Journal of Pharmaceutical Analysis 14 (2024) 100940

Contents lists available at ScienceDirect

Journal of Pharmaceutical Analysis

journal homepage: www.elsevier.com/locate/jpa

Original article

Paeoniflorin ameliorates chronic colitis via the DR3 signaling pathway in group 3 innate lymphoid cells

Shaowei Huang [a,1], Xueqian Xie [a,1], Bo Xu [a], Zengfeng Pan [a], Junjie Liang [a], Meiling Zhang [a], Simin Pan [c], Xiaojing Wang [a], Meng Zhao [a], Qing Wang [a], Jinyan Chen [b], Yanyang Li [a], Lian Zhou [a,**], Xia Luo [a,*]

[a] School of Pharmaceutical Sciences, Guangzhou University of Chinese Medicine, 510006, China
[b] School of Basic Medicine, Guangzhou University of Chinese Medicine, 510006, China
[c] First Clinical Medical College, Guangzhou University of Chinese Medicine, 510000, China

ARTICLE INFO

Article history:
Received 29 August 2023
Received in revised form
22 January 2024
Accepted 28 January 2024
Available online 1 February 2024

Keywords:
Paeoniflorin
Ulcerative colitis
Intestinal mucosal barrier
DR3 signaling pathway
Group 3 innate lymphoid cells

ABSTRACT

Inhibiting the death receptor 3 (DR3) signaling pathway in group 3 innate lymphoid cells (ILC3s) presents a promising approach for promoting mucosal repair in individuals with ulcerative colitis (UC). Paeoniflorin, a prominent component of *Paeonia lactiflora* Pall., has demonstrated the ability to restore barrier function in UC mice, but the precise mechanism remains unclear. In this study, we aimed to delve into whether paeoniflorin may promote intestinal mucosal repair in chronic colitis by inhibiting DR3 signaling in ILC3s. C57BL/6 mice were subjected to random allocation into 7 distinct groups, namely the control group, the 2 % dextran sodium sulfate (DSS) group, the paeoniflorin groups (25, 50, and 100 mg/kg), the anti-tumor necrosis factor-like ligand 1A (anti-TL1A) antibody group, and the IgG group. We detected the expression of DR3 signaling pathway proteins and the proportion of ILC3s in the mouse colon using Western blot and flow cytometry, respectively. Meanwhile, DR3-overexpressing MNK-3 cells and 2 % DSS-induced $Rag1^{-/-}$ mice were used for verification. The results showed that paeoniflorin alleviated DSS-induced chronic colitis and repaired the intestinal mucosal barrier. Simultaneously, paeoniflorin inhibited the DR3 signaling pathway in ILC3s and regulated the content of cytokines (Interleukin-17A, Granulocyte-macrophage colony stimulating factor, and Interleukin-22). Alternatively, paeoniflorin directly inhibited the DR3 signaling pathway in ILC3s to repair mucosal damage independently of the adaptive immune system. We additionally confirmed that paeoniflorin-conditioned medium (CM) restored the expression of tight junctions in Caco-2 cells via coculture. In conclusion, paeoniflorin ameliorates chronic colitis by enhancing the intestinal barrier in an ILC3-dependent manner, and its mechanism is associated with the inhibition of the DR3 signaling pathway.

1. Introduction

Ulcerative colitis (UC) is a chronic inflammatory bowel disease. and its pathogenesis is mainly related to genes, diet, and intestinal flora disturbance, and is finally characterized as intestinal mucosal barrier damage by an abnormal immune response. Currently, the main therapeutic approaches for UC focus on inhibiting the inflammation of damaged mucosa, that is, treating UC with anti-inflammatory agents and suppressing the immune response [1]. Although these therapies have specific clinical efficacy, they still need to solve the problem of UC persistence and recurrence [2]. Recent studies have found that restoring the integrity of the intestinal mucosal epithelium could effectively relieve UC symptoms and even strengthen the therapeutic effect of drugs [3]. Hence, achieving a strong intestinal barrier is considered a crucial breakthrough in the treatment of UC.

In the past few years, accumulating evidence has supported that group 3 innate lymphoid cells (ILC3s) can protect the intestinal barrier by secreting interleukin (IL)-22. IL-22, mainly produced by natural cytotoxic receptor $(NCR)^{+}$ ILC3s, repairs the intestinal mucosa by promoting the proliferation of goblet cells and Paneth's cells, participating in the formation of intestinal mucous layer, and promoting the expression of intestinal epithelial tight junctions (TJs). However, in patients with UC and in

* Corresponding author.
** Corresponding author.
E-mail addresses: zl@gzucm.edu.cn (L. Zhou), luoxia@gzucm.edu.cn (X. Luo).
Peer review under responsibility of Xi'an Jiaotong University.
[1] Both authors contributed equally to this work.

https://doi.org/10.1016/j.jpha.2024.01.008

dextran sodium sulfate (DSS)-induced UC mice, a significant reduction in NCR^+ ILC3 in the colon was accompanied by an increase in the fraction of NCR^- ILC3. NCR^- ILC3s interact with intestinal epithelial cells and promote the secretion of inflammatory cytokines (tumor necrosis factor-α (TNF-α), IL-1β, and IL-6) to aggravate barrier damage by secreting IL-17A and granulocyte-macrophage colony stimulating factor (GM-CSF). In addition, some scholars have reversed colonic inflammation in UC mice by adoptive transfer of IL-22^+ ILC3. These findings suggest that regulating ILC3 function in the gut is an essential strategy to promote the repair of UC mucosal damage.

Recent studies have confirmed that the membrane death receptor 3 (DR3)-mediated pathway is one of the influential pathways in regulating the function of ILC3s [4]. Activation of DR3 by tumor necrosis factor-like ligand 1A (TL1A) leads to phosphorylation of its downstream molecules p38 mitogen-activated protein kinase (MAPK) and nuclear factor-kappaB (NF-κB) p65, promoting the production of inflammatory factors GM-CSF and IL-17A and disrupting the intestinal barrier [5]. In addition, agonistic DR3 antibodies can increase the production of GM-CSF in intestinal ILC3s by activating the DR3 signaling pathway, which further recruit neutrophils and disrupts intestinal immune homeostasis [4]. Next, it promotes the occurrence and development of intestinal inflammation in mice; moreover, the activation of the DR3 signaling pathway is accompanied by a reduction in ILC3s, especially IL-22^+ ILC3s. DR3-Fc inducer, an agent that blocks the binding of TL1A to DR3 on the surface of ILC3s, significantly improves mucosal ulcers in colitis mice [6], indicating that DR3 signaling is an essential mechanism regulating the immune response of ILC3s.

Paeoniflorin, one of the main components of *Paeonia lactiflora* Pall., has attracted the attention of scholars due to its low toxicity and great anti-inflammatory activity. Presently, preparations with paeoniflorin as the main component have been used in clinical trials to treat rheumatoid arthritis, and significant curative effects have been achieved [7]. Likewise, previous studies have shown that paeoniflorin improved intestinal inflammation in acute colitis mice [8,9]. However, the efficacy and mechanism of paeoniflorin in chronic colitis remain to be confirmed. In this study, we aimed to explore the immunological mechanism of paeoniflorin in DSS-induced chronic colitis mice.

2. Materials and methods

2.1. Reagents

Paeoniflorin (>98% purity) was obtained from Chengdu Pufei De Biotech Co., Ltd. (Chengdu, China). Dextran sulfate sodium salt (DSS, CAS: 9011-18-1) was obtained from MP Biomedicals (Santa Ana, CA, USA). MAb anti-mouse TL1A (TNFSF15) and MAb polyclonal Armenian hamster IgG were provided by BioXcell (West Lebanon, NH, USA). The CY3 labeled Enter1432 FISH probe was provided by Guangzhou Exon Biotechnology Co., Ltd (Guangzhou, China). β-Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Bioss (Beijing, China). Claudin-1 antibody was purchased from Santa Cruz (Dallas, TX, USA). Occludin and ZO-1 antibodies were obtained from Proteintech (Wuhan, China). PerCP/Cyanine5.5-IL-22 antibody, AF700-CD45 antibody, and APC-CD335 (NKp46) antibody were obtained from Biolegend (Santiago, CA, USA). FITC-lineage and PE-Cyanine7-ROR gamma (t) were purchased from Tonbo (Santiago, CA, USA) and eBioscience (Santiago, CA, USA), respectively. Luria-Bertani (LB) solid medium was purchased from Solarbio Life Science Co., Ltd (Beijing, China). LV-Tnfrsf25 (73359-1), and all transfection reagents were obtained from Shanghai Genechem Co., Ltd. (Shanghai, China).

2.2. Animals and treatments

Wild-type (WT) male C57BL/6 mice (6–8 weeks) were obtained from Guangzhou University of Chinese Medicine (Guangzhou, China; SYXK (Yue) 2018-0002), and male $Rag1^{-/-}$ mice on the C57BL/6 background were procured from GemPharmatech Co., Ltd. (Nanjing, China; SYXK (Su) 2018-0008). Mice were kept in an animal house under specific pathogen-free (SPF) conditions, and all animal testing protocols were approved by the Ethics Committee of Guangzhou University of Chinese Medicine (Approval number: ZYD-2021-116).

WT mice were subjected to random allocation into 7 groups (10 mice/group): 2% DSS group, paeoniflorin groups (25, 50, 100 mg/kg), anti-TL1A antibody (10 mg/kg) group, IgG antibody (10 mg/kg) group, and control group. Except for the control group, mice in other groups drank 2% DSS freely for five days, followed by sterile distilled water for two days, for a total of 4 cycles (Fig. 1A). Beginning on the sixth day, paeoniflorin was administered orally to the mice daily. Meanwhile, TL1A is the only ligand of DR3. Anti-TL1A antibody neutralized the stimulatory molecule TL1A and avoided the activation of the DR3 signal. Therefore, we chose the anti-TL1A antibody as the positive control and IgG as the isotype control of the anti-TL1A antibody. Anti-TL1A (10 mg/kg) or IgG (10 mg/kg) was injected intraperitoneally on the 6th, 13th, 20th, and 27th days. $Rag1^{-/-}$ mice were subjected to random allocation into four groups (8 mice/group): the 2% DSS group, paeoniflorin (100 mg/kg) group, anti-TL1A antibody (10 mg/kg) group, and control group. The modeling and administration methods were the same as before. Body weight and disease activity index (DAI) scores were evaluated [10].

On the 29th day, we collected peripheral blood, spleen, colon, and thymus. Hemoglobin (HGB) content, white blood cell (WBC) and red blood cell (RBC) number, as well as the proportions of monocytes (Mon), granulocytes (Gran), and lymphocytes (Lymph) were determined according to a previously described methodology [11]. Additionally, the spleen index and thymus index were also evaluated in the same manner.

2.3. Hematoxylin and eosin (HE) staining

First, 1 cm of the distal colon was fixed overnight with 4% paraformaldehyde. Then, the samples were dehydrated, paraffin-embedded and sliced into 4 μm thick sections for morphological observations after being stained with HE. Histological scores were assessed previously [12].

2.4. Enzyme-linked immunosorbent assay (ELISA)

Serum lipopolysaccharide (LPS) was detected by an ELISA kit (Jiangsu Meimian Industrial Co., Ltd., Yancheng, China) following the manufacturer's instructions.

2.5. Small animal imaging

Mice were fasted for 12 h the day before sampling. Then, 50 mg/kg FITC-Dextran (Sigma-Aldrich, St. Louis, MO, USA) was administered orally to the mice. After 4 h, a small animal imager (Berthold LB983, Bad Wildbad, Germany) was used to measure the fluorescence distribution in the mice.

2.6. Fluorescence in situ hybridization (FISH)

The opportunistic pathogen Enterobacteriaceae of colon mucosa in paraffin sections was stained with a Cy3-labeled Enter 1432 FISH probe according to the instructions of the FISH kit (Guangzhou exon biological technology co., LTD, Guangzhou, China).

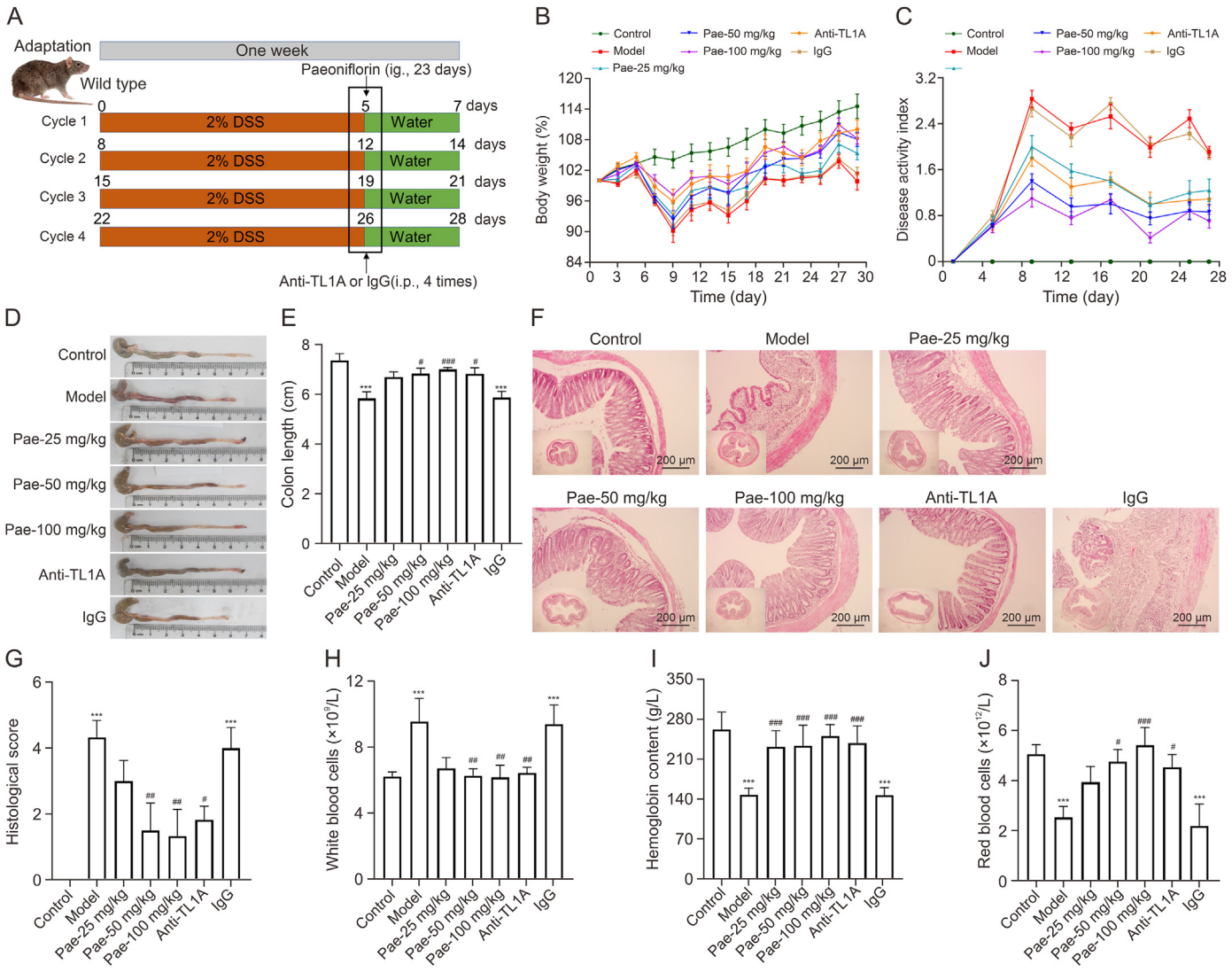

Fig. 1. Paeoniflorin alleviated dextran sodium sulfate (DSS)-induced chronic colitis in mice. (A) Experiment design. (B) Changes in body weight in different groups. (C) Changes in disease activity index (DAI) scores in different groups. (D) Representative colon images. (E) Changes in colon length in different groups. (F) Representative hematoxylin-eosin staining images of colon tissue. (G) Changes in colon histological scores in different groups. (H–J) Changes in white blood cells number (H), hemoglobin content (I), and red blood cells number (J) in different groups. $n = 6–8$ per group. $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. model group. Pae: paeoniflorin; anti-TL1A: anti-tumor necrosis factor-like ligand 1A.

2.7. Spleens and mesenteric lymph nodes (MLNs) for bacterial culture

Spleens and MLNs were separated from mice under sterile conditions and homogenized with 500 μL of 0.9% physiological saline. Next, 10 μL of homogenate was transferred onto LB solid medium and incubated at 37 °C for 24 h. The colony forming units (CFUs) were then examined and counted.

2.8. Western blot (WB)

We extracted the total proteins from colons or cells through radio immunoprecipitation assay (RIPA) lysis buffer containing 1% phosphatase inhibitor and 1% phenyl methane sulfonyl fluoride (PMSF). The extracted protein (20 μg) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were sealed with 5% bovine albumin (BSA) at room temperature for 2 h and incubated with primary antibodies against phospho-p38 MAPK, claudin-1, β-actin, DR3, IL-17A, occludin, tumor necrosis factor receptor-associated death domain protein (TRADD), p38 MAPK, ZO-1, NF-κB p65, and phospho-NF-κB p65. A chemiluminescence imager (Tanon 5200, Tanon, Shanghai, China) was used to detect all protein bands with an ECLPlus kit (Millipore, Boston, MA, USA) and the gray values were quantified using ImageJ software.

2.9. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA (1 μg) extracted from colons or cells using TRIzol (Solarbio, Beijing, China) was reversely transcribed into cDNA with M-MLV reverse transcriptase (Vazyme, Nanjing, China). The resulting cDNA was then subjected to real-time PCR analysis on the CFX96 system (Bio-Rad, Hercules, CA, USA) using 2× SYBR Green qPCR Master Mix. The reaction conditions and cycling parameters were set according to the instructions. All primers are shown in Table S1.

2.10. Alcian blue staining

The change in colonic mucus in paraffin sections was determined by the alcian blue staining kit (Beyotime, Shanghai, China), as directed by the instructions.

2.11. Flow cytometry analysis

On the 29th day, cells were isolated from the colonic lamina propria and subjected to different treatments according to previous reports [11]. Briefly, the small pieces of colons were incubated in phosphate buffer saline (PBS) containing 1% double antibody (penicillin/streptomycin), 1 mmol/L dithiothreitol (DTT), and 1 mmol/L ethylene diamine tetraacetic acid (EDTA) two times at 37 °C for 10 min under shaking. Then, the pieces were digested with 1 mg/mL collagenase IV at 37 °C for 1 h under shaking. Subsequently, the suspensions were collected after 100 μm and 70 μm filtration. After that, the lamina propria lymphocytes were separated by the concentration difference between 80% Percoll and 40% Percoll at 800 *g*, 20 min, 20 °C (speed up 4, speed down 1). The lamina propria lymphocytes were blocked with CD16/32 antibody for 5 min and stained with antibodies (surface markers such as lineage, CD45, Nkp46, DR3) for 30 min on ice. Finally, we used the Foxp3/Transcription factor staining buffer kit (Thermo Fisher Scientific, Waltham, MA, USA) for cell fixation and permeability, and staining (permeabilization staining markers such as retinoic acid receptor-related orphan receptor gamma t (RORγt) and IL-22) followed by flow cytometry analysis (BD LSRFortessa, Franklin Lakes, NJ, USA).

2.12. Cell culture, cytotoxicity assessment and treatments

Caco-2 cells and MNK-3 cells were cultured according to previous reports [11]. To assess the toxic effect of paeoniflorin on cells, cell counting kit-8 (CCK-8) assays were conducted. MNK-3 cells were grouped into 6 different groups including paeoniflorin (25, 50, 100 μM) groups, a recombinant mouse TL1A (rmTL1A) group, a control group, and an anti-TL1A antibody group. 6 h later, 100 ng/mL rmTL1A was added to MNK-3 cells and incubated for 18 h.

2.13. Stable DR3-overexpressing cell line and treatments

In this experiment, we plated 8000 MNK-3 cells in 48-well plates and cultured them for 24 h. Afterward, we transfected the cells with different titers (MOI: 10, 40, 70, 100) of green fluorescent lentivirus labeled with green fluorescent protein (GFP) and two auxiliary transfection reagents (Histrans G A and Histrans G P) for 12 h. After cultured for 72 h in Dulbecco's modified Eagle medium (DMEM), the cells were observed with both a fluorescence microscope (OLYMPUS BX53, Olympus, Tokyo, Japan) and flow cytometry (BD Accuri C5, BD Biosciences, Franklin Lakes, NJ, USA). Next, we used puromycin for purification, eliminating any remaining normal MNK-3 cells. DR3-overexpressing MNK-3 cells (DR3-MNK-3) were identified by flow cytometry and WB. DR3-MNK-3 cells were grouped into 2 groups (paeoniflorin (100 μM) and control groups) and cultured for 24 h. Finally, DR3 pathway proteins were detected by WB.

2.14. Coculture of paeoniflorin-conditioned media (CM) and Caco-2 cells

The supernatants of MNK-3 cells from the above six groups were added to Caco-2 cells and incubated for 24 h. Similarly, Caco-2 cells were cultured with normal control (NC) -MNK-3 and DR3-MNK-3 cell supernatants for 24 h. Next, WB was performed to detect TJ proteins in Caco-2 cells.

2.15. Molecular docking

Paeoniflorin and DR3 protein (PDB ID: 5YGS) were molecularly docked using AutoDock Vina software. To visualize the docking results, PyMOL 2.1 software was utilized. We analyzed and scored the interaction between paeoniflorin and DR3.

2.16. Immunohistochemistry (IHC) and Immunofluorescence (IF) staining

The expression of DR3 protein in the colonic paraffin profile was determined using an IHC kit provided by Bioss (Beijing, China). The colon paraffin sections were deparaffinized and antigenically repaired with citric acid (3 M, pH = 6.0) solution for 8 min, followed by natural cooling to room temperature. After three washes with PBS, the sections were blocked with 10% goat serum for 30 min at room temperature. Subsequently, the sections were incubated with primary antibodies (ZO-1, Occludin, and Claudin-1) overnight at 4°C in a humidified chamber. The following day, Alexa Fluor 488-labeled goat anti-rabbit and cy3-labeled anti-mouse fluorescent secondary antibodies were applied and incubated for 1 h at room temperature. Following three washes with PBS, the slides were sealed with an anti-fluorescence quencher containing 4',6-diamidino-2-phenylindole (DAPI) and imaged under a fluorescence microscope. In addition, MNK-3 cells were fixed by 4% paraformaldehyde and subjected to IF staining for NF-κB p65 and DR3 using the same method.

2.17. Cellular thermal shift assay (CETSA)

MNK-3 cells (1×10^6 cells/mL) were cultured with DMEM in a 10 cm dish and treated with or without paeoniflorin (100 μM) for 4 h. Next, MNK-3 cells were lysed using RIPA buffer containing 1% protease inhibitor cocktail. The lysate was split into 6 tubes and heated at 47, 52, 57, 62, and 67 °C for 3 min. After 3 cycles of freeze–thawing in liquid nitrogen for 1 min each time, the lysate was centrifuged (4 °C, 12,000 rpm) for 20 min, and the supernatant was detected by WB.

2.18. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 software. An unpaired Student's *t*-test was employed to compare two groups, while one-way analysis of variance (ANOVA) was used to compare multiple groups followed by Dunnett's post hoc test. The mean ± standard deviation (SD) values are presented as the results, and statistical significance was considered at $P < 0.05$.

3. Results

3.1. Paeoniflorin alleviated DSS-induced chronic colitis in mice

DSS-induced chronic colitis is a classic model for studying ulcerative colitis, and we first assessed the efficacy of paeoniflorin in chronic colitis. Anti-TL1A antibody, used for clinical treatment of UC, could neutralize the stimulatory molecule TL1A and avoid activating the DR3 signal [13]. Thus, we chose the anti-TL1A antibody as the positive control and IgG as the isotype control of the anti-TL1A antibody. As shown in Fig. 1B, paeoniflorin and the anti-TL1A antibody improved weight loss in mice with chronic colitis. The disease activity index (DAI) score, an authoritative indicator reflecting colitis severity [14], decreased significantly after treatment with paeoniflorin and the anti-TL1A antibody (Fig. 1C). The IgG and model groups showed noticeable redness and swelling in the colon, and its length was significantly shortened, which was

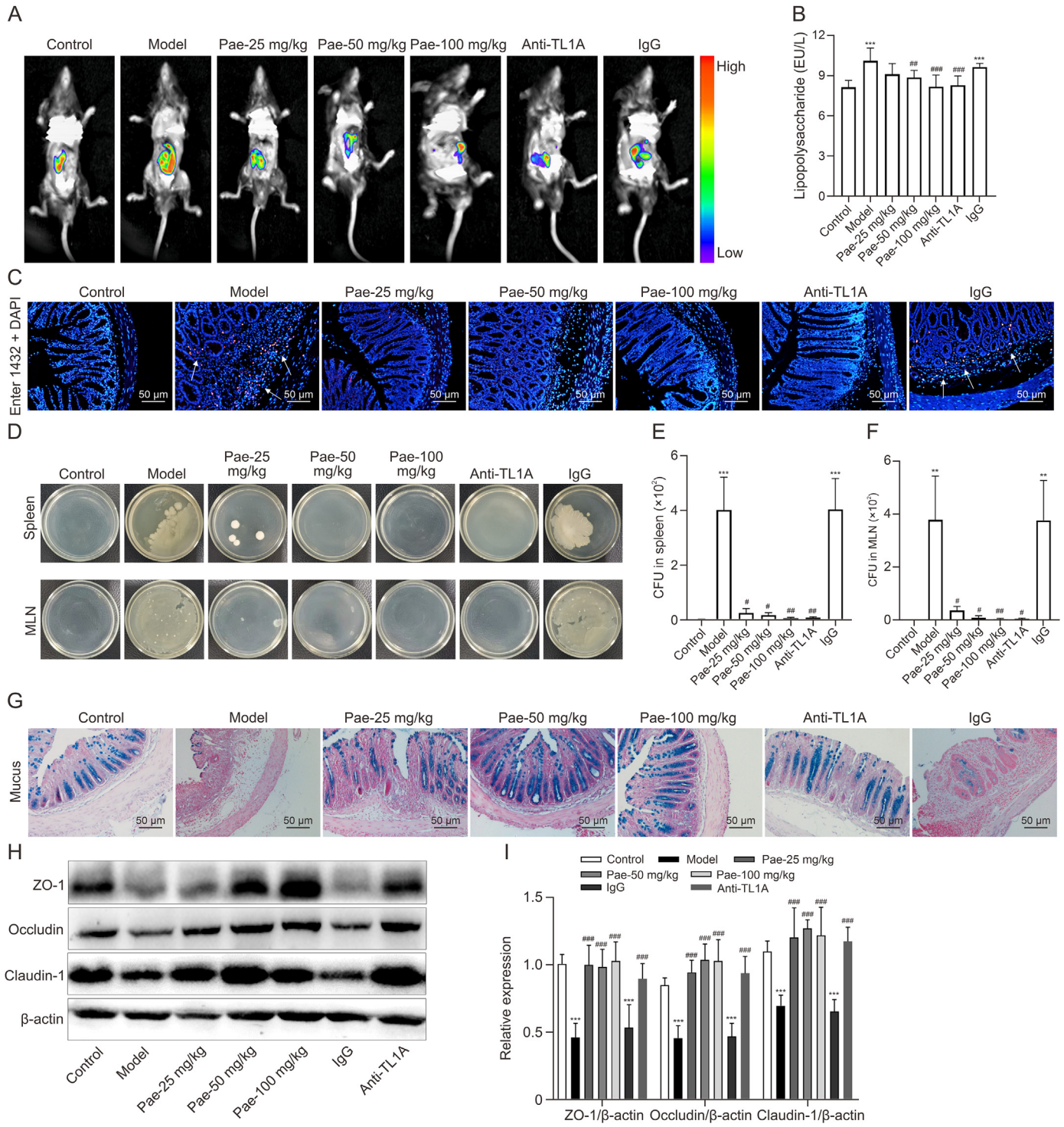

Fig. 2. Paeoniflorin restored intestinal barrier function in mice with chronic colitis. (A) *In vivo* imaging of FITC-Dextran. (B) Changes in serum lipopolysaccharide (LPS) content in different groups. (C) Bacterial infiltration (red) of colon mucosa in different groups. (D) Bacterial translocation of the spleen and mesenteric lymph node (MLN) in different groups. (E, F) Changes in CFU in the spleen (E) and MLN (F) in different groups. (G) Mucin (alcian blue). (H) Representative Western blot images of Claudin-1, Occludin, and ZO-1. (I) Changes in colon Claudin-1, Occludin, and ZO-1 proteins in different groups. $n = 4–6$ per group. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. model group. CFU: colony forming unit; FITC: fluorescein isothiocyanate; Pae: paeoniflorin; anti-TL1A: anti-tumor necrosis factor-like ligand 1A.

reversed by paeoniflorin and anti-TL1A antibody (Figs. 1D and E). Moreover, HE staining revealed the disappearance of colonic glands, disordered intestinal epithelial cells, and severe inflammatory cell infiltration in the model group (Figs. 1F and G). The proportion of white blood cells in peripheral blood was also significantly increased (Fig. 1H). Additionally, these symptoms were mitigated by treatment with paeoniflorin and anti-TL1A antibody. In addition, paeoniflorin and the anti-TL1A antibody restored the decreased red blood cells number and hemoglobin content caused by hematochezia in mice (Figs. 1I and J). The above results all

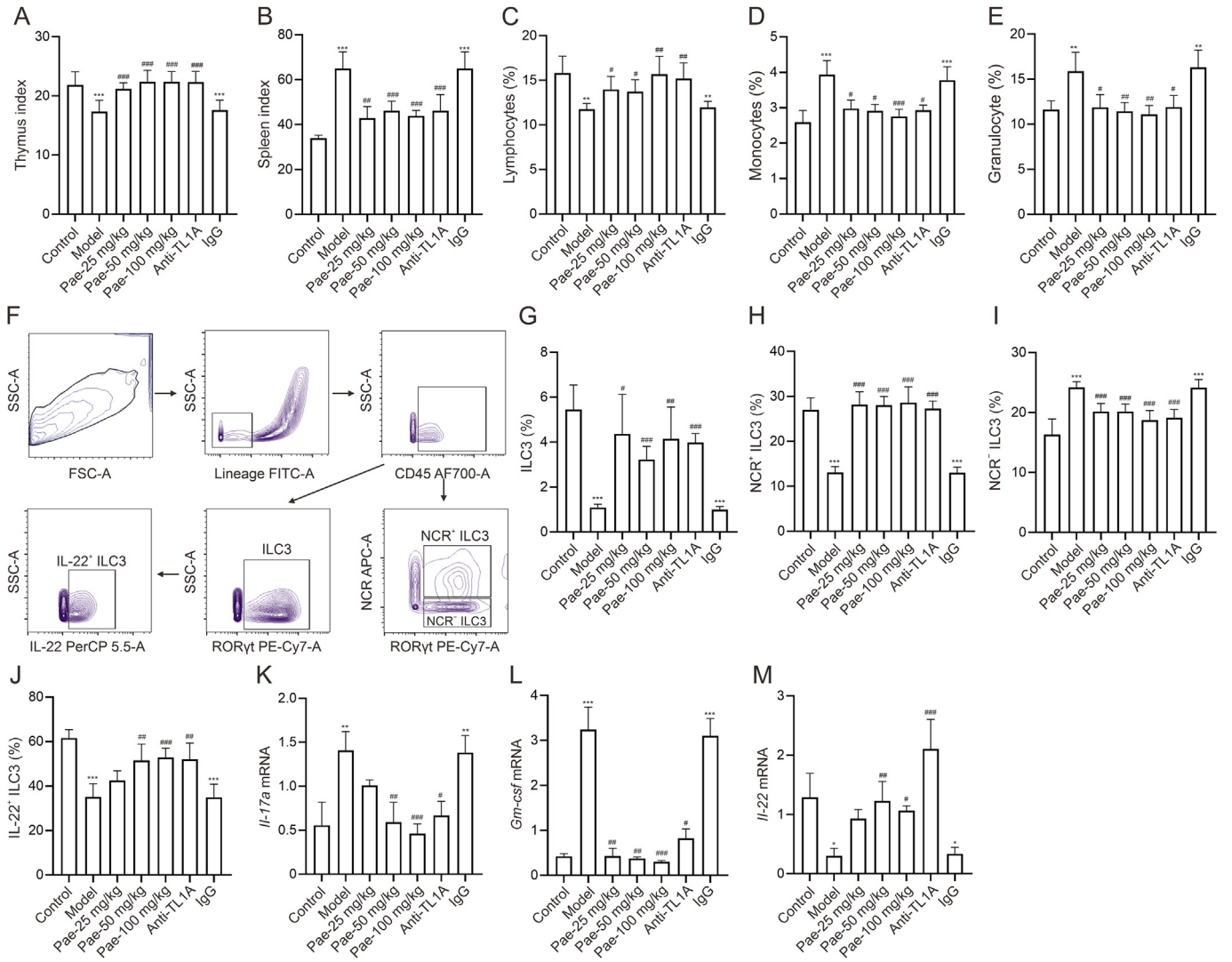

Fig. 3. Paeoniflorin restored immune function and regulated group 3 innate lymphoid cells (ILC3) function. (A, B) Changes in the thymus index (A) and spleen index (B) in different groups. (C) Changes in lymphocytes, (D) monocytes , and (E) granulocytes in peripheral blood. (F) Gating strategy of flow cytometry. (G–J) Changes in the proportion of ILC3s (G), natural cytotoxic receptor NCR)$^{+}$ ILC3s (H), NCR^{-} ILC3 (I), and interleukin (IL)-22^{+} ILC3s (J) in the colon of each group. (K–M) Changes in interleukin-17a (*Il-17a*) (K), granulocyte-macrophage colony stimulating factor (*Gm-csf*) (L), and *Il-22* (M) mRNA expression in the colon of each group. n = 5–6 per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. model group. Pae: paeoniflorin; anti-TL1A: anti-tumor necrosis factor-like ligand 1A.

indicated that paeoniflorin has an ameliorative effect on chronic colitis, and the anti-TL1A antibody also alleviated the inflammatory symptoms. There was no significant difference between the IgG and model groups, revealing that IgG does not affect the efficacy of the anti-TL1A antibody.

3.2. Paeoniflorin restored barrier function in chronic colitis mice

Relapse in UC patients is usually accompanied by an increase in intestinal permeability [15]. Small animal imager results showed that the fluorescence distribution in the paeoniflorin and anti-TL1A antibody groups was concentrated, suggesting that paeoniflorin and anti-TL1A antibody reduced intestinal permeability (Fig. 2A). Meanwhile, DSS treatment caused a marked increase in serum LPS, a sensitive marker of intestinal permeability in mice [16], which could also be reversed by paeoniflorin and anti-TL1A antibody (Fig. 2B). In addition, increased intestinal permeability results in bacterial invasion of the intestinal epithelium and has even been detected in the MLN and spleen of UC patients. Consistent with previous studies [12], opportunistic pathogens were also detected in colitis mouse colonic mucosa, MLNs, and spleens (Figs. 2C–F). In contrast, bacterial infiltration and translocation were reversed after treatment with paeoniflorin and the anti-TL1A antibody. Additionally, the reduction in intestinal mucin and the destruction of TJs are essential factors that increase intestinal permeability [17]. In contrast with the IgG and model groups, the colonic blue area in the groups of anti-TL1A antibody and different doses of paeoniflorin were significantly increased, indicating that the anti-TL1A antibody and paeoniflorin promote the secretion of intestinal mucin (Fig. 2G). This observation may be strongly related to the promotion of goblet cell proliferation. Additionally, paeoniflorin dose-dependently restored the expression of colon TJ proteins (Figs. 2H and I). Similarly, after treatment with the anti-TL1A antibody, the expression of colon TJ proteins showed a significant increase in comparison with the IgG group and the model group (Figs. 2H and I), which indicated that avoiding the activation of DR3 signaling

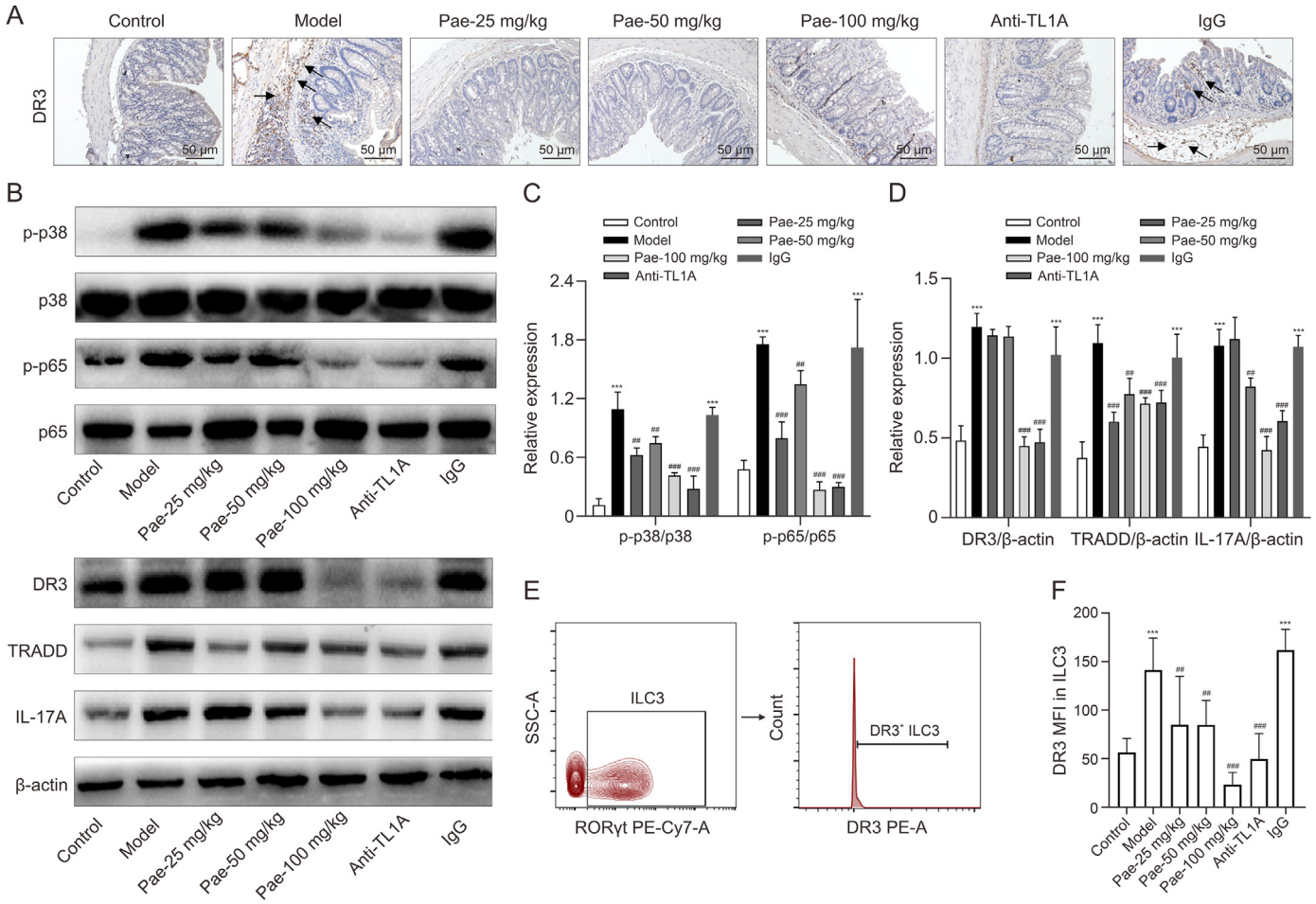

Fig. 4. Paeoniflorin inhibited the death receptor 3 (DR3) signaling pathway in chronic colitis mice. (A) Representative immunohistochemical staining images of DR3 in the colon. (B) Representative Western blot images of DR3 signaling pathway proteins. (C) Changes in the phosphorylation levels of p38 and p65 proteins in different groups. (D) Changes in colon DR3, tumor necrosis factor receptor-associated death domain protein (TRADD), and interleukin (IL)-17A proteins in different groups. (E) Gating strategy of flow cytometry. (F) Mean fluorescence intensity (MFI) of DR3 in group 3 innate lymphoid cells (ILC3s). $n = 4–6$ per group. $^{***}P < 0.001$ vs. control group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. model group. Pae: paeoniflorin; anti-TL1A: anti-tumor necrosis factor-like ligand 1A; p-p38: phospho-p38; p-p65: phospho-p65.

could effectively enhance the renovate of intestinal mucosa. These data also reflect that paeoniflorin was able to restore intestinal integrity and barrier function in mice with chronic colitis.

3.3. Paeoniflorin regulated ILC3 function in chronic colitis mice

A chronic abnormal immune response in the gut is one of the main causes of intestinal barrier damage [18]. From this, we assessed whether paeoniflorin and anti-TL1A antibody could modulate immune function in mice. Paeoniflorin and anti-TL1A prevented spleen enlargement and thymus atrophy in chronic colitis mice, reducing the Mon and Gran percentages in peripheral blood while restoring the lymph proportion (Figs. 3A–E). These results provide preliminary evidence that paeoniflorin and anti-TL1A antibody could regulate the immune function of mice. ILC3, a crucial cell regulating intestinal mucosal immunity, has been vividly compared by scholars as a “bridge” between adaptive immunity and innate immunity. Therefore, the effects of paeoniflorin and the anti-TL1A antibody on ILC3 function and its effector were examined. Flow cytometry and qRT–PCR results confirmed that both could restore the percentage of ILC3s, IL-22^{+} ILC3s and NCR^{+} ILC3s while reducing the percentage of NCR^{-} ILC3s (Figs. 3F–J). In addition, they also controlled the mRNA levels of *Il-22, Il-17a* and *Gm-csf* to normal ranges (Figs. 3K–M). These data suggest that paeoniflorin and the anti-TL1A antibody regulate the function of ILC3s.

3.4. Paeoniflorin inhibited DR3 signaling in chronic colitis mice

To clarify whether the regulation of ILC3 function by paeoniflorin is related to the inhibition of DR3 signaling, we investigated the expression of proteins involved in this pathway in the colon of mice. The immunohistochemistry results showed that paeoniflorin could inhibit the expression of DR3 protein in the colon (Fig. 4A). As shown in Figs. 4B–D, the DR3 protein and its downstream pathway proteins TRADD and IL-17A were significantly reduced in each dose group after paeoniflorin treatment. Similarly, significant reductions were observed in the phosphorylation of p38 and p65 proteins. Moreover, flow cytometry results displayed that the mean fluorescence intensity of DR3 protein on colon ILC3 cells was considerably lower in the paeoniflorin group than that in the model group (Figs. 4E and F, $P < 0.01$ or $P < 0.001$), indicating that paeoniflorin inhibited the DR3 signaling pathway on colonic ILC3 cells in chronic colitis mice. The anti-TL1A antibody had the same effect as paeoniflorin, which also demonstrated that the positive effect of paeoniflorin on barrier function was achieved by modulating ILC3s through the DR3 signaling pathway.

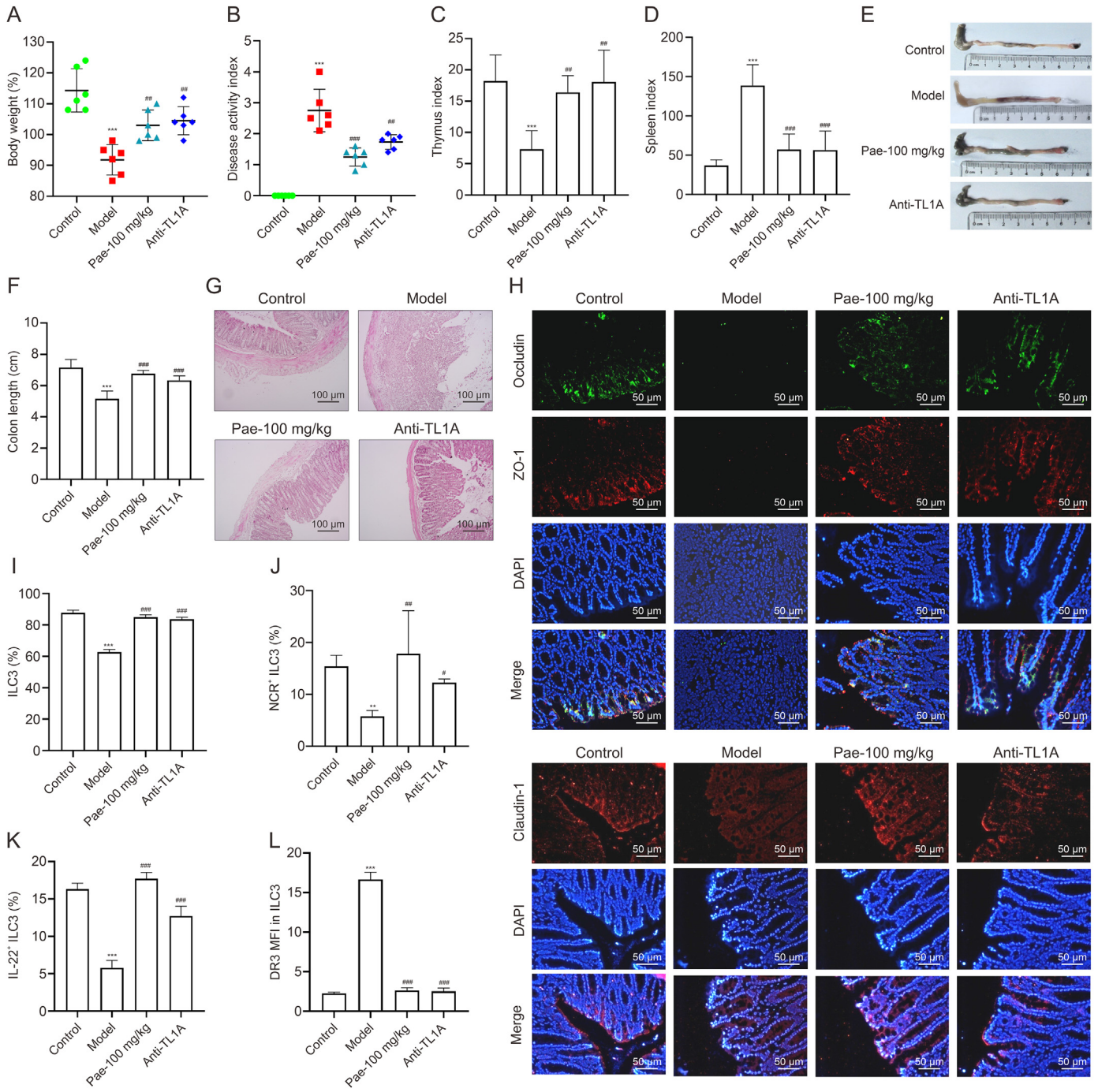

Fig. 5. Paeoniflorin protected the intestinal barrier through the death receptor 3 (DR3)/group 3 innate lymphoid cells (ILC3s) pathway (Rag1$^{-/-}$ mice). (A–F) Changes in body weight (A), disease activity index score (B), thymus index (C), spleen index (D), and colon length (E, F) in different groups on day 29. (G) Representative hematoxylin-eosin (HE) staining images of colon tissue. (H) Representative immunofluorescence images of ZO-1, Occludin, and Claudin-1. (I–K) Changes in the proportion of ILC3s (I), natural cytotoxic receptor (NCR)$^+$ ILC3s (J), and interleukin (IL)-22$^+$ ILC3s (K) in the colon of each group. (L) Mean fluorescence intensity (MFI) of DR3 in ILC3s (flow cytometry). $n = 4–6$ per group. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. model group. Pae: paeoniflorin; anti-TL1A: anti-tumor necrosis factor-like ligand 1A; DAPI: 4',6-diamidino-2-phenylindole.

3.5. Paeoniflorin protected the intestinal barrier through the DR3/ILC3 pathway

DR3 is expressed not only on ILC3s but also on T and B cells [19]. To clarify whether the repair effect of paeoniflorin on the intestinal barrier is exerted by affecting ILC3s rather than relying on the adaptive immune system, Rag1$^{-/-}$ mice (deficient in T and B lymphocytes) were used for additional verification. The results revealed that paeoniflorin improved the extraintestinal manifestations and inflammatory symptoms of chronic colitis (Figs. 5A–G) and restored the expression of TJ proteins (Fig. 5H). Meanwhile, the anti-TL1A antibody had the same effect as paeoniflorin, indicating that paeoniflorin played a role in repairing the intestinal mucosa of chronic colitis by affecting other immune cells other than T and B lymphocytes. Based on our previous findings, we carried out further investigations using flow cytometry to examine the effects

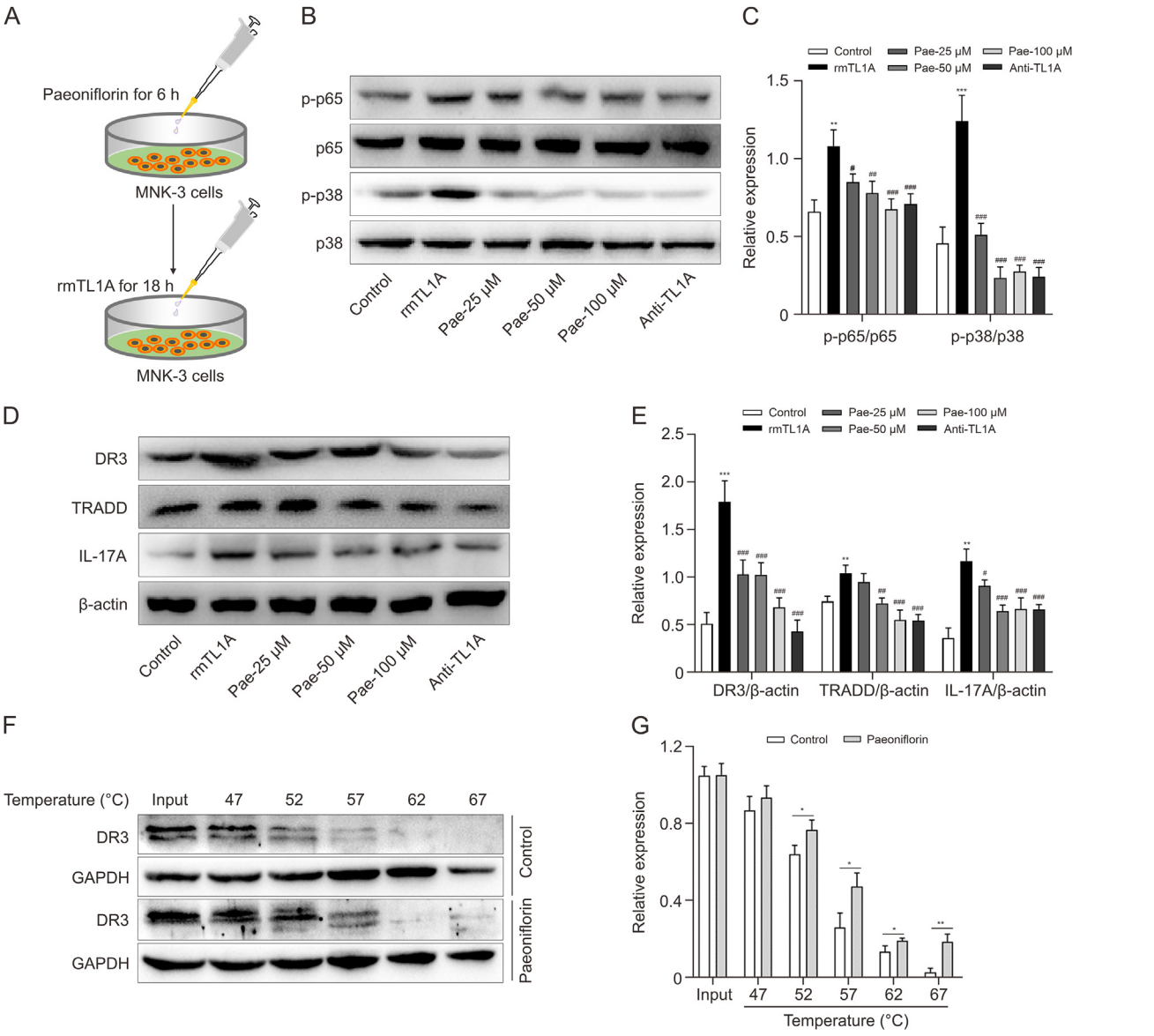

Fig. 6. Paeoniflorin inhibited the death receptor 3 (DR3) signaling pathway in MNK-3 cells. (A) Experimental design (MNK-3 cells). (B, C) Changes in the phosphorylation levels of p38 and p65 proteins in different groups. (D, E) Changes in colon DR3, tumor necrosis factor receptor-associated death domain protein (TRADD), and interleukin (IL)-17A protein expression in different groups. (F) Representative Western blot images of DR3 proteins. (G) Changes in colon DR3 protein expression in different groups. $n = 3$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. rmTL1A group. Pae: paeoniflorin; anti-TL1A: anti-tumor necrosis factor-like ligand 1A ; p-p38: phospho-p38; p-p65: phospho-p65; rmTL1A: recombinant mouse TL1A; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

of paeoniflorin on ILC3s. Our results revealed that paeoniflorin effectively restored the ratio of ILC3s, IL-22^{+} ILC3s, and NCR^{+} ILC3s while also reducing the protein expression of DR3 in ILC3s (Figs. 5I–L). After administration of the anti-TL1A antibody, the above symptoms were also significantly restored, suggesting that blocking the combination of TL1A and DR3 may restore ILC3 function. It has also been shown that the positive effect of paeoniflorin on ILC3 function is related to the inhibition of DR3 signaling.

3.6. Paeoniflorin inhibited the DR3 signaling pathway in MNK-3 cells

Based on the animal experimental results mentioned above, we employed recombinant factor TL1A to stimulate MNK-3 cells, which is an effective cell line for studying ILC3s [20]. By doing so, we set up an *in vitro* model to investigate the effect of paeoniflorin on the DR3 signaling pathway. First, CCK-8 was used to screen the nontoxic dose of paeoniflorin in MNK-3 cells, and the results showed that paeoniflorin below 100 μM did not inhibit the proliferation of MNK-3 cells (Fig. S1). Subsequently, we found that nontoxic doses (25, 50, and 100 μM) of paeoniflorin significantly inhibited the expression of the DR3 downstream signaling pathway proteins TRADD and IL-17A and phosphorylation of the p65 and p38 proteins in MNK-3 cells, as well as the anti-TL1A antibody (Figs. 6A–E). In addition, the molecular docking model (Fig. S2) predicted that paeoniflorin binds to DR3 in three binding types, including hydrophobic interactions (PHE A: 157, TRP A: 63, TRP A: 231, and PRO A: 155), π-stacking (TRP A: 341) and hydrogen bonds (GlU A: 157, GLU A: 45, ASN A: 13, GLU A: 112 and LYS A: 16). The binding energy of paeoniflorin is −10.1 kcal/mol (Table S2). Meanwhile, the experimental results of CETSA, a method that can directly measure how efficiently a drug binds to its target [21], revealed that MNK-3 cells incubated with paeoniflorin exhibited a higher expression of DR3 protein compared to the control group, as evidenced by the

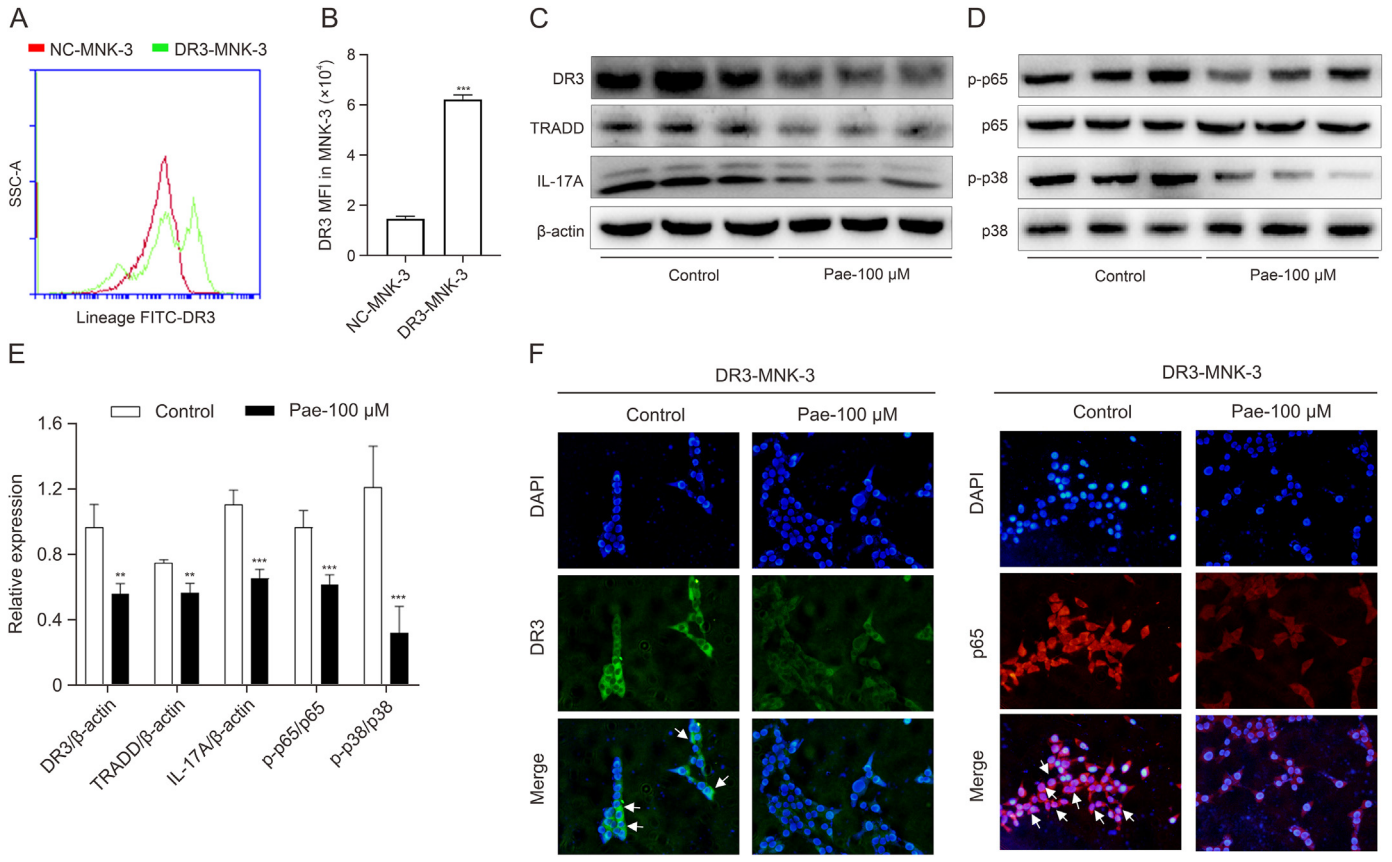

Fig. 7. Paeoniflorin inhibited the death receptor 3 (DR3) signaling pathway in DR3-overexpressing MNK-3 cells (DR3-MNK-3). (A) DR3 in normal control (NC)-MNK-3 or DR3-MNK-3 cells (flow cytometry analysis). (B) Mean fluorescence intensity (MFI) of DR3. (C) Representative Western blot images of tumor necrosis factor receptor-associated death domain protein (TRADD), DR3, and interleukin (IL)-17A proteins in DR3-MNK-3 cells. (D) Representative Western blot images of phospho-p65 (p-p65), p65, phospho-p38 (p-p38), and p38 in DR3-MNK-3 cells. (E) Changes in colon DR3, TRADD, and IL-17A protein expression, and the phosphorylation levels of p38 and p65 proteins in different groups. (F) Expression of DR3 (left) in DR3-MNK-3 and expression of p65 in the nucleus of DR3-MNK-3. $n = 3$ per group. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group. Pae: paeoniflorin; DAPI: 4',6-diamidino-2-phenylindole.

observed increase in temperature (Figs. 6F and G). These findings also confirmed the results obtained through molecular docking.

To further elucidate whether paeoniflorin could inhibit the DR3 signaling pathway, we constructed a DR3-overexpressing MNK-3 cell line (DR3-MNK-3). In the present study, we utilized the efficient introduction of the DR3-overexpressing virus into cells via lentiviral transfection. Additionally, after optimizing the transfection conditions and purifying the cells with puromycin, the transfection efficiency exceeded 93% Fig. S3A and B(Figs. S3A and B). Subsequently, WB determined that the expression of the DR3 downstream pathway proteins TRADD, IL-17A, and p-p38 was significantly increased (Fig. S3C). Additionally, the mean fluorescence intensity of DR3 in MNK-3 cells was enhanced, indicating that the stably transfected DR3-MNK-3 was successfully constructed (Figs. 7A and B). Nevertheless, paeoniflorin inhibited the elevated expression of TRADD, p-p38, p-p65, and IL-17A in DR3-MNK-3 cells (Figs. 7C–E). The IF results also demonstrated that the fluorescence intensity of DR3 protein and the nuclear translocation of p65 were significantly weakened after treatment with the paeoniflorin (Fig. 7F). These data demonstrated that paeoniflorin could inhibit the DR3 signaling pathway in MNK-3 cells.

3.7. Paeoniflorin-CM restored the expression of TJs in Caco-2 cells

To set up a coculture system of CM with Caco-2 cells, we treated with Caco-2 cells using two cell supernatants separately. Compared with normal MNK-3 cells, the supernatant from DR3-MNK-3 significantly reduced the expression of Occludin and Claudin-1, which indirectly indicated that activating the DR3 signaling pathway in ILC3s resulted in the disruption of the intestinal barrier (Figs. 8A–D, $P < 0.05$).

To further elucidate whether paeoniflorin has a beneficial effect on the intestinal barrier by regulating the DR3 signaling pathway on ILC3s directly, we consolidated a coculture system including Caco-2 cells and paeoniflorin-conditioned medium (CM) (Fig. 8E). First, we employed the CCK-8 assay to determine the nontoxic dose of paeoniflorin for Caco-2 cells. Consistent with previous findings, paeoniflorin showed no inhibitory effect on the proliferation of Caco-2 cells at concentrations below 100 μM (Fig. S4). Therefore, a coculture system was established with nontoxic doses (25, 50, and 100 μM) of paeoniflorin. The expression levels of TJ in Caco-2 cells were significantly higher after incubation with paeoniflorin-CM and anti-TL1A-CM than after incubation with rmTL1A-CM ($P < 0.001$, Figs. 8F and G). The findings suggest that paeoniflorin was able to regulate the secretion of inflammatory mediators by inhibiting the DR3 pathway, which in turn improved TJ proteins in Caco-2 cells.

4. Discussion

More recently, increasing evidence has focused on the crucial role of the intestinal barrier in the pathogenesis of various diseases. The findings suggest that there is a connection between

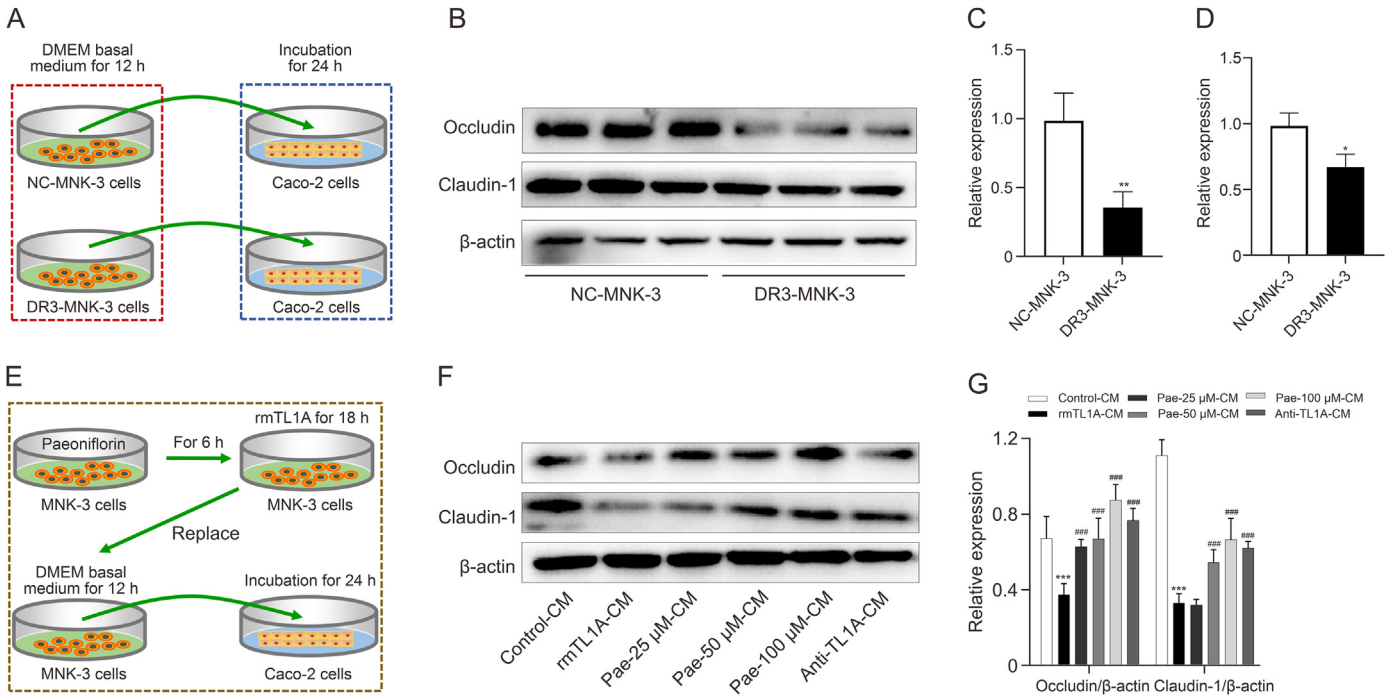

Fig. 8. Paeoniflorin-conditioned medium restored the expression of tight junctions (TJs) in Caco-2 cells. (A) Experimental design. (B) Representative Western blot images of Claudin-1 and Occludin. (C, D) Changes in Occludin (C) and Claudin-1 (D) proteins in different groups. (E) Experimental design (preparation method of paeoniflorin (Pae)-conditioned medium (CM)). (F) Representative Western blot images of Occludin and Claudin-1. (G) Changes in the levels of Occludin and Claudin-1 proteins in different groups. n = 3 per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. rmTL1A-CM group. DMEM: Dulbecco's modified Eagle's medium; NC: normal control; Pae: paeoniflorin; DR3: death receptor 3; rmTL1A: recombinant mouse TL1A.

autoimmune diseases, such as UC, Crohn's disease, and other systemic diseases, and dysfunction of the intestinal barrier [22,23]. However, it has not been conclusively established whether intestinal mucosal injury is, in fact, an initiating or secondary factor. Some studies have found that drug treatment when the intestinal mucosa is intact is more conducive to improving UC [24]. The clinical goal of treating UC has shifted to repairing the damaged intestinal mucosal, indicating that repairing the intestinal mucosal is an essential therapeutic strategy for UC. Additionally, IL-22, secreted by ILC3s, is a major effector in repairing intestinal mucosal damage. Therefore, our study uses mucosal repair as an entry point for the treatment of intestinal luminal antigen-driven diseases and explores the immune mechanisms of paeoniflorin in the treatment of UC based on the ILC3s.

Under normal physiological conditions, the integrated intestinal epithelium paly a dominant role in preventing excessive immune activation in response to intestinal luminal antigens, thereby maintaining intestinal immune homeostasis. When intestinal barrier function is impaired, pathogenic antigens penetrate the intestinal mucosa and disrupt the delicate balance of the intestinal immune system. This disruption leads to chronic and abnormal immune responses, ultimately contributing to the course of UC [25]. At the same time, the increased intestinal permeability creates conditions for bacterial invasion from the lumen into the lamina propria, followed by emerging pathogen-associated molecular patterns (PAMPs) such as LPS in the serum. It even transfers to the secondary lymphoid organs through blood circulation [12], which was also confirmed in DSS-induced chronic colitis mice. Moreover, after administering FITC-Dextran, the fluorescence was scattered in the chronic colitis mice, which further proved that intestinal permeability increased [26]. In this study, paeoniflorin was found to have a significant effect on enhancing intestinal barrier function. In addition, the reduction or deletion of mucin and intestinal epithelial TJ proteins is the main reason for the increased intestinal permeability. Through a number of experiments, we determined that paeoniflorin restored intestinal TJ proteins and mucin, implying that its positive regulatory effect on intestinal barrier function may be achieved through this pathway.

Research in the past 10 years has shown that ILC3s, a type of immune cell enriched in the intestinal mucosa, play a leading role in repairing the intestinal epithelium and maintaining immune balance [27]. Recent studies have used single-cell sequencing to determine the transformation of immune cells in the peripheral blood and colon of patients with UC during active and remission phases, and it has been found that the proportion of ILC3s decreases significantly [28]. Moreover, ILC3-deficient mice developed severe intestinal mucosal damage and induced colitis [29], suggesting that restoring the ratio of intestinal ILC3s could help maintain intestinal barrier function and protect mice from colitis. The regulatory effect of ILC3s on the intestinal immune microenvironment is primarily achieved through the secretion of the cytokines GM-CSF, IL-17A, and IL-22. Considering all of the above, we examined the ratio of ILC3s and their effectors to further study the mechanism of paeoniflorin. As anticipated, paeoniflorin restored ILC3 cell subsets and their effector effectors in colitis mice, indicating that paeoniflorin regulated the function of ILC3s.

It has been found that the DR3 agonist α-DR3 did not exacerbate DSS-induced colitis in the absence of ILC3s, suggesting that activating DR3 signaling on ILC3s plays a key role in triggering inflammatory storms [4]. Next, *in vivo* and *in vitro* experiments were performed to determine whether paeoniflorin exerts this function related to the regulation of DR3 signaling. Interestingly, paeoniflorin inhibited the DR3 signaling pathway, which was further confirmed by changes in the DR3 pathway proteins in ILC3s and

MNK-3 cells, in particular. Moreover, it has been documented that ligand-induced receptor activation can be simulated by overexpression of receptors without ligands [30,31]. We found that paeoniflorin could suppress the high expression of DR3 pathway proteins in DR3-MNK-3 cells, providing further evidence that paeoniflorin modulates ILC3 function through the DR3 signaling pathway. However, we found that paeoniflorin also inhibited the expression of DR3 mRNA in the colon (Fig. S5) and DR3 protein in DR3-MNK-3 cells, which may be due to feedback regulation after the inhibition of the DR3 signaling pathway. The activation of MAPK and NF-κB downstream signals of DR3 can promote the expression of TL1A and form feedback regulation.

Although studies have confirmed that paeoniflorin inhibits the DR3 signaling pathway in ILC3s, it is worth noting that DR3 is also expressed in T and B lymphocytes. The Rag1$^{-/-}$ mouse, a mouse deficient in T and B lymphocytes, has recently gained significant attention as a valuable tool for studying the function of ILC3s [32,33]. In contrast to WT mice, ILC3s are enriched deeply in the gut mucosa of Rag1$^{-/-}$ mice. Experiments have confirmed that the proportion of colonic ILC3s increased from 0.5 % or 1 % to approximately 30 % (data not shown). This also reflects that the presence of T and B lymphocytes has an inhibitory effect on the development and proliferation of ILC3s. In addition, T cells also secrete IL-22 and IL-17A, and using Rag1$^{-/-}$ mice was able to rule out drug-induced repair of intestinal epithelium through T cells. Moreover, the DR3 agonist α-DR3 aggravated DSS-induced colitis independently of the adaptive immune system. Consistent with the results in WT mice, paeoniflorin still inhibited the DR3 pathway in ILC3s and restored intestinal mucosal injury in Rag1$^{-/-}$ mice. Additionally, the anti-TL1A antibody had the same effect as paeoniflorin. All of these findings suggest that blocking the combination of TL1A and DR3 could restore ILC3 function and confirm that the regulatory effect of paeoniflorin on ILC3s is related to the inhibition of DR3 signaling.

Indeed, the relationship between targeting DR3 signaling on ILC3s and intestinal mucosal repair has been confirmed [4]. However, whether paeoniflorin acts directly or indirectly on the intestinal mucosa requires additional validation. We constructed a coincubation system between paeoniflorin-CM and Caco-2 cells, and this method was established in our previous study [34]. To accurately reflect the effect of paeoniflorin in regulating intestinal barrier function through ILC3 activation, we treated MNK-3 cells with paeoniflorin and collected their CM, which was then cocultured with Caco-2 cells. The findings revealed that TL1A-CM reduced the expression of Claudin-1 and Occludin in Caco-2 cells, while paeoniflorin-CM reversed this decrease. It is also implied that the effect of paeoniflorin on the barrier is achieved by preventing the DR3 signaling pathway in ILC3 cells. To verify that activation of DR3 signaling caused intestinal epithelial barrier damage, we conducted additional experiments in which we incubated the supernatant of DR3- MNK-3 cells or NC-MNK-3 cells with Caco-2 cells, showing that DR3-MNK-3 cells reduced the expression of Claudin-1 and Occludin in Caco-2 cells, which also provides supporting evidence for the regulatory effect of paeoniflorin. However, there are still limitations in this study, and the combination of a DR3 agonist and paeoniflorin has not been validated in animal experiments. Although molecular docking and CETSA experiments have been used to demonstrate that paeoniflorin can bind to DR3, the specific binding sites still need to be studied further.

5. Conclusion

In summary, our study demonstrates that the repair effect of paeoniflorin on intestinal mucosal injury depends on ILC3s and that its mechanism is related to the inhibition of the DR3 signaling pathway. This confirms that targeting the DR3 pathway in ILC3s is essential for repairing intestinal mucosal damage in UC. Consequently, this study lays the groundwork for the clinical application of paeoniflorin in UC.

CRediT author statement

Shaowei Huang: Project administration, Data curation, Writing - Original draft preparation, Project administration, and Data curation; **Xueqian Xie**: Formal analysis, Project administration, and Formal analysis; **Bo Xu**: Data curation, and Writing - Original draft preparation; **Zengfeng Pan**: Methodology, and Validation; **Junjie Liang**: Methodology, and Validation; **Meiling Zhang**: Software, and Validation; **Simin Pan**: Investigation; **Xiaojing Wang**: Validation; **Meng zhao**: Formal analysis; **Qing Wang**: Writing - Reviewing and Editing; **Jinyan Chen**: Validation; **Yanyang Li**: Methodology; **Lian Zhou**: Funding acquisition, and Project administration; **Xia Luo**; Writing - Reviewing and Editing, and Project administration.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant No.: 82074092), Natural Science Foundation of Guangdong Province, China (Grant No.: 2021A1515012219), and Guangzhou University of Chinese Medicine “Double First-Class” and High-level University Discipline Collaborative Innovation Team Project, China (Grant No.: 2021xk81), and Graduate Research Innovation Project of Guangzhou University of Chinese Medicine, China. Finally, we appreciate the School of Basic Medicine, Guangzhou University of Chinese Medicine for technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2024.01.008.

References

[1] T. Raine, S. Bonovas, J. Burisch, et al., ECCO guidelines on therapeutics in ulcerative colitis: Medical treatment, J. Crohns Colitis 16 (2022) 2–17.
[2] R.P. Hirten, B.E. Sands, New therapeutics for ulcerative colitis, Annu. Rev. Med. 72 (2021) 199–213.
[3] T. Rath, R. Atreya, J. Bodenschatz, et al., Intestinal barrier healing is superior to endoscopic and histologic remission for predicting major adverse outcomes in inflammatory bowel disease: The prospective ERIca trial, Gastroenterology 164 (2023) 241–255.
[4] J. Li, W. Shi, H. Sun, et al., Activation of DR3 signaling causes loss of ILC3s and exacerbates intestinal inflammation, Nat. Commun. 10 (2019), 3371.
[5] N. Porquet, A. Poirier, F. Houle, et al., Survival advantages conferred to colon cancer cells by E-selectin-induced activation of the PI3K-NFκB survival axis downstream of death receptor-3, BMC Cancer 11 (2011), 285.
[6] J.G. Castellanos, V. Woo, M. Viladomiu, et al., Microbiota-induced TNF-like ligand 1A drives group 3 innate lymphoid cell-mediated barrier protection and intestinal T cell activation during colitis, Immunity 49 (2018) 1077–1089.e5.
[7] L. Chen, H. Qi, D. Jiang, et al., The new use of an ancient remedy: A double-blinded randomized study on the treatment of rheumatoid arthritis, Am. J. Chin. Med. 41 (2013) 263–280.
[8] X. Luo, X. Wang, S. Huang, et al., Paeoniflorin ameliorates experimental colitis by inhibiting gram-positive bacteria-dependent MDP-NOD2 pathway, Int. Immunopharmacol. 90 (2021), 107224.
[9] J. Li, S. Ren, M. Li, et al., Paeoniflorin protects against dextran sulfate sodium (DSS)-induced colitis in mice through inhibition of inflammation and eosinophil infiltration, Int. Immunopharmacol. 97 (2021), 107667.
[10] H.S. Cooper, S.N. Murthy, R.S. Shah, et al., Clinicopathologic study of dextran sulfate sodium experimental murine colitis, Lab. Invest. 69 (1993) 238–249.
[11] S. Huang, X. Wang, X. Xie, et al., Dahuang Mudan Decoction repairs intestinal barrier in chronic colitic mice by regulating the function of ILC3, J. Ethnopharmacol. 299 (2022), 115652.

[12] S. Huang, Y. Fu, B. Xu, et al., Wogonoside alleviates colitis by improving intestinal epithelial barrier function via the MLCK/pMLC2 pathway, Phytomedicine 68 (2020), 153179.
[13] S. Danese, M. Klopocka, E.J. Scherl, et al., Anti-TL1A antibody PF-06480605 safety and efficacy for ulcerative colitis: A phase 2a single-arm study, Clin. Gastroenterol. Hepatol. 19 (2021) 2324–2332.e6.
[14] G. Esposito, E. Capoccia, F. Turco, et al., Palmitoylethanolamide improves colon inflammation through an enteric glia/toll like receptor 4-dependent PPAR-α activation, Gut 63 (2014) 1300–1312.
[15] J. Chang, R.W. Leong, V.C. Wasinger, et al., Impaired intestinal permeability contributes to ongoing bowel symptoms in patients with inflammatory bowel disease and mucosal healing, Gastroenterology 153 (2017) 723–731.e1.
[16] R.F. Loeser, L. Arbeeva, K. Kelley, et al., Association of increased serum lipopolysaccharide, but not microbial dysbiosis, with obesity-related osteoarthritis, Arthritis Rheumatol. 74 (2022) 227–236.
[17] C.H.T. Hall, J.S. Lee, E.M. Murphy, et al., Creatine transporter, reduced in colon tissues from patients with inflammatory bowel diseases, regulates energy balance in intestinal epithelial cells, epithelial integrity, and barrier function, Gastroenterology 159 (2020) 984–998.e1.
[18] Y. Kurashima, H. Kiyono, Mucosal ecological network of epithelium and immune cells for gut homeostasis and tissue healing, Annu. Rev. Immunol. 35 (2017) 119–147.
[19] F. Meylan, A.C. Richard, R.M. Siegel, TL1A and DR3, a TNF family ligand-receptor pair that promotes lymphocyte costimulation, mucosal hyperplasia, and autoimmune inflammation, Immunol. Rev. 244 (2011) 188–196.
[20] D.S.J. Allan, C.L. Kirkham, O.A. Aguilar, et al., An in vitro model of innate lymphoid cell function and differentiation, Mucosal Immunol. 8 (2015) 340–351.
[21] D.M. Molina, R. Jafari, M. Ignatushchenko, et al., Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay, Science 341 (2013) 84–87.
[22] M. Camilleri, K. Madsen, R. Spiller, et al., Intestinal barrier function in health and gastrointestinal disease, Neuro Gastroenterol. Motil. 24 (2012) 503–512.
[23] G. de Palma, M.D. Lynch, J. Lu, et al., Transplantation of fecal microbiota from patients with irritable bowel syndrome alters gut function and behavior in recipient mice, Sci. Transl. Med. 9 (2017), eaaf6397.
[24] E.F. Stange, Improvement of a 'leaky' intestinal barrier, Dig. Dis. 35 (2017) 21–24.
[25] L. Thoo, M. Noti, P. Krebs, Keep calm: The intestinal barrier at the interface of peace and war, Cell Death Dis. 10 (2019), 849.
[26] T. Breugelmans, H. Van Spaendonk, J.G. De Man, et al., In-depth study of transmembrane mucins in association with intestinal barrier dysfunction during the course of T cell transfer and DSS-induced colitis, J. Crohns Colitis 14 (2020) 974–994.
[27] B. Zeng, S. Shi, G. Ashworth, et al., ILC3 function as a double-edged sword in inflammatory bowel diseases, Cell Death Dis. 10 (2019), 315.
[28] V. Mitsialis, S. Wall, P. Liu, et al., Single-cell analyses of colon and blood reveal distinct immune cell signatures of ulcerative colitis and Crohn' disease, Gastroenterology 159 (2020) 591–608.e10.
[29] P. Aparicio-Domingo, M. Romera-Hernandez, J.J. Karrich, et al., Type 3 innate lymphoid cells maintain intestinal epithelial stem cells after tissue damage, J. Exp. Med. 212 (2015) 1783–1791.
[30] W.B. Su, Y.H. Chang, W. Lin, et al., Differential regulation of interleukin-8 gene transcription by death receptor 3 (DR3) and type I TNF receptor (TNFRI), Exp. Cell Res. 312 (2006) 266–277.
[31] D. Wallach, E.E. Varfolomeev, N.L. Malinin, et al., Tumor necrosis factor receptor and Fas signaling mechanisms, Annu. Rev. Immunol. 17 (1999) 331–367.
[32] B. Liu, B. Ye, X. Zhu, et al., An inducible circular RNA circKcnt2 inhibits ILC3 activation to facilitate colitis resolution, Nat. Commun. 11 (2020), 4076.
[33] W. Zhou, G.F. Sonnenberg, Activation and suppression of group 3 innate lymphoid cells in the gut, Trends Immunol. 41 (2020) 721–733.
[34] Y.Y. Li, X.J. Wang, Y.L. Su, et al., Baicalein ameliorates ulcerative colitis by improving intestinal epithelial barrier via AhR/IL-22 pathway in ILC3s, Acta Pharmacol. Sin. 43 (2022) 1495–1507.