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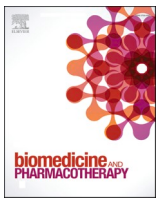

Linderanine C regulates macrophage polarization by inhibiting the MAPK signaling pathway against ulcerative colitis

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ABSTRACT

Ulcerative colitis (UC) is a chronic non-specific inflammatory disease involving the mucosa and submucosa of the rectum and colon. *Lindera aggregate* (Sims) Kosterm is a traditional Chinese herb used for thousands of years in the treatment of gastrointestinal diseases. Previously, we have demonstrated that the extracts of *Lindera aggregate have* good anti-UC effects, but their pharmacodynamic active components have not been fully clarified. Therefore, we explored the therapeutic effect of Linderanine C (LDC), a characteristic component of *Lindera aggregata*, on UC and its mechanism in this study. Firstly, we found that LDC could significantly reduce the disease activity index of UC and improve shortened colon and pathological changes *in vivo*. Colon tissue transcriptomics suggested that the anti-UC effect of LDC might be related to its anti-inflammatory activity. Cellular experiments revealed that LDC could inhibit the expression of the M1 cell marker CD86 in RAW264.7 cells, reduce the production of inflammatory mediators such as IL-6 and TNF-α, and have good anti-inflammatory activity *in vitro*. Cellular transcriptomics reveal the potential involvement of the MAPK signaling pathway in the anti-inflammatory effect of LDC. The co-culture assay confirmed that LDC could significantly reduce inflammation-mediated intestinal epithelial cell injury. In conclusion, LDC was able to inhibit macrophage M1 polarization and reduce inflammatory mediator production by inhibiting the MAPK signaling pathway, effectively improving UC.

1. Introduction

Ulcerative colitis (UC) is a chronic inflammatory disease of the stomach and intestinal tract, with typical clinical symptoms of mucopurulent blood stools and abdominal pain. Epidemiological surveys show that ulcerative colitis used to be regarded as a disease of the western countries, however, with the process of industrialization and lifestyle changes, its incidence has increased significantly worldwide, especially in the new Asian industrial countries[1]. For instance, the highest incidence of IBD in the Asia-Pacific region has been reported at 9.31 per 100,000 persons in India[2]. It severely affects patients' quality of life, and even triggers life-threatening adverse events, such as

Abbreviations: UC, Ulcerative colitis; LDC, Linderanine C; LPS, lipopolysaccharide; DSS, dextran sodium sulfate; NC, normal control; MC, model control; SASP, Sulfasalazine; GPCR, G-coupled protein receptors; DAI, disease activity index; H&E, hematoxylin and eosin; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1β; NO, nitric oxide; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differential expression genes; PAS, periodic acid-schiff; PCA, principal component analysis; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; MFI, mean fluorescence intensity; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; FBS, fetal bovine serum; DMEM, Dulbecco's Modified Eagle Medium; HRP, Horseradish peroxidase; PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide.

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infectious and neoplastic ones[3]. Although the exact pathogenesis of UC has not been fully clarified to date, the mainstream belief is that it is closely related to intestinal immune dysfunction. Macrophages as crucial effector cells of the innate immune system with high plasticity and multifunctionality, which are essential for the maintenance of immune homeostasis in intestinal tissues[4]. During acute enteritis, macrophages play a dominant role in the development of intestinal inflammation[5,6]. The invasion of intestinal microorganisms and metabolites such as lipopolysaccharide (LPS) into the intestinal mucosal lamina propria induces macrophages to polarize towards the M1 pro-inflammatory phenotype, secreting large quantities of inflammatory cytokines and chemokines, such as TNF-α and IL-8, and recruiting a large number of immune cells to infiltrate e the intestinal tissue[7]. Excessive immune response can lead to extensive intestinal epithelial cell damage, which in turn leads to tissue repair imbalance, triggering UC. The current pharmacological treatment of UC mainly includes 5-aminosalicylic acids, glucocorticosteroids, etc[8]. Although these drugs can temporarily control the symptoms, their long-term efficacy is not ideal, and there are many side effects. Numerous studies have shown that natural medicine has good anti-UC pharmacological activity with less toxic side effects, which have greater advantages and potential [9–11].

Ancient Chinese medical book record that *Lindera aggregate* has the effect of warming the kidneys, dispersing cold, activating qi to relieve pain, and is commonly used in the treatment of gastrointestinal diseases [12]. Modern pharmacological studies have found that *Lindera aggregate* has analgesic and antispasmodic pharmacological effects, which can improve symptoms such as abdominal distension, dyspepsia, and regurgitation[13]. Previous studies have found that *Lindera aggregate* is effective in improving symptoms and histopathology in several UC models *in vivo* and inhibiting the production of inflammatory mediators by macrophages *in vitro*, but the specific pharmacological active substances remain unclear[14]. Therefore, we carried out a screening study of the anti-inflammatory active components of *Lindera aggregata* by the LPS-induced RAW264.7 inflammatory model. We found that Linderanine C has a good anti-inflammatory effect, which is one of the anti-inflammatory active components of *Lindera aggregata*. LDC is a characterized sesquiterpene lactone in *Lindera aggregata*, however, few studies of its pharmacological activity have been reported so far. Therefore, we explored the anti-inflammatory and anti-UC pharmacological effects of LDC and its mechanism. Our research could promote its subsequent development and utilization, and further confirm and enrich the pharmacologically active substance of *Lindera aggregata*.

2. Materials and methods

2.1. Reagents

Linderanine C, a standard chemical substances were purchased from Chengdu MUST Bio-Technology (Chengdu, China); Lipopolysaccharide (LPS, Escherichia coli O111:B4) and Sulfasalazine (SASP) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA); Dextran sulfate sodium (DSS, MW 36000–50000) was procured from MP Biomedical (OH, USA); CD25-APC, Foxp3-PE antibodies were purchased from BD Biosciences (CA, United States); Stat3, Phospho-Stat3 (Tyr705), ERK, Phospho-ERK(Thr202/Tyr204), JNK, Phospho-JNK (Tyr1034/1035), p38, Phospho-p38, α-Tubulin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-GAPDH polyclonal antibody was purchased from HUABIO Co. (Hangzhou, China); a Multicolor protein marker was purchased from Absin Bioscience Inc. (Shanghai, China); Other chemical products used were of the analytical grade available.

2.2. Mice

C57BL/6 J mice were obtained from Hangzhou Medical College Experimental Animal Center (Hangzhou, China). Mice were maintained in an environmentally clean barrier system with a temperature of 20–25℃, humidity of 50–60 %, and a light period of 12 hours. In this study, Animals were cared for humanely according to Hangzhou Medical College's institutional animal ethics guidelines (Approval NO. ZJCLA-IACUC-20020145) .

2.3. Disease models and administration of LDC

After one week of isolation and acclimatization, 30 male mice were randomized into 5 groups: normal control (NC), model control (MC), positive control (SASP, 200 mg/kg), LDC low-dose group (LDC-L, 6 mg/kg) and LDC high-dose group (LDC-H, 20 mg/kg). Except the NC group, the other groups will be given 2.5 % DSS prepared in autoclaved water for 9 days. The DSS water solution is renewed every two days. The mice were subjected to continuous gavage of LDC, SASP, or 0.5 % CMC-Na solution for 9 days. The water intake of each group was observed by measuring the residual water volume. All mice were sacrificed on 10th day.

2.4. Body weight, fecal composition, and fecal occult blood

During the experimental period, the general status, body weight, survival status, fecal pattern and occult blood of mice in each group were monitored and recorded daily. The conditions of weight loss were scored as follows: no loss scored 0, 1–5 % scored 1, 5–10 % scored 2, 10–20 % scored 3, > 20 % scored 4. Stool consistency was based on the following scale: normal stool (dark brown, hard oval) was 0, loose stool was 1, watery diarrhea was 2, sticky diarrhea was 3, and severe watery diarrhea was 4. Occult blood in the stool was evaluated on the following scale: absence of blood was 0, presence of blood was 2, and hemorrhage was 4. The disease activity index is obtained by adding the above three scores.

2.5. Colonic morphology, length, and histopathological examination

After the last dose, the mice fasted for 12-hours but drank water normally. The mice were sacrificed after weighting. The entire colon was measured for morphology and length and washed with 0.9 % sodium chloride solution before being stored in 10 % formalin. Periodic Acid-Schiff (PAS), Hematoxylin and Eosin (H&E), and Masson staining were performed and observed under a microscope.

2.6. Transcriptomics study

RAW264.7 cells and colon tissues were sequenced using the platform of Majorbio Biotech (Shanghai, China). The data were analyzed online through the cloud platform. The differential expression genes (DEGs) were identified by up-regulated fold change > 2 or down-regulated fold change < 0.5, p adjust value < 0.05.

2.7. Treg in mesenteric lymph node detection

We removed the mesenteric lymph nodes (MLNs) from the sacrificed mice, and detected the Treg ratio of $CD4^+$T cells by flow cytometry with staining anti-CD4, anti-CD25, and anti-Foxp3 antibody.

2.8. Cell lines and culture

RAW264.7 cells and intestinal epithelial cells MODE-K are respectively cultivated in Dulbecco's modified Eagle's medium (DMEM) and 1640 medium (Gibco). The above media were accompanied by 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin (Gibco). The cells were maintained in a cell incubator with air humidification and 5 % CO_2 at 37 ℃.

2.9. Cell viability

The viability of RAW264.7 and MODE-K cells was determined using the Cell Counting Kit 8 assay (CCK-8). The above two types of cells were separately used in 96 well culture plates at a density of 1×10^5 cells/mL and pretreated with different concentrations of LDC. Then 10 μL of cck-8 was added to each well and incubated for 4 hours. Finally, quantification was performed using a Bio-Tek Synergy HT Multi-Detection Microplate Reader. with detection at 450 nm.

2.10. Macrophage polarization and inflammation medium assay

RAW264.7 cells were inoculated into 24-well culture plates at a density of 5×10^5 cells/mL. The cells were then treated with 1 μg/mL LPS and varying degrees concentrations of LDC for a total of 48 hours. Inflammatory mediators in cell culture supernatants were analyzed by Cytometric Bead Array (BD Bioscience, CA, USA) and total nitric oxide detection kits (Beyotime Bio-tech, Shanghai, China). RAW264.7 cells were collected, washed, and incubated with anti-CD16/32 antibody for 15 min, followed by incubation with anti-CD86 antibody for 30 min at room temperature and protected from light. The cells were also incubated with fluorescent antibodies against IL-6 and TNF-α and stood for 30 minutes at room temperature avoiding light. Finally, detection by flow cytometry.

2.11. Cell co-culture in the transwell co-culture system

MODE-K cells were seeded on the lower chamber of the 24-well transwell co-culture system at a density of 5×10^5 cells/mL, while RAW264.7 cells were seeded in the upper chamber at 5×10^5 cells/mL. Then the cells were stimulated with 1 μg/mL LPS and various concentrations of LDC and incubated for 48 h total. The apoptotic cells of MODE-K and RAW264.7 cells were detected by flow cytometry.

2.12. Real Time quantitative polymerase chain reaction (RT-qPCR)

RAW264.7 cells were pretreated with LDC or solvent and then stimulated with LPS for 12 hours. The colon was quickly removed and washed with a pre-cooled saline solution. Complete RNA was purified from RAW264.7 cells and colons with the help of a column total RNA purification kit (Accurate Biotechnology, Hunan, Chang Sha, China). cDNAs were employed to reverse transcribe the total RNA into cDNA using a SureScript™ First-Strand cDNA synthesis kit. (Accurate Biotechnology, Hunan, Chang Sha, China). The RT-qPCR was done on a 7500 Real-Time PCR System (Applied Biosystems, USA) as per the instructions using BlazeTaq™ SYBR Green qPCR Mix 2.0 reagent (AG11733, Accurate Biotechnology) The Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ algorithm for target genes, and GAPDH was used as a control to calculate relative expression. The PCR primers were synthesized by Ykang Biotech (Hangzhou, China), and the sequences of primers are listed in Supplementary Table 1.

2.13. Western blotting

Cell samples obtained by lysis with RIPA containing protease inhibitors were centrifuged at 13,000 × g (FA-45–24–11, Eppendorf) for 15 min at 4℃. For tissue samples, the colon was rinsed twice with pre-cooled saline. 1 cm length of mouse colon tissue was lysed with 1 mL of RIPA solution and transferred to a pre-cooled homogenizer. The colon tissue lysate was centrifuged at 4 ℃ for 15 min at 13,000 × g. The total protein concentration was evaluated using a BCA protein assay kit. (Thermo-fisher, MA, USA). Then, the samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene fluoride membranes. The membrane was blocked with 3 % BSA for 60 min at room temperature on a shaker with slow shaking, then incubated with primary antibody overnight at 4°C. The membrane was incubated with a secondary antibody in an incubation box for 60 min at room temperature. The membrane was placed in a chemiluminescent imager and the ECL luminescent solution was added uniformly. Western blot quantifications were analyzed by ImageJ software.

2.14. Statistical analyses

Statistical analyses and graphical displays were performed using GraphPad Prism software. All quantitative results were expressed as mean ± standard deviation (SD) and statistical differences were assessed by one-way analysis of variance (ANOVA) followed by post-hoc LSD (Homogeneity) or Games-Howell (Heterogeneity). p-value <0.05 was considered statistically significant.

3. Results

3.1. LDC significantly improves symptoms and colorectal histopathology in UC models

Acute UC model induced by DSS was used to evaluate the anti-UC activity of LDC *in vivo* (Fig. 1A). DSS induction resulted in decreased body weight and increased disease activity index (DAI) in mice, confirming that the UC model was successfully constructed. Compared with the MC group, body weight loss and DAI were ameliorated in the SASP group and the LDC intervention groups (Fig. 1B-D). Histopathological examination showed significant shortening of colon length and oedema of the colonic mucosa in the MC group; H&E staining showed that the epithelial crypts were damaged and the mucosal inflammation was severe in the MC group; PAS staining showed that the mucus secretion function of intestinal epithelial cells was impaired; and the results of Masson staining showed that the degree of fibrosis of tissues was increased in the MC group (Fig. 1G). Compared with the MC group, the LDC intervention groups and the SASP group could significantly alleviate the shortening of colon length and oedema (Fig. 1E and F). There was a significant decrease in inflammatory cell infiltration, crypt damage, and tissue fibrosis. Above all, it was shown that LDC could effectively improve the symptoms and tissue lesions in the UC model mice.

3.2. The transcriptome suggests that the anti-UC effect of LDC is associated with the modulation inflammatory signaling pathway

To explore the potential mechanism of LDC against UC, we performed transcriptomics on colon tissues (Fig. 2A). The differentially expressed genes (DEGs) volcano map showed 313 DEGs (266 genes down-regulated and 47 genes up-regulated) between the LDC group and the MC group (Fig. 2B). Principal component analysis (PCA) showed clear grouping of the NC, MC and LDC groups, indicating that there were significant differences among the three groups. The LDC group was closer to the NC group than the MC group, indicating that LDC improved gene expression in the colon tissue of the UC model mice, had a good anti-UC effect (Fig. 2C). KEGG signaling pathway enrichment analysis showed that the DEGs between the LDC and MC group was highly enriched in inflammation-related pathways such as cytokine-cytokine receptor interactions and IL-17 signaling at the level of Organismal Systems (OS) (Fig. 2D). The results of qPCR, western blot and flow cytometry showed that compared with the MC group, IL-6 and IL-1β mRNA expression and stat3 phosphorylation were significantly reduced in the colonic tissues of the LDC group (Fig. 2E-G). The ratio of Treg cells to $CD4^+$ T cells in mesenteric lymph nodes was significantly reduced in the LDC intervention groups (Fig. 2H and I). The above results suggest that LDC can inhibit the expression of inflammatory mediators such as IL-6 and stat3 phosphorylation, and regulate the IL-17 signaling pathway, which hints LDC may have an anti-inflammatory effect that needs to be studied.

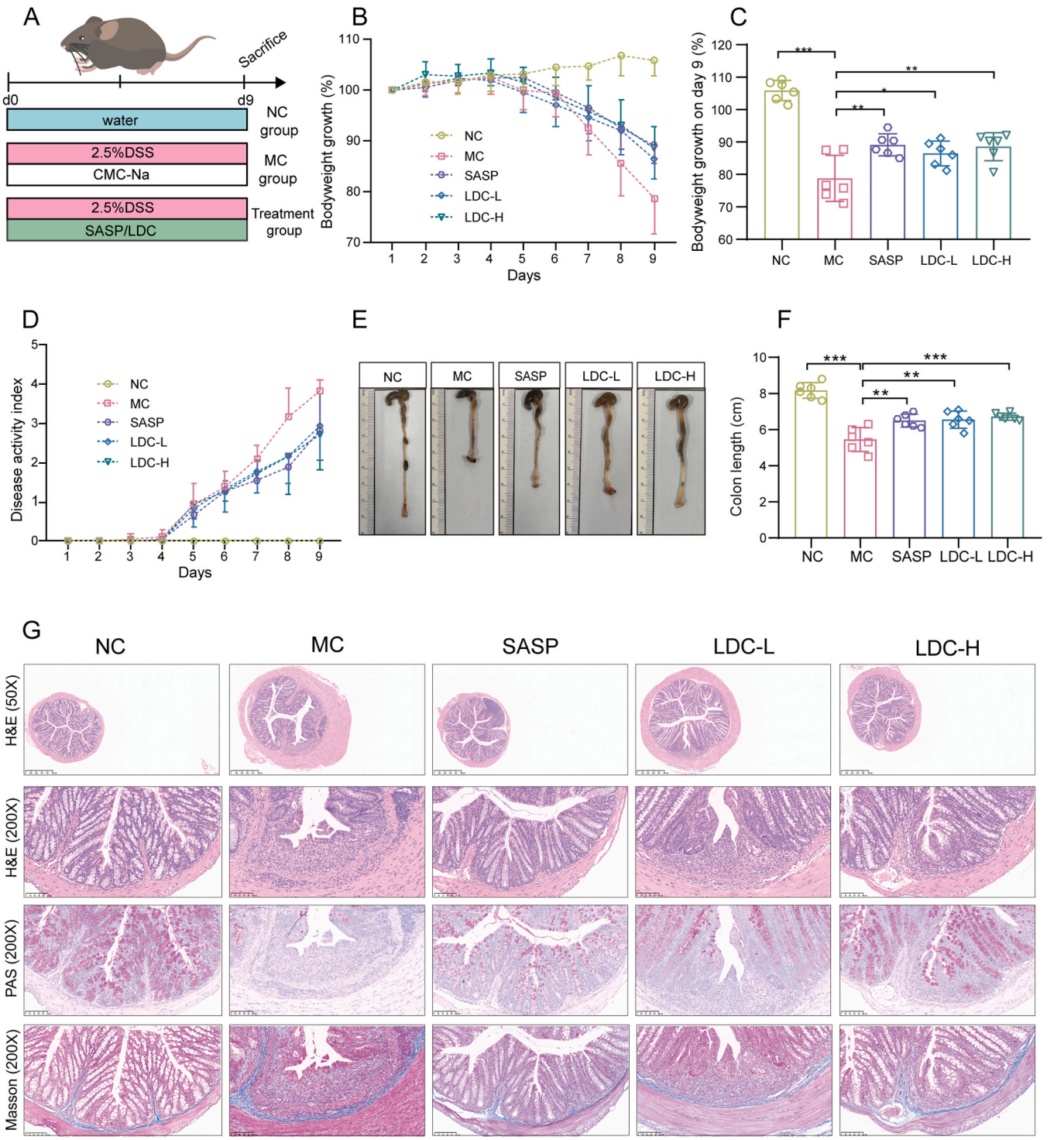

Fig. 1. LDC significantly improves symptoms and colorectal histopathology in UC model. (A) Establishment of murine colitis model and administration of Linderanine C (B) Body weight change. (C) Statistical analysis of body weight. (D) Disease activity index. (E) The gross morphology and length of colon of mice. (F) Statistical analysis of the length of colon of mice. (G) Representative microscopic images of H&E-stained, Masson-stained and Periodic Acid-Schiff stained colon sections.

3.3. LDC inhibits macrophage M1 polarization and inflammatory mediators production

The LPS-induced cellular model was used to assess the anti-inflammatory activity of LDC *in vitro*. Firstly, we mapped the cytotoxicity of LDC on RAW264.7 cells using the CCK-8 assay, and found that LDC (100, 200, and 400 μmol/L) was not significantly cytotoxic (Fig. 3A and B). Subsequently, ELISA assays showed that LPS stimulation increased inflammatory mediators in the supernatant, while LDC treatment significantly reduced the expression of IL-6, TNF-α, and NO (Fig. 3 C-D). Flow cytometry was used to detect macrophage M1 type marker CD86 in RAW264.7 cells. The mean fluorescence intensity (MFI) of CD86 was increased under LPS induction, which was significantly reduced under LDC intervention (Fig. 3G and H). Likewise, qPCR results

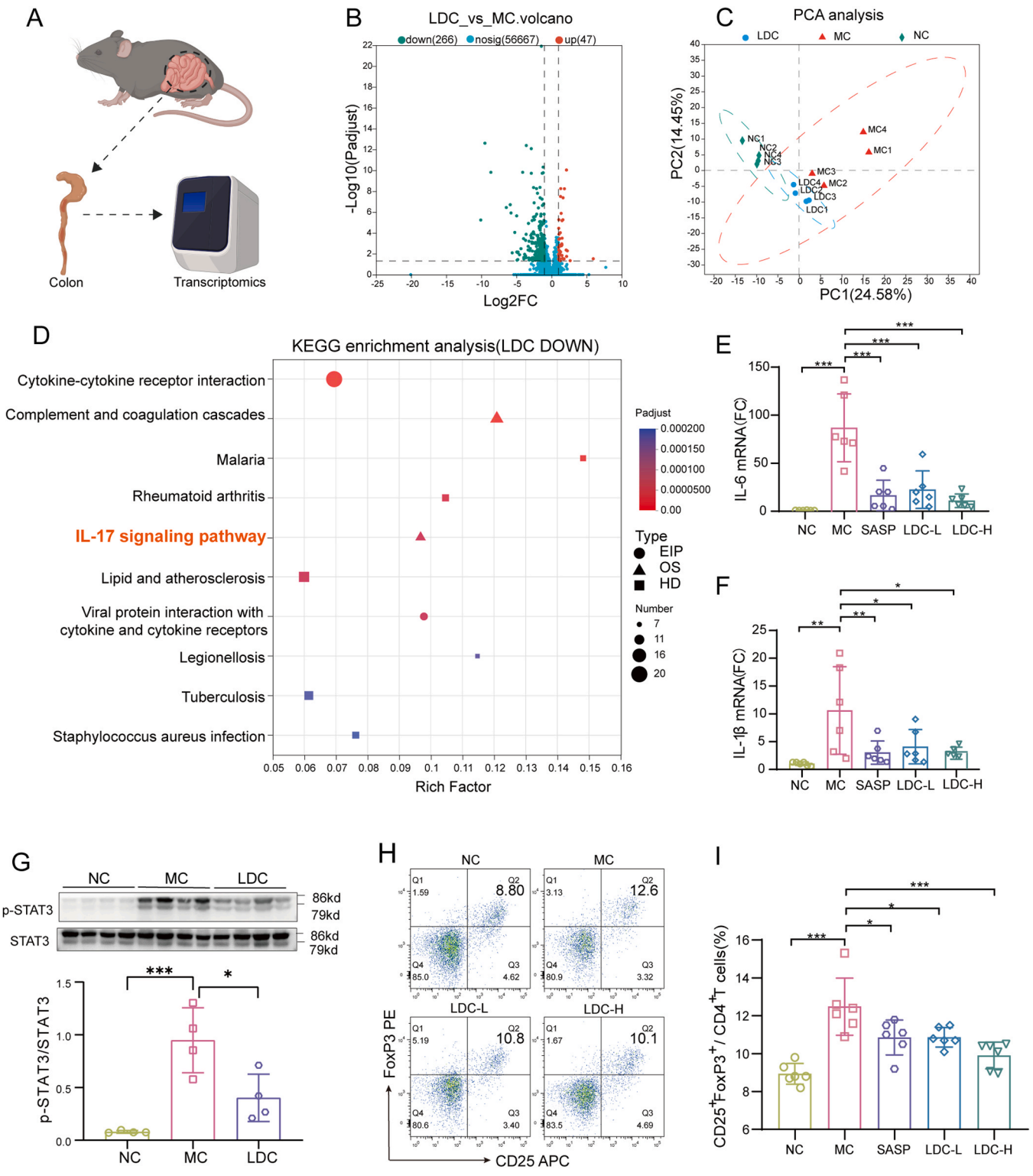

Fig. 2. The transcriptome suggests that the anti-UC effect of LDC is associated with modulation inflammatory signaling pathway. (A) Schematic representation of the tissue transcriptome. (B) Volcano plot of LDC group vs MC group. (C) Principal component analysis of the NC, MC and LDC groups. (D) KEGG signaling pathway enrichment analysis of DEGs between the LDC and MC groups. (E, F) Relative mRNA expression of IL-6 and IL-1β in tissue. (G) Western blot and Densitometry analysis of phospho-STAT3. (H, I) Representative scatter diagram and statistical results of the Treg cells by flow cytometry.

validated that LDC treatment can significantly inhibit the expression of IL-6 and TNF-α mRNA expression (Fig. 3I and J). We further verified the protective effect of LDC on RAW264.7 cells by using Annexin V/PI apoptosis assay, and the results showed that LDC (200 and 400 μmol/L) was able to significantly reduce the proportion of apoptotic cells compared to the LPS group (Fig. 3K and L). In summary, LDC exerts direct anti-inflammatory activity by inhibiting macrophage polarization and modulating the production of inflammatory mediators *in vitro*.

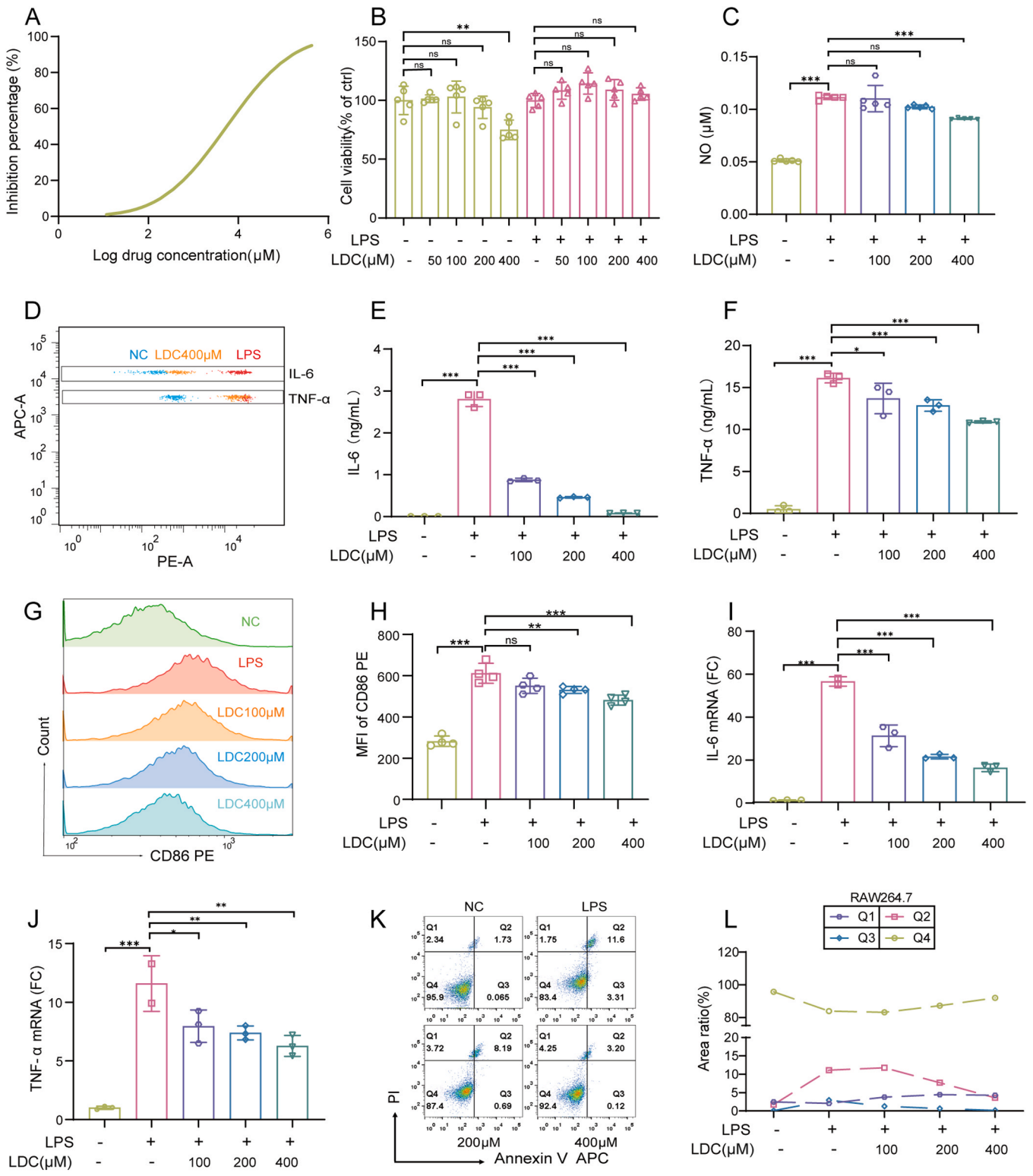

Fig. 3. LDC inhibits macrophage M1 polarization and inflammatory mediators production. (A)The inhibitory curves of LDC on RAW 264.7cells. (B) The cell viability of normal and LPS-stimulated RAW264.7 cells intervened with LDC. (C) The content of Nitric oxide of each group. (D) Representative scatter diagram of IL-6 and TNF-αdetected by Cytometric Bead Array.(E, F) The content of IL-6 and TNF-α of each group. (G) Representative histogram of CD86 detected by flow cytometry. (H) The mean fluorescence intensity (MFI) of CD86 of each group (I, J) Relative mRNA expression of IL-6 and TNF-α. (K, L) Annexin V/PI apoptosis assay and statistical results of LDC on RAW264.7 cells.

3.4. Transcriptomics reveal the potential involvement of the GPCR-mediated MAPK signaling pathway in the anti-inflammatory effect of LDC

Transcriptome assay was used to explore the anti-inflammatory mechanism of LDC. The volcano plot of DEGs showed that 67 genes were down-regulated and 19 genes were up-regulated between the LDC group and the LPS group (Fig. 4B). The results of PCA showed that the LDC group were closer to the NC group than the LPS group, indicated

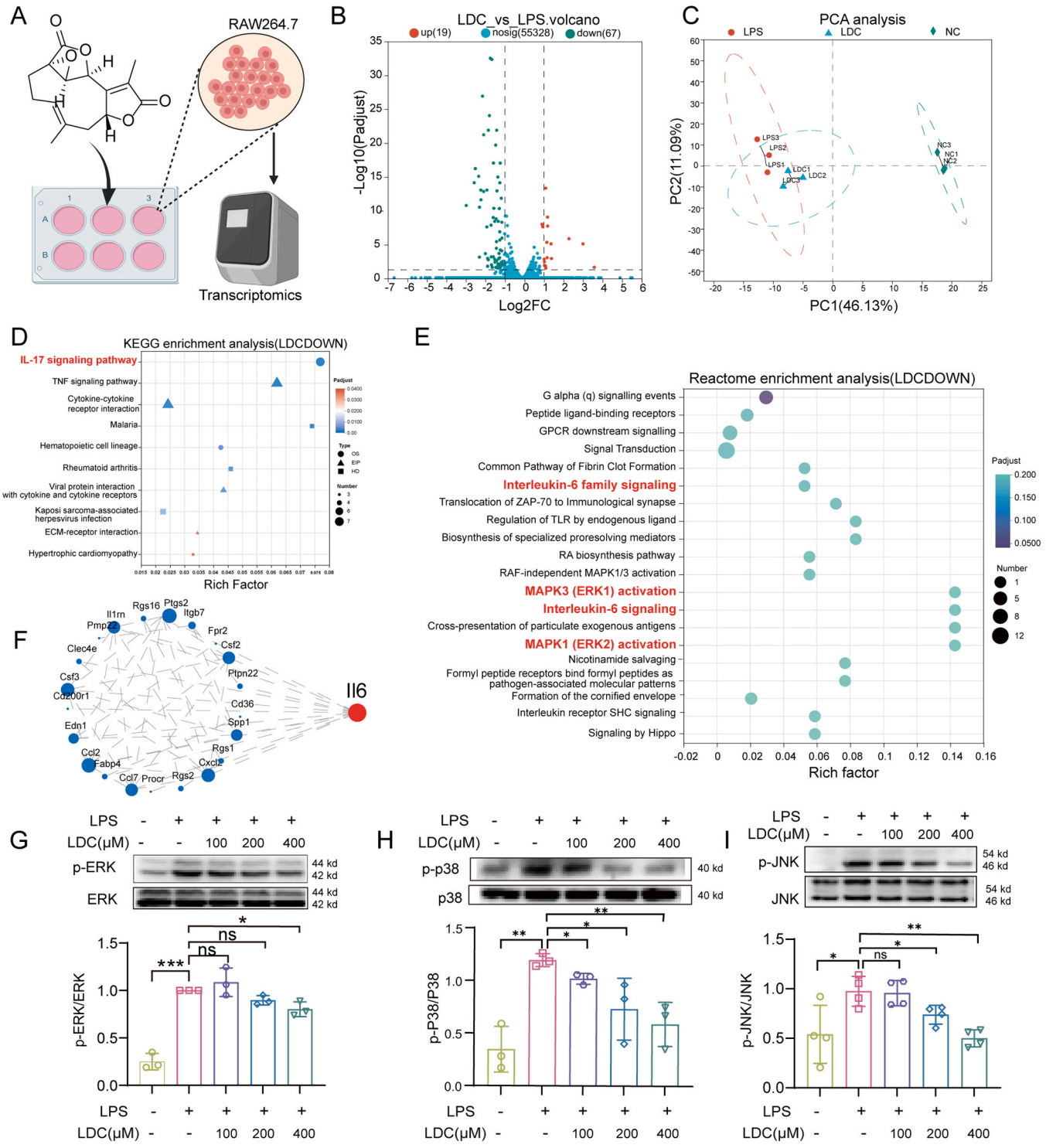

Fig. 4. Transcriptomics reveal potential involvement of GPCR-mediated MAPK signaling pathway in the anti-inflammatory effect of LDC. (A)Schematic representation of the RAW 264.7cells transcriptome. (B) Volcano plot of DEGs between the LDC group and LPS group. (C) Principal component analysis of NC, LPS and LDC groups. (D) KEGG signaling pathway enrichment analysis of DEGs between the LDC and LPS groups. (E) Reactome enrichment analysis of DEGs between the LDC and LPS groups. (F)Protein-Protein Interaction Networks. (G-I) Western blot and densitometry analysis of phospho-ERK/ERK, phospho-P38/P38 and phospho-JNK/JNK.

that LDC can modify gene expression induced by LPS (Fig. 4C). KEGG signaling pathway enrichment analysis showed that the DEGs between the LDC and LPS group was highly enriched in the IL-17 signaling pathway (TOP1) (Fig. 4D), which is consistent with transcriptomics on colon tissues. Protein interaction analysis showed that IL-6 plays a key role in the anti-inflammatory effect of LDC (Fig. 4F) . Reactome enrichment analysis showed that the DEGs between the LDC and LPS group were highly enriched in the G alpha (q) signaling pathway, Mitogen Activated Protein Kinase (MAPK) signaling pathway, and IL-6 signaling pathway (Fig. 4E). Thus, we speculated that LDC might inhibit the production of inflammatory mediators such as IL-6 by regulating GPCR/MAPK signaling pathway. Western blot was used to validate these transcriptomics results. We found that compared with the LPS group, the phosphorylation levels of ERK, p38, and JNK were

significantly reduced in the LDC group (Fig. 4G-I). The above results suggest that the anti-inflammatory activity of LDC may be related to the regulation of the GPCR-mediated MAPK signaling pathway.

3.5. LDC protects intestinal epithelial cells by inhibiting macrophage inflammatory mediators production in co-cultured system

To simulate inflammatory mediators mediate epithelial cell damage under UC state, we co-cultured RAW264.7 and MODE-K cells in transwell system *in vitro* (Fig. 5D). Firstly, we mapped the cytotoxicity of LDC on MODE-K cells using the CCK8 assay and found that it had no significant cytotoxic concentration up to 300 μmol/L (Fig. 5A). The results of apoptosis detection assay showed that the proportion of live cells (Q4) in the LDC group was not significantly reduced compared with the NC group, validating that LDC (300 μmol/L) did not damage MODE-K cells (Fig. 5B and C). In a co-culture system, LPS can induce RAW264.7 cells inflammatory mediators production. A large number of inflammatory mediators not only damage intestinal epithelial cells but

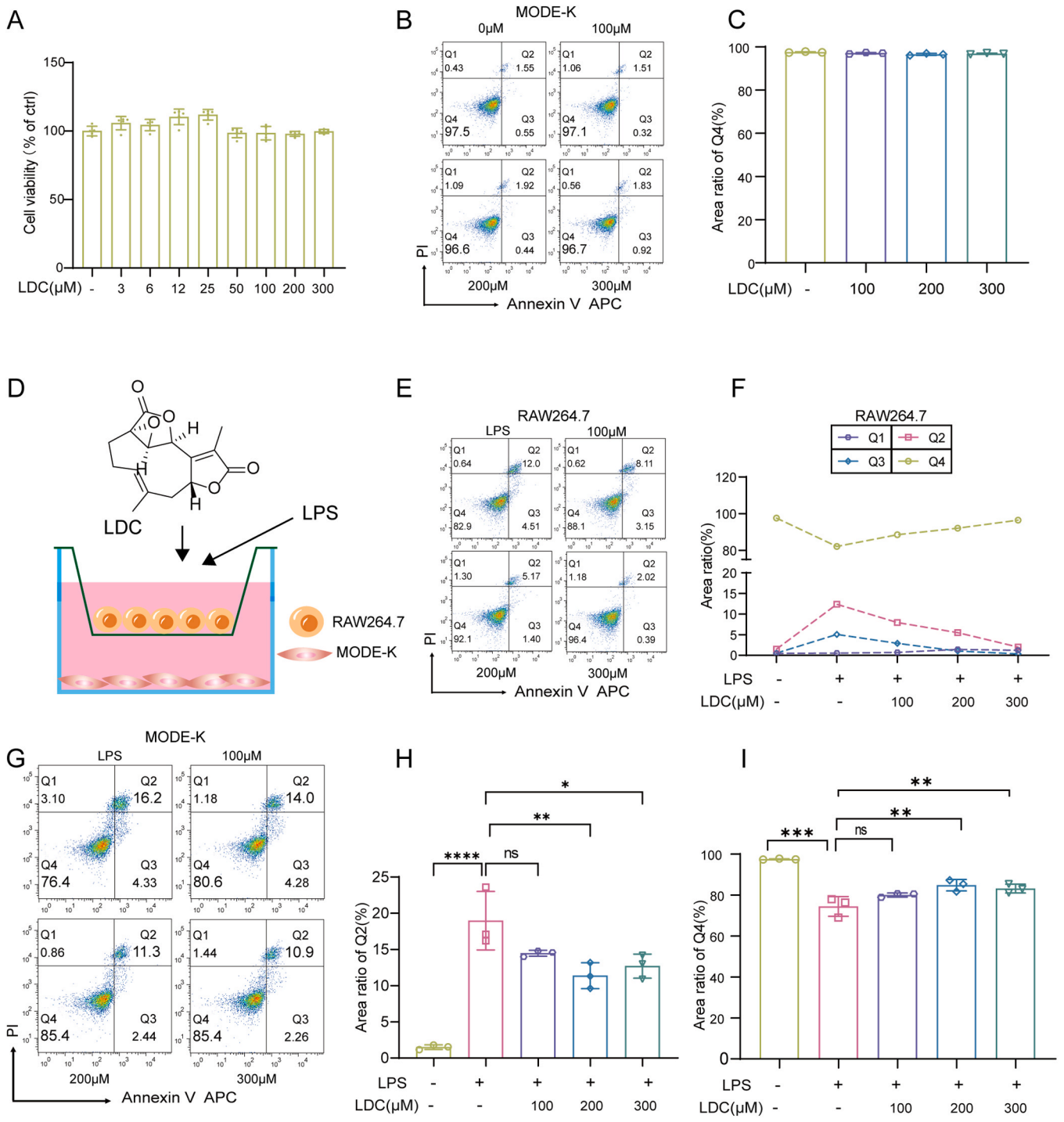

Fig. 5. LDC alleviates intestinal epithelial cells damage by inhibiting macrophage inflammatory mediators production in co-cultured system. (A)The cell viability of MODE-K cells intervened with LDC. (B, C) Annexin V/PI apoptosis assay and statistical results of LDC on MODE-K cells. (D) Schematic representation of co-cultured RAW264.7 and MODE-K cells in transwell system. (E, F) Annexin V/PI apoptosis assay and statistical results of LDC on RAW264.7 cells. (G-I) Annexin V/PI apoptosis assay and statistical results of LDC on MODE-K cells.

damage the macrophage itself. Flow cytometry results confirmed that LPS can induce MODE-K and RAW264.7 cells apoptosis. On the one hand, the percentage of apoptotic RAW264.7 cells was about 20 % in the LPS group, which decreased to about 8 % in the LDC group. Similarly, the percentage of live RAW264.7 cells (Q4) was increased in the LDC group with dose-dependent (Fig. 5E and F) On the other hand, the percentage of apoptotic MODE-K cells was more than 20 % in the LPS group, which decreased in the LDC group with dose-dependent (Fig. 5G-I). These results suggest that LDC can alleviate intestinal epithelial cell damage by inhibiting the production of inflammatory mediators through macrophage M1 polarization.

4. Discussion

Ulcerative colitis is a chronic inflammatory disease that results from a combination of genetic susceptibility, mucosal barrier dysregulation, and dysregulated immune response[15]. Macrophages are a major class of intrinsic immune cells with a high degree of plasticity and multifunctionality; on the one hand, they can polarize towards M1-type macrophages under inducing conditions such as LPS[16]. M1-type macrophages can produce pro-inflammatory cytokines such as TNF-α, IL-6, and reactive oxygen species, maintaining the microinflammatory state of the gut. On the other hand, they can also polarize towards M2-like macrophages under induced conditions such as IL-4. M2-like macrophages could induce intestinal inflammation to subside, promoting intestinal mucosal healing and maintaining intestinal tissue homeostasis by producing anti-inflammatory cytokines such as IL-10, TGF-β, and PGE2[17]. Therefore, macrophages are considered to be major players in establishing and maintaining intestinal homeostasis. Initially, intestinal microbes and metabolites such as LPS invade the intestinal mucosal lamina propria tissue, inducing polarization of macrophage towards the M1 pro-inflammatory phenotype[18]. M1-type macrophages can secrete large amounts of inflammatory cytokines resulting in extensive intestinal epithelial cell damage, which in turn leads to tissue repair imbalance, triggering UC[19]. Increasing evidence supports that targeted therapy of macrophage polarization can re-establish the intestinal immune microenvironment and restore tissue homeostasis, which is an effective strategy for UC treatment[20].

Previously, we had confirmed that *Lindera aggregata* has anti-inflammation and anti-UC effects. Further studies revealed that LDC was able to significantly improve the histopathology and disease index of DSS-induced UC mice, but the mechanism was unclear. We employed a transcriptomics assay to further validate the anti-UC effect of LDC and explore its mechanism. Transcriptomics PCA analysis showed that LDC was more similar to NC group than MC group in gene expression, confirming the protective effect of LDC against UC. Enrichment analysis suggested that the anti-UC effect of LDC might be related to the regulation of the IL-17 signaling pathway. qPCR and western blot results confirmed that LDC could significantly inhibit the expression of IL-6 and IL-1β mRNA and the phosphorylation of STAT3 in colon tissues. Flow cytometry assay showed that LDC was able to reduce the ratio of Treg cells in mesenteric lymph nodes. IL-17 is a pro-inflammatory cytokine produced mainly by Th17 cells[21]. Naïve T cells are differentiated into Th17 cells upon IL-6 stimulation, and modulation of the IL-6/STAT3 pathway can effectively regulate the Th17/Treg balance[22]. Therefore, we speculated that LDC could reduce IL-6 production, inhibit phosphorylated of STAT3, and modulate the IL-17 signaling pathway, thereby reducing intestinal inflammation, indicating that LDC may improve UC through anti-inflammatory effects.

Subsequently, we employed the LPS-induced RAW264.7 cell model in vitro to explore the anti-inflammatory activity of LDC and its mechanism. We found that LDC could significantly decrease the expression of CD86, a marker of M1-type macrophage, and could inhibit macrophage M1 polarization. Likewise, qPCR and ELISA assay confirmed that LDC could inhibit the expression of pro-inflammatory factors IL-6 and TNF-α. The above results suggest that LDC can inhibit LPS-induced macrophage M1 polarization directly, and has a good anti-inflammatory effect, but the mechanism of action remains unknown. We further explored the anti-inflammatory mechanism of LDC using transcriptomics examination. Transcriptomics indicated that LDC could improve the expression of inflammatory genes induced by LPS, and the KEGG signaling pathway enrichment analysis showed that the DEGs were highly enriched in the IL-17 signaling pathway (Top1), which was consistent with the results of the transcriptomic enrichment of the colonic tissues. According to Reactome enrichment analysis, DEGs were highly enriched in G alpha(q) signaling events, MAPK activation, and IL-6 family signaling. GPCRs as the largest cell-surface receptors in eukaryotes, having diversity plays a significant role in cells[23]. When GPCRs are stimulated by external signals in macrophages, the cascade activates MAPK signaling pathway to mediate the production of inflammatory mediators, such as IL-6[24]. Activation of the MAPK signaling pathway involves the phosphorylation of JNK, ERK, and p38, which can further activate transcription factors in the nucleus that are involved in activities such as cell growth, stress, and inflammatory responses[25]. We examined the expression and activation of related proteins in MAPK signaling pathway and found that LDC significantly reduced the phosphorylation levels of ERK, P38, and JNK, indicating that LDC can inhibit the MAPK pathway activation[26]. Taken together, the above results suggest that LDC can inhibit macrophage M1 differentiation and reduce the expression of inflammatory mediators through the regulation of the GPCR-mediated MAPK signaling pathway, which has a good anti-inflammatory effect.

During intestinal inflammation, M1 macrophages secrete large amounts of pro-inflammatory factors such as TNF-α, IL-6, inducible nitric oxide synthase (iNOS), and reactive oxidants (ROS)[27]. The overproduction of ROS can induce lipid peroxidation, protein dysfunction, and DNA mutations resulting in structural protein disruption in the epithelial cells, and impairment of the intestinal mucosal epithelial barrier[28]. The large amount of TNF-α secreted by macrophages exerts various pro-inflammatory functions by binding to the receptors TNFR1 and TNFR2, such as causing the death of intestinal epithelial cells and destroying the intestinal barrier[29]. Therefore, macrophage polarization plays a crucial role in UC disease development and prevention. In order to simulate the damage of intestinal epithelial cells mediated by inflammatory mediators in the UC state, we adopted RAW264.7 and MODE-K cells co-culture model used transwell assay to verify the anti-UC mechanism of LDC. Flow results showed that LPS can induce intestinal epithelial cell damage by activating macrophage inflammatory effects in co-cultured system. We found that LDC could protect intestinal epithelial cells by inhibiting macrophage polarization and the release of inflammatory mediators. In summary, LDC is able to protect intestinal epithelial cells damage by inhibiting inflammation, having an anti-UC effect.

5. Conclusion

Linderanine C regulates the MAPK signaling pathway, inhibits macrophage M1 polarization, reducing inflammatory mediator levels, and has anti-inflammatory and anti-UC pharmacological effects.

CRediT authorship contribution statement

Lulu Zeng: Software, Investigation, Data curation. **Cailu Lin:** Software, Investigation, Data curation. **Yuan Shi:** Software, Data curation. **Shijie Hu:** Validation, Software. **Mengyao Lan:** Writing – original draft, Software, Investigation, Data curation, Conceptualization. **mincong Huang:** Writing – review & editing, Project administration, Funding acquisition, Data curation. **Xin Liu:** Funding acquisition, Formal analysis, Data curation. **Yan Zhao:** Validation, Software. **Guang Liang:** Writing – review & editing, Funding acquisition, Conceptualization. **Jinfeng Sun:** Investigation, Funding acquisition, Formal analysis.

Declaration of Competing Interest

The authors have no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117239.

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