis; OTU: Operational taxonomic unit; SEM: Standard error of mean; ANOVA: One-way analysis of variance; RDA/CCA: redundancy analysis/canonical correlation analysis; MIF: Macrophage migration inhibitory

factor; RIPA: Radio immunoprecipitation assay; SPF: specific pathogen-free; IACUC: Institutional animal care and use committee; ER: endoplasmic reticulum; ROCK: Rho kinase.

Declarations

Ethics approval and consent to participate

All the animal experiments were approved by the Institutional Animal Care and Use Committee of Jiangxi University of Traditional Chinese Medicine.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used in the current study are available from the corresponding author on reasonable request.

Competing interests

All authors declare that they have no conflicts of interest.

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Authors' contributions

Y-BZ and D-YL conceived and designed the experiments; JL, X-KL, Z-PK, M-XW and H-MZ performed the experiments; D-YL and H-MZ contributed reagents/ materials/ analytical tools; JL, Y-BZ and D-YL analyzed the data; Y-BZ and D-YL wrote the paper.

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Not applicable.

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Figures

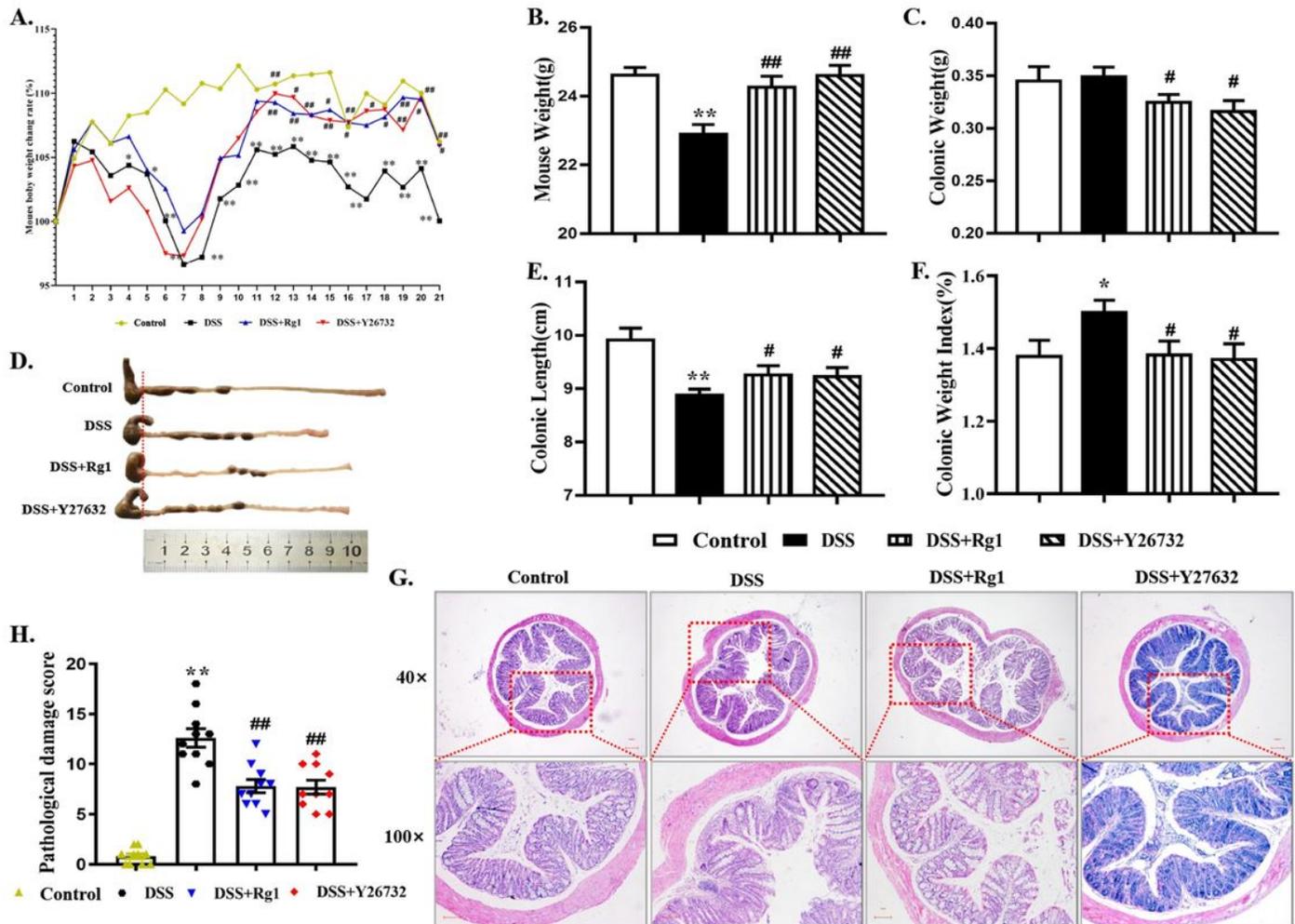


Figure 1

Therapeutic evaluation of Ginsenoside Rg1 on DSS-induced colitis mice. (A) Mouse body weight change rate. (B) Mouse weight. (C) Colonic weight. (D) Changes in colonic length by naked eye. (E) Colonic length. (F) Colonic weight index. (G) Histological appearance of the colons from individual groups of mice; the colonic sections were stained with hematoxylin and eosin. (H) Pathological damage score. Data are representative images or as the mean \pm SEM (n = 8 mice per group). *P < 0.05 and **P < 0.01 compared to the Control group, #P < 0.05 and ##P < 0.01 compared to the DSS group.

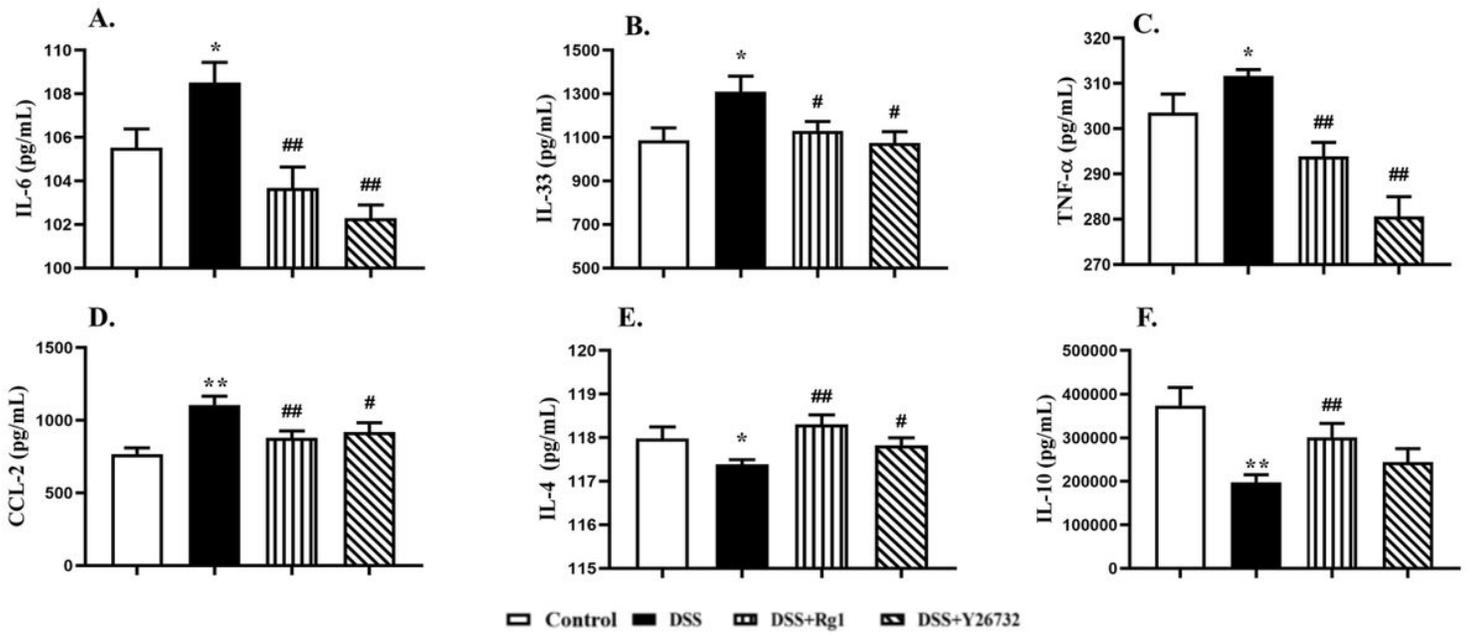


Figure 2

Ginsenoside Rg1 regulated the expression of inflammatory cytokines in colonic tissues of colitis mice. The levels of pro-inflammatory cytokines, (A) IL-6, (B) IL-33, (C) TNF- α , and (D) CCL-2 and the levels of anti-inflammatory cytokines, (E) IL-4 and (F) IL-10 were measured by Elisa. Data are presented as mean \pm SEM (n = 8). Data are representative images or as the mean \pm SEM (n = 8 mice per group). *P < 0.05 and **P < 0.01 compared to the Control group, #P < 0.05 and ##P < 0.01 compared to the DSS group.

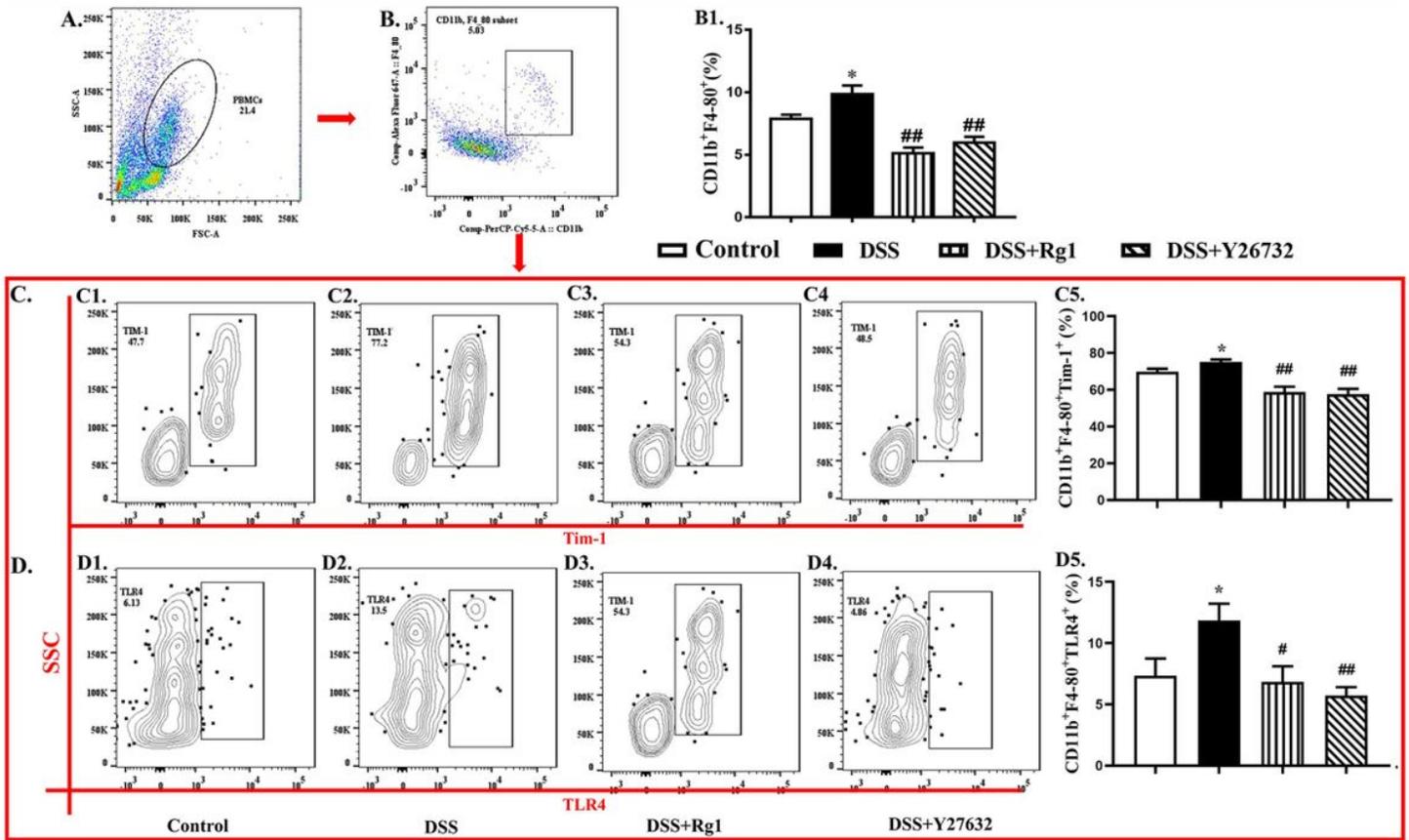


Figure 3

Ginsenoside Rg1 inhibited the activation of macrophage in colitis mice. (A) Total lymphocytes in peripheral blood. (B) Double-positive F4/80 and CD11b cells. (C) Flow cytometry analysis of Tim-1⁺F4/80⁺CD11b⁺ macrophage frequencies; C1–C4 represent Tim-1⁺F4/80⁺CD11b⁺ macrophage in the Control, DSS, DSS + Rg1, and DSS + Y26732 groups in order; C5: statistical analysis of Tim-1⁺F4/80⁺CD11b⁺ macrophage in these 4 groups. (D) Flow cytometry analysis of TLR4⁺F4/80⁺CD11b⁺ macrophage frequencies; D1–D4 represent TLR4⁺F4/80⁺CD11b⁺ macrophage in the Control, DSS, DSS + Rg1, and DSS + Y26732 groups in order; D5: statistical analysis of TLR4⁺F4/80⁺CD11b⁺ macrophage in these 4 groups. Data are representative images or as the mean ± SEM (n = 8 mice per group). *P < 0.05 and **P < 0.01 compared to the Control group, #P < 0.05 and ##P < 0.01 compared to the DSS group.

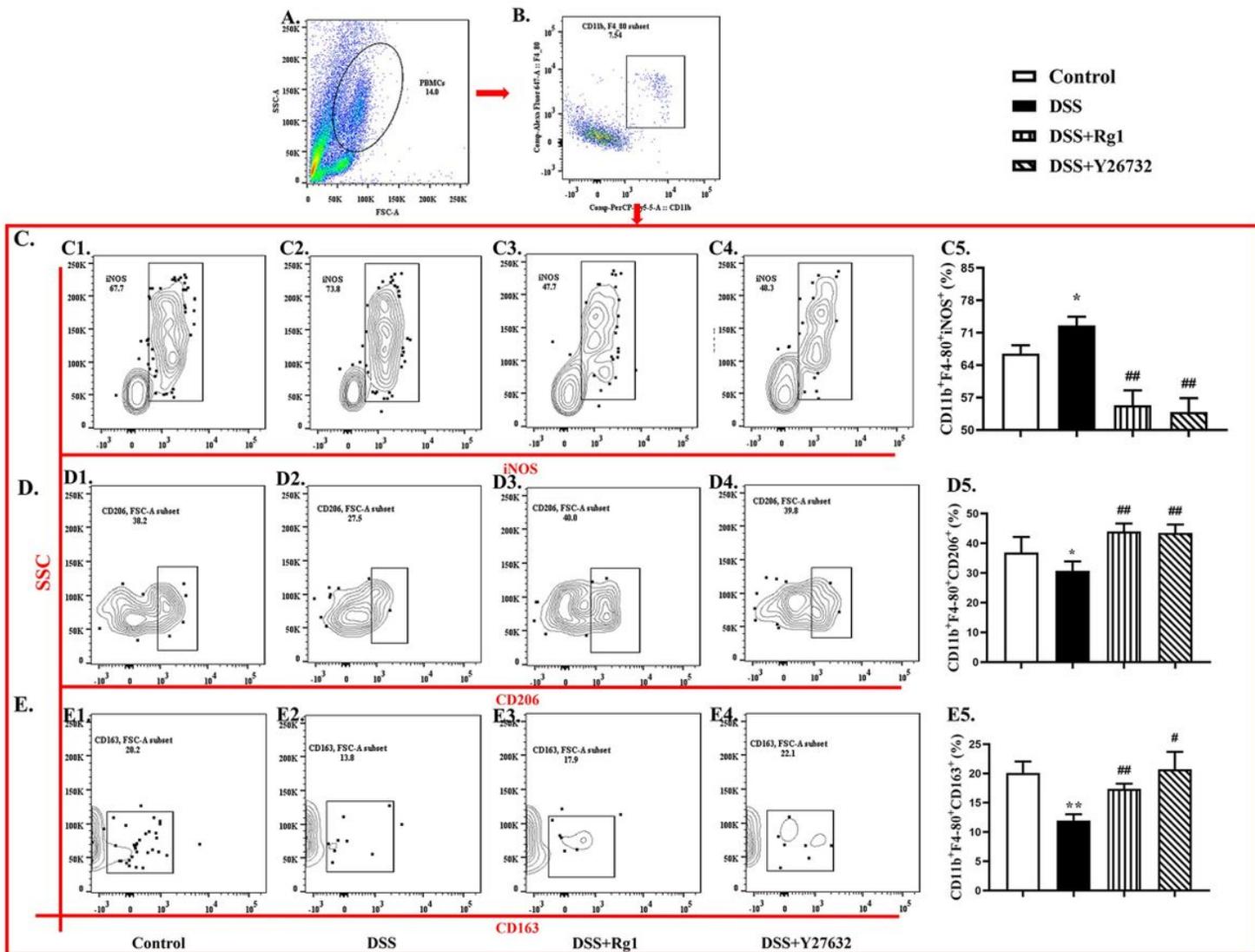


Figure 4

Ginsenoside Rg1 regulated M2 macrophage polarisation in colitis mice. (A) Peripheral blood mononuclear cells (PBMCs). (B) Double-positive F4/80 and CD11b cells. (C) Flow cytometry analysis of iNOS+F4/80+CD11b+ macrophage frequencies; C1–C4 represent iNOS+F4/80+CD11b+ macrophage in the Control, DSS, DSS + Rg1, and DSS + Y27632 groups in order; C5: statistical analysis of iNOS+F4/80+CD11b+ macrophage in these 4 groups. (D) Flow cytometry analysis of CD163+F4/80+CD11b+ macrophage frequencies; D1–D4 represent CD163+F4/80+CD11b+ macrophage in the Control, DSS, DSS + Rg1, and DSS + Y27632 groups in order; D5: statistical analysis of CD163+F4/80+CD11b+ macrophage in these 4 groups. (E) Flow cytometry analysis of CD206+F4/80+CD11b+ macrophage frequencies; E1–E4 represent CD206+F4/80+CD11b+ macrophage in the Control, DSS, DSS + Rg1, and DSS + Y27632 groups in order; E5: statistical analysis of CD206+F4/80+CD11b+ macrophage in these 4 groups. Data are representative images or as the mean \pm SEM (n = 8 mice per group). *P < 0.05 and **P < 0.01 compared to the Control group, #P < 0.05 and ##P < 0.01 compared to the DSS group.

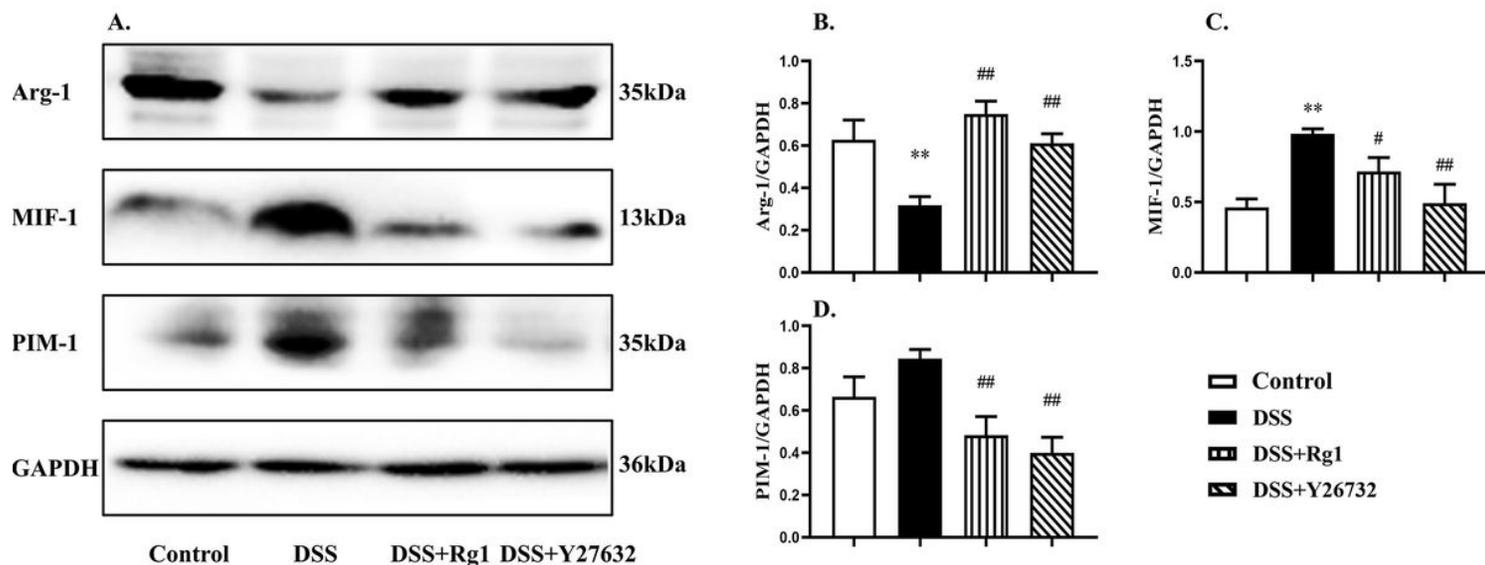


Figure 5

Ginsenoside Rg1 inhibited regulated the expression of macrophage related protein in colitis mice. (A) Western blotting of major proteins in the macrophage related moleculars, such as Arg-1, MIF-1 and PIM-1. (B) Quantitative analysis of Arg-1. (C) Quantitative analysis of MIF-1. (D) Quantitative analysis of PIM-1. Data are presented as mean \pm SEM (n = 8). #p <0.05 and ##p <0.01 versus the Control group, *p <0.05 and **p <0.01 versus the DSS group.

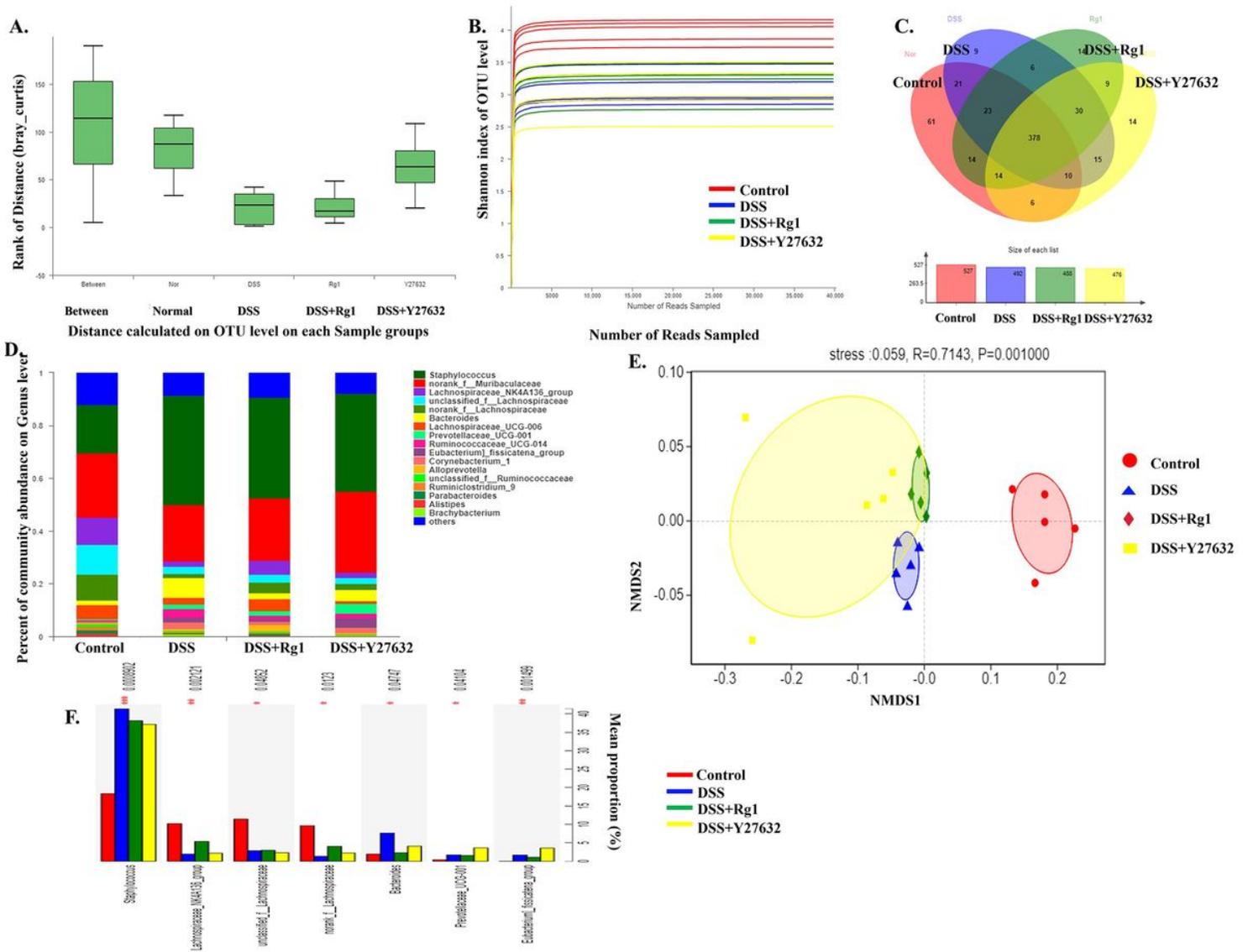


Figure 6

Ginsenoside Rg1 improved the structure of intestinal flora in colitis mice. (A) Rank of Distance (bray_curtis). (B) Shannon index of OTU level. (C) The Venn diagram depicts operational taxonomic units (OTUs) that differed in each group. (D) Percent of community abundance on Genus level. (E) NMDS diagram of multi-level samples at the OTU level based on the Bray_Curtis algorithm. (F) Differential analysis among these four groups at the species level.

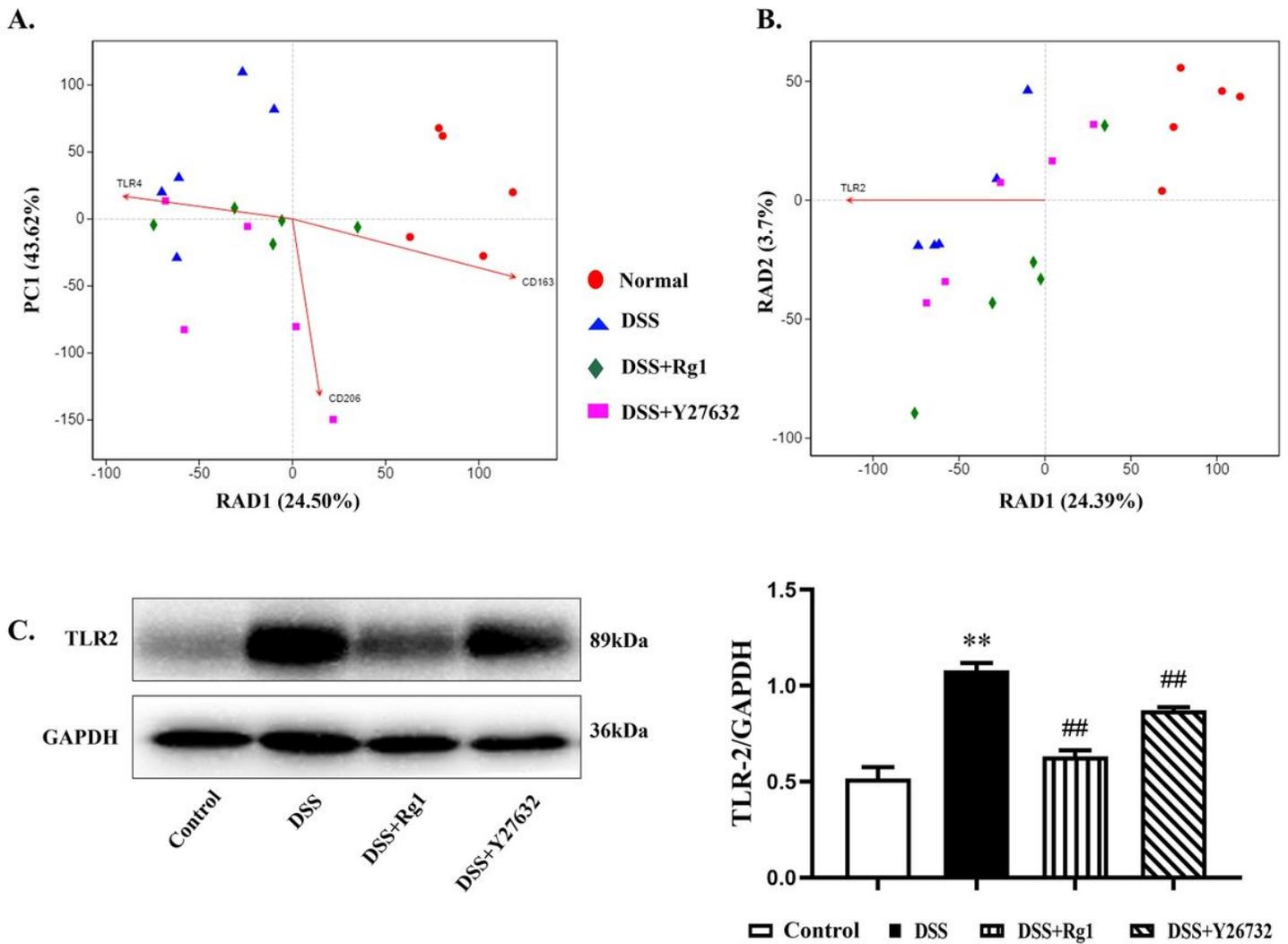


Figure 7

Ginsenoside Rg1 regulated correlation with the crosstalk among macrophage and gut microbiota in colitis mice. (A) Correlation between TLR4, CD163 and CD206 and microbial flora structure displayed by distance-based redundancy analysis (db-RDA) diagram. (B) Correlation between TLR2 and microbial flora structure displayed by distance-based redundancy analysis (db-RDA) diagram. (C) The expression levels of TLR2 protein in colon tissue were detected by Western Blotting. Data are presented as mean \pm SEM (n = 8). #p < 0.05 and ##p < 0.01 versus the Control group, *p < 0.05 and **p < 0.01 versus the DSS group.

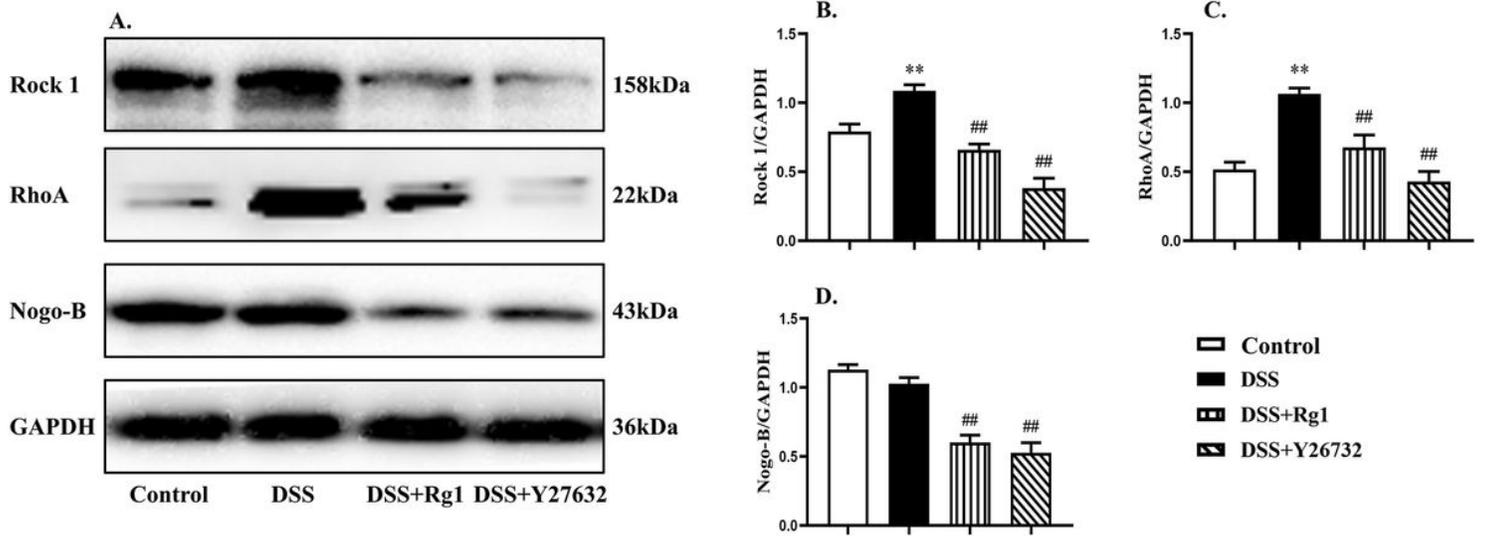


Figure 8

Ginsenoside Rg1 inhibited the activation of Nogo-B/RhoA signal pathway. (A) Western blotting of major proteins in Nogo-B/RhoA signal pathway, including Rock 1, RhoA and Nogo-B. (B) Quantitative analysis of Rock 1. (C) Quantitative analysis of RhoA. (D) Quantitative analysis of Nogo-B. Data are presented as mean \pm SEM (n = 8). #p < 0.05 and ##p < 0.01 versus the Control group, *p < 0.05 and **p < 0.01 versus the DSS group.