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ORIGINAL RESEARCH

Jianpi Lishi Jiedu Decoction (JLJD) Inhibit Th17 Cell Differentiation via the Jak/Stat3/RORγt Pathway in Colorectal Adenomas

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Background: Jianpi Lishi Jiedu Decoction (JLJD) are used in China to prevent colorectal adenoma recurrence, but their molecular mechanism is unclear.

Objective: Evaluate JLJD's preventive and therapeutic effects on colorectal adenomas and elucidate its molecular mechanisms.

Methods: JLJD components were identified via HPLC. The *Apc*$^{Min/+}$ mouse model assessed therapeutic efficacy. Effects on colorectal tissue proliferation/apoptosis were analyzed. Flow cytometry evaluated Th17 cells; ELISA quantified inflammatory cytokines (IL-17, IL-6, IL-1β, IL-18). Jak2/Stat3/RORγt pathway proteins were detected by Western blot (WB) and immunohistochemistry (IHC). A Jak2 activator (RO8191) validated functional targets. Key components (chlorogenic acid, atractylenolide I) were tested in vitro for: (1) non-toxic concentrations (MTT), (2) IL-17A levels, (3) Th17 differentiation, (4) p-Stat3/RORγt expression (WB).

Results: JLJD significantly reduced adenoma number and progression in *Apc*$^{Min/+}$ mice. Anti-Ki67 IHC showed suppressed proliferation; TUNEL assay confirmed induced apoptosis. ELISA indicated JLJD significantly decreased pro-inflammatory cytokine levels. WB/IHC demonstrated JLJD inhibited Th17 cell differentiation by downregulating Jak2/Stat3/RORγt pathway proteins in colon tissues and mesenteric lymph nodes. RO8191 abrogated JLJD's anti-adenoma effects, reversed Th17 suppression, and nullified pathway inhibition. In vitro, chlorogenic acid and atractylenolide I significantly reduced IL-17A, reversed Th17 expansion, and decreased p-Stat3/RORγt expression.

Conclusion: JLJD inhibits the differentiation of Th17 cells by suppressing the Jak2/Stat3/RORγ pathway, thereby exerting an inhibitory effect on colorectal adenomas.

Keywords: Jianpi Lishi Jiedu decoction, colorectal adenomas, Th17 cell differentiation, Jak2/Stat3/RORγt pathway, *Apc*$^{Min/+}$ mouse model

Introduction

By the year 2020, it was estimated that around 19.3 million new cancer cases were identified worldwide. Colorectal cancer (CRC), making up nearly 10% of these cases, held the position of the third most commonly diagnosed cancer.[1] Precancerous lesions associated with CRC primarily include colorectal adenomas (CRA), ulcerative colitis, and hereditary conditions.[2] Among these, CRA is the most common, comprising 85–90% or more of the precancerous lesions associated with CRC.[3] The "adenoma-carcinoma sequence" is currently recognised as one of the key tumorigenesis pathways for CRC.[4] Advanced endoscopic techniques, such as endoscopic mucosal resection (EMR) and endoscopic

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submucosal dissection (ESD), have been developed for polyp removal.[5] However, due to their side effects and high costs, it is crucial to develop therapeutic agents for intervening in colorectal adenomas.

Inflammation plays a critical role in cancer development.[6] Infection sites, chronic stimulation, and inflammation serve as the origin of many malignant tumors.The inflammatory cell-regulated tumor microenvironment critically contributes to cancer progression by promoting cell proliferation, survival, and migration.[7] The relationship between inflammation and colorectal tumours is supported by evidence showing that inflammatory bowel disease predisposes individuals to CRC. At the same time, anti-inflammatory drugs such as aspirin,[8] celecoxib,[9] and rofecoxib[10] have proven effective in preventing the progression of human colorectal adenomas. Levels of IL-17A are elevated in human CRC compared to adjacent unaffected colon tissues.[11] Studies have indicated that cytokine IL-17 levels, typically associated with Th17 responses, are elevated in adenomatous polyps and serum from mice with polyps.[12,13] Naive helper T cells from CRC patients are more prone to be induced into the Th17 pathway.[14] The Th17 cell differentiation pathway generally maintains a state of dynamic equilibrium under normal conditions. Following TCR (T cell receptor) engagement with Th0 cells, the cytokines TGF-β/IL-1, in conjunction with IL-6/IL-21, induce STAT3 activation to initiate Th17 cell differentiation.[15] The downstream transcriptional factors of TCR cooperate with STAT3 to activate the key transcriptional program of Th17 cells, where the lineage-specific transcription factor RORγt orchestrates the differentiation of Th0 to Th17 cells.[16] Disruption of this balance, leading to excessive Th17 cell differentiation, plays a key role in the progression of colorectal adenomas to CRC.[17] The JAK-STAT signaling pathway is a critical route for Th17 cell differentiation.[18] It is well known that aberrant activation of the JAK/STAT3 pathway is associated with the malignant development of various cancers, such as hepatocellular carcinoma, pancreatic cancer, gastric cancer, and breast cancer.[19–22] Studies have reported that activation of JAK2/STAT3 can lead to the failure of inhibition of malignant phenotypes in NSCLC cells.[23]

JLJD, a traditional Chinese medicine for treating colorectal adenomas, is composed of Radix Codonopsis, Stir-fried Rhizoma Atractylodis Macrocephalae, Rhizoma Smilacis Glabrae, Caulis Lonicerae, Fructus Forsythiae, and Radix Cynanchi Atrati. JLJD aligns with the pathogenesis of spleen deficiency with dampness-toxicity in post-operative colorectal adenoma patients, achieving the therapeutic goals of fortifying the spleen, replenishing deficiency to support healthy qi, while resolving dampness and eliminating toxins to remove pathogenic factors. The efficacy and safety of JLJD have been confirmed in our previously published randomised, double-blind clinical trial.[24,25] At 12-month follow-up, the JLJD group demonstrated significantly reduced recurrence rates (26.83%, 11/41) versus controls (52.50%, 21/40). However, the mechanisms and functional targets of JLJD require further investigation. The therapeutic efficacy of JLJD may derive from synergistic interactions among its multiple bioactive constituents. Chlorogenic acid[26] (extracted from Lonicera japonica) and atractylenolide I[27] (derived from Atractylodes macrocephala), as key components of JLJD.

Here, we report that JLJD inhibits the occurrence of colorectal adenomas by suppressing Th17 cell differentiation, thereby reducing the secretion of associated inflammatory factors. Mechanistically, JLJD inhibits Jak phosphorylation, suppressing the Jak-STAT pathway and preventing excessive Th17 cell differentiation. In summary, we explored the mechanisms and anti-adenoma effects of JLJD, identifying it as a potential novel therapy for the prevention and treatment of colorectal tumours.

Materials and Methods

Reagents

A concentrated Chinese herbal decoction for strengthening the spleen, removing dampness, and detoxification. (Manufactured by Jiangyin Tianjiang Pharmaceutical Co., batch number: 20201101). Its main ingredients include Codonopsis pilosula (15 g), Smilax glabra (30 g), roasted Atractylodes macrocephala (10 g), Forsythia suspensa (15 g), Lonicera japonica stem (30 g), and Cynanchum atratum (10 g). Prepared as a concentrated decoction in the Traditional Chinese Medicine Preparation Room of Nanjing Hospital of Integrated Traditional Chinese and Western Medicine, with a specification of 100 mL per bottle. Each millilitre of the liquid corresponds to 1.1 g of raw medicinal materials. Stored at 4°C.Aspirin, CAS Number: 50-78-2, manufactured by Bayer, Product Number: HJ20160685.The Annexin V FITC apoptosis detection kit was purchased from Beyotime Biotechnology Co., Ltd. The ELISA kit was purchased from Wuhan Saipei Biotechnology Co., Ltd. RO8191 was purchased from Sigma, CAS Number SML1200. The TUNEL cell apoptosis detection kit (red fluorescence) was purchased from Beyotime, CAS Number: C1089.

HPLC Detection

Precisely measure 1 mL of the extract and add 1 mL of methanol solution. Vortex for 120 seconds, ultrasonicate for 5 minutes in an ice-water bath, and centrifuge at 4°C and 13,000 r/min for 10 minutes. Collect the supernatant and dilute it 10-fold and 100-fold with 50% methanol solution, then transfer it to autosampler vials for analysis.

High-performance liquid chromatography (HPLC) was used to determine the contents of eight components in JLJD: codonopyranoside, atractylenolide I, filipenduloside, forsythiaside, forsythin A, chlorogenic acid, strychnine, and cynandione A.

Chromatographic Conditions

A Thermo Hypersil GOLD column (2.1 × 100 mm, 1.9 μm) was used for the analysis. The mobile phase comprised two components: phase A, which contained water with 0.1% formic acid, and phase B, consisting of acetonitrile. Gradient elution was carried out with the following conditions: 0–1 minute, 5% B; 6 minutes, 99% B; and 99% B until 8 minutes. The column temperature was kept at 35°C.

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Animal Handling and Experimental Protocol

SPF-grade *Apc*$^{Min/+}$ mice were purchased from Jiangsu Hua Chuang Xin Nuo Pharmaceutical Technology Co., Ltd. The mice were 4 weeks old, weighing 16–20 g, and were housed under controlled conditions with a temperature range of 20–23°C, relative humidity of 45% ± 5%, and a 12-hour light/dark cycle. All male *Apc*$^{Min/+}$ mice were fed a high-fat diet and divided into four groups (model group, high-dose group, low-dose group, and aspirin group) with 12 mice in each group. The high-fat diet consisted of 60% kcal from fat, including butter (35 g/kg), lard (30 g/kg), and corn oil (80 g/kg). A 12-week high-fat diet was used to induce colorectal adenomas. Drug administration began in the 8th week of the high-fat diet and continued for 4 weeks.[28] In brief, based on the human equivalent dose (HED), the JLJDL/H groups received intragastric administration at doses of 4.5 g/kg and 13.5 g/kg, respectively, while the aspirin group was treated with 15 mg/kg daily via gavage. *Apc*$^{Min/+}$ mice in the intervention group received 30 mg/kg RO8191, a JAK2 activator,[29] orally for 4 weeks in alignment with the traditional Chinese medicine administration schedule.[29] The blank group of C57BL/6J$^{+/+}$ wild-type mice were orally administered an equal volume of sterile isotonic saline and fed a normal diet. Disease signs were monitored daily, and body weight was recorded weekly.

In the final month of the study, polyps in the colon and rectum were observed. To analyse the polyps, the colon tissue was opened longitudinally from the distal to the proximal end and thoroughly rinsed with cooled PBS. The tissue was then flattened on filter paper for polyp counting. This task was performed by two independent researchers using a high-magnification Olympus microscope, blinded to the treatment groups. The experimental protocol was approved by the Animal Experimentation Ethics Committee of Nanjing University of Chinese Medicine (Ethics Approval No: 202501A001).

Histology and Immunohistochemistry

Mice were euthanised by cervical dislocation. Following euthanasia, the entire intestine was carefully removed, washed with cold PBS, and longitudinally opened. Tumours were counted, and their locations were recorded to determine adenoma incidence. Tissue samples were fixed in 10% formalin and subsequently embedded in paraffin. Hematoxylin and eosin (H&E) staining was carried out, and pathological assessment was performed by a pathologist who was blinded to the experimental groups. Histological evaluation was conducted by a board-certified pathologist, as described previously.

Immunohistochemical staining was performed on mouse tissue samples to assess total Ki67 expression (anti-mouse Ki67, Abcam). All staining procedures employed horseradish peroxidase-conjugated antibodies, followed by development with 3,3-diaminobenzidine (DAB) as the substrate and counterstaining with hematoxylin.

Lymphocyte Preparation

Mesenteric lymph nodes (MLN) were isolated from *Apc*$^{Min/+}$ and WT mice. Freshly isolated MLN were immediately immersed in ice-cold sterile PBS. Adherent adipose and connective tissues were carefully dissected from the MLN surface using sterile forceps and scissors, and the nodes were transferred to a sterile dish containing RPMI 1640 medium. Mechanical homogenization was performed using a sterile mortar and pestle to disrupt the MLN tissue until complete cellular release was achieved. The resulting cell suspension was incubated in RPMI 1640 medium supplemented with 0.5 mg/mL collagenase VIII (Sigma-Aldrich) under gentle agitation (100 rpm) at 37°C for 15–30 min to enzymatically digest the extracellular matrix and maximize single-cell yield.[30]

The digested suspension was filtered through a 70 μm cell strainer to remove undigested debris, followed by centrifugation at 400 × g for 10 min. The supernatant was discarded, and the cell pellet was resuspended in PBS containing 2% FBS for two sequential washes to eliminate residual enzymes and impurities. All procedures were conducted on ice to preserve cell viability.

Cell Viability Assay

To evaluate the effects of Chlorogenic acid and Atractylenolide I on cell viability, a quantitative analysis was conducted using the MTT method. In the experiment, cells were seeded in 96-well plates at a density of 5000 cells per well and incubated in a culture incubator for 24 hours to ensure complete cell adhesion. Subsequently, the cells were treated with different concentrations of Chlorogenic acid (0, 12.5, 25, 50, 100, 200, 400, 600, 800, and 1000 μg/mL) and Atractylenolide I (0, 2.5, 5, 10, 20, 40, 60, 80, and 100 μM) for 48 hours. The absorbance values (OD values) of each group were detected by the MTT assay according to the kit instructions. Cell viability was expressed as a percentage, calculated by the formula: Cell viability (%) = [(OD of drug-treated group - OD of blank control group) / (OD of untreated group - OD of blank control group)] × 100%. All data were statistically analyzed using GraphPad Prism software.

Isolation and Induction of Primary Th17 Cells

Male C57BL/6 mice were euthanized under intraperitoneal anesthesia, and their spleens were harvested. The spleens were mechanically dissociated on a cell strainer to generate a single-cell suspension. Red blood cells were lysed using a red blood cell lysis buffer. Naive CD4+ T lymphocytes were subsequently isolated from the splenocytes by magnetic-activated cell sorting (MACS), following the manufacturer's instructions for the mouse naive CD4+ T cell isolation kit (Miltenyi Biotec, Germany). The purified naive CD4+ T cells were seeded into 96-well plates pre-coated with anti-CD3 and anti-CD28 antibodies at a density of approximately 1 × 10^6 cells per well. To induce Treg differentiation, the cells were cultured in medium supplemented with 5 ng/mL IL-2 and 5 ng/mL TGF-β. After successful induction of Treg cells, an inflammatory environment was simulated by stimulating the cells with 50 ng/mL IL-6 and 10 ng/mL IL-1β. In the drug treatment group, the specified drugs were added to the culture. For the control and model groups, an equivalent volume of culture medium solvent was used instead.

TUNEL Assay

To identify apoptotic cells, the Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay, provided by Roche Applied Science (Mannheim, Germany), was employed. The percentage of cells showing positive staining was calculated in five randomly chosen regions per tumour. For each mouse, a minimum of three tumours were selected at random for analysis.

Flow Cytometry Analysis

Mesenteric lymph nodes were processed to create a single-cell suspension by passing them through a 70 μm cell strainer. For Th17 cell staining, the cells were initially incubated with APC-CD4 antibody for 30 minutes. This was followed by permeabilisation and further incubation with PC5.5-CD3, and FITC-IL-17A antibodies at 4°C for one hour, all conducted in darkness. The resulting cells were examined using a BD Calibur flow cytometer, and the data were subsequently analysed with FlowJo version 10 software.

Western Blot

Proteins were extracted with RIPA buffer and separated by SDS-PAGE gel electrophoresis. Afterwards, the proteins were transferred onto a PVDF membrane and incubated with the corresponding primary antibody. Subsequently, the membrane was incubated for 1 hour with an HRP-conjugated secondary antibody. Signal intensity was quantified using an ECL kit (Biosharp) and analysed with the Bio-Rad GelDoc XR+ system.

Statistical Analysis

Statistical differences among groups were evaluated using a one-way ANOVA, with a significance threshold set at $p < 0.05$. Results are presented as the mean ± standard deviation (SD).

Result

Phytochemical Analysis of JLJD

The content of eight major compounds in JLJD was determined using HPLC analysis. Table 1 lists the detailed contents of the eight identified compounds, and Figure 1 presents the chromatograms of the standard samples and JLJD.

JLJD Inhibits Adenomas

We first sought to determine whether the traditional Chinese medicine JLJD has beneficial effects on intestinal tumorigenesis in *Apc*$^{Min/+}$ mice. No significant differences in body weight were observed between the experimental groups and the control group, indicating that the drug did not exhibit any toxic effects on the mice. (Figure 2A). *Apc*$^{Min/+}$ mice treated with JLJD showed a significant reduction in adenomas, with the high-dose group exhibiting a more pronounced reduction, even surpassing the reduction observed in the aspirin-treated group (Figure 2B and C). JLJD treatment improved the pathological features observed in *Apc*$^{Min/+}$ mice. Untreated *Apc*$^{Min/+}$ mice had control tumours histologically identified as polyps with severe atypia, while polyps from JLJD-treated mice exhibited significantly reduced dysplastic changes (Figure 2D).

JLJD Inhibits Colorectal Adenoma Proliferation and Promotes Adenoma Apoptosis

Since Ki67 is a marker of cell proliferation, we examined Ki67 expression levels in intestinal tissues from the control group and tumours from *Apc*$^{Min/+}$ mice using immunohistochemistry. Compared with the control group, Ki67 levels were significantly elevated in *Apc*$^{Min/+}$ mice. Treatment with JLJD significantly reduced Ki67 expression, with the high-dose group showing greater reductions compared to the aspirin group. (Figure 3A and B). To further investigate the mechanism of JLJD's effects on intestinal tumour development, we evaluated tumour cell apoptosis (Figure 3C and D). Apoptosis was assessed using TUNEL staining. Compared to the control group, tumour cell apoptosis was significantly increased in *Apc*$^{Min/+}$ mice, with further increases observed in JLJD-treated mice. These findings suggest that JLJD promotes cell apoptosis, which may play a role in inhibiting tumour development in *Apc*$^{Min/+}$ mice.

Table 1 Weights of Reference Standards and Final Dissolved Volumes

Number	Name	Weight (mg)	Volume (mL)
CM54	Lobetyolin	0.9	1.7
CM59	Atractylenolide I	3.7	1
CM60	Filipendulin	1.1	1
CM61	Forsythoside	1.3	1.5
CM62	Forsythiaside A	5.7	1
CM63	Chlorogenic Acid	0.8	1
CM64	Strychnoside	1.3	1
CM65	Cynarasaponin A	0.9	1

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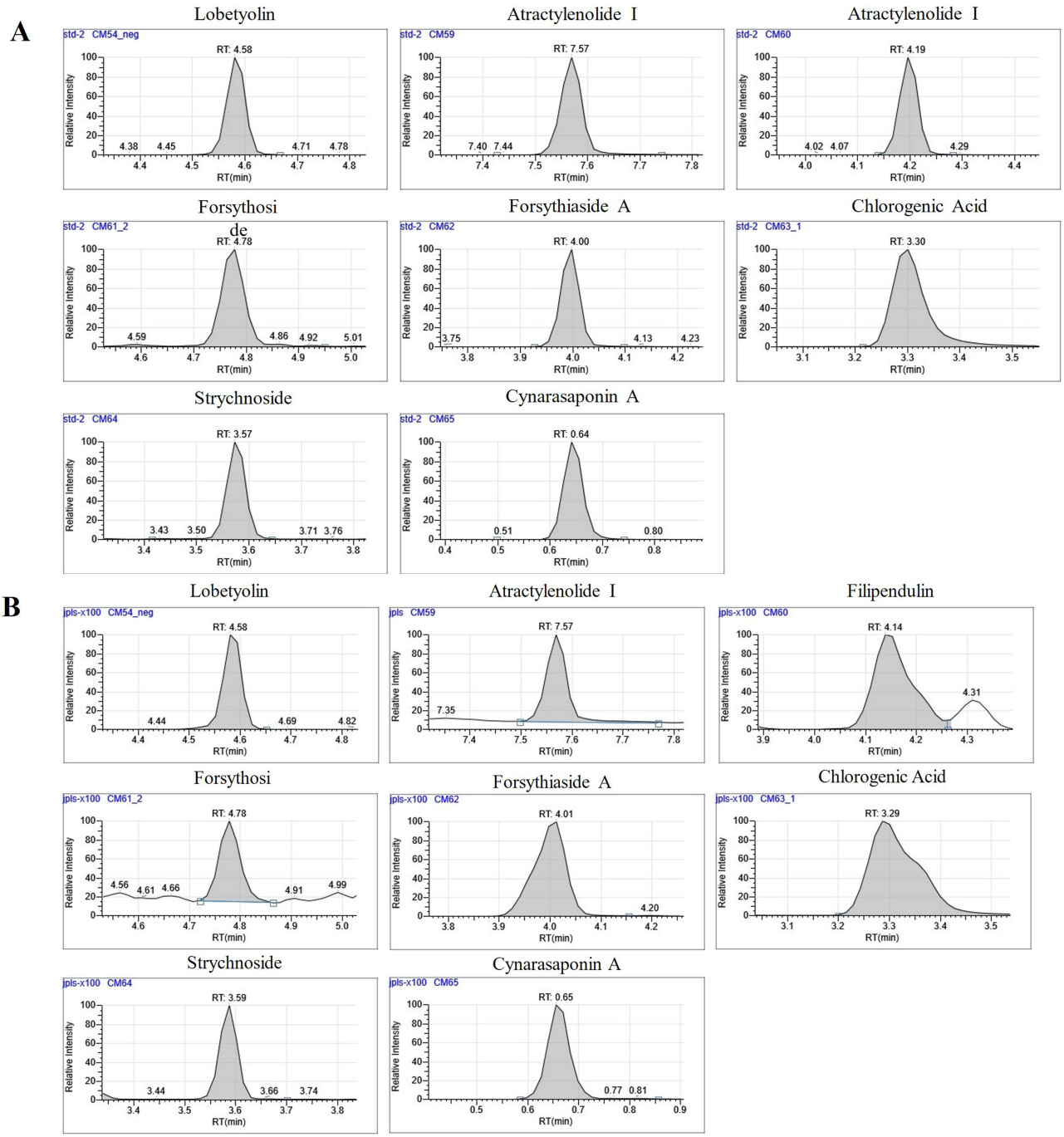

Figure 1 The contents of the reference standards (**A**) and samples (**B**).

JLJD Suppresses Colorectal Tumour Inflammatory Factors and Corrects the Increase of Th17 Cells in Apc$^{Min/+}$ Mice

An MTT assay was performed to evaluate the effects of chlorogenic acid and atractylenolide I on the viability of Treg cells. Drug concentrations were screened using the MTT method, and the selected concentrations for chlorogenic acid were 50, 100, and 200 μg/mL, while those for atractylenolide I were 15, 30, and 60 μM (Figure 4A and B).

We also found that JLJD treatment suppressed Th17 cell-associated inflammatory responses in the colonic tissues of mice. The levels of classic pro-inflammatory cytokines in tissue homogenate supernatants were measured using ELISA. The levels of IL-17, IL-6, IL-1β, and IL-18 were significantly elevated in *Apc*$^{Min/+}$ mice compared to control mice, and

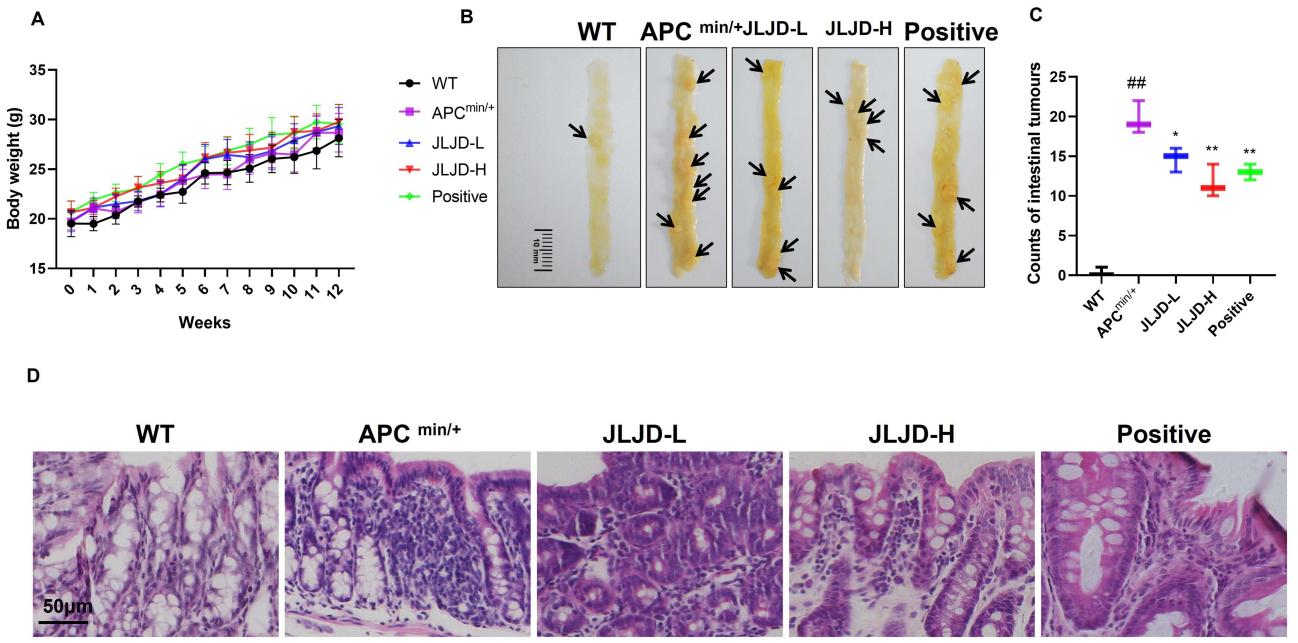

Figure 2 JLJD Treatment Reduces Intestinal Tumorigenesis in *Apc*$^{Min/+}$ Mice. (**A**) Body weight differences among the groups of mice. (**B** and **C**) Adenoma formation and tumorigenesis. (**D**) H&E staining of colon tissue. All data are presented as mean ± SD: ###*P* < 0.01 vs WT group; **P* < 0.05, ***P* < 0.01 vs *Apc*$^{Min/+}$ group (one-way ANOVA).

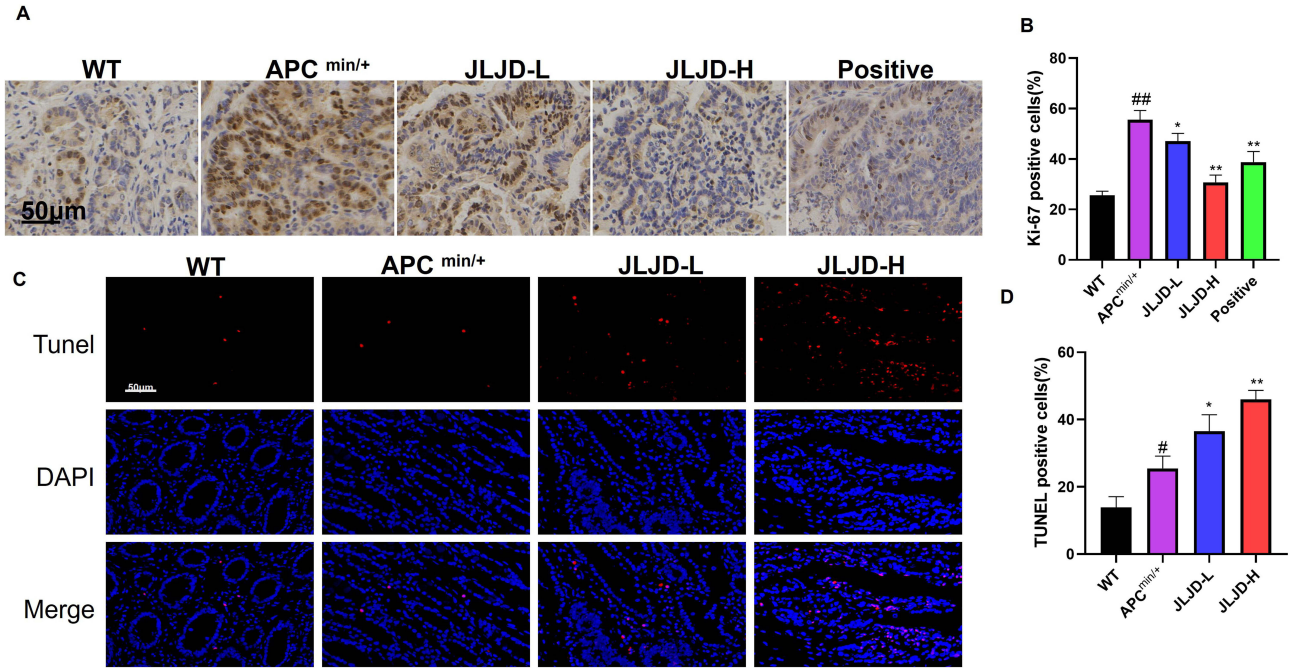

Figure 3 (**A** and **B**) Immunohistochemical staining with anti-Ki67 antibody. Scale bar: 50 μm. (**C** and **D**): JLJD increases apoptosis in tumour tissues of *Apc*$^{Min/+}$ mice. Colon segments were processed for TUNEL assay, and data were quantified as the average percentage of positive cells in five randomly selected areas per sample. Scale bar: 50 μm. The data are expressed as mean ± SD, based on three animals per experimental group, and analysed using one-way ANOVA. Statistical significance was indicated as #*P* < 0.05, ##*P* < 0.01 when compared to the WT group, and **P* < 0.05, ***P* < 0.01 when compared to the *Apc*$^{Min/+}$ group.

these levels were significantly reduced following JLJD treatment (Figure 4C–F). Meanwhile, following the intervention with chlorogenic acid and atractylenolide I, the levels of IL-17 cytokine were significantly decreased in a concentration-dependent manner (Figure 4G and H). In conclusion, The results demonstrated that JLJD inhibited the inflammatory response induced in *Apc*$^{Min/+}$ mice and cells.

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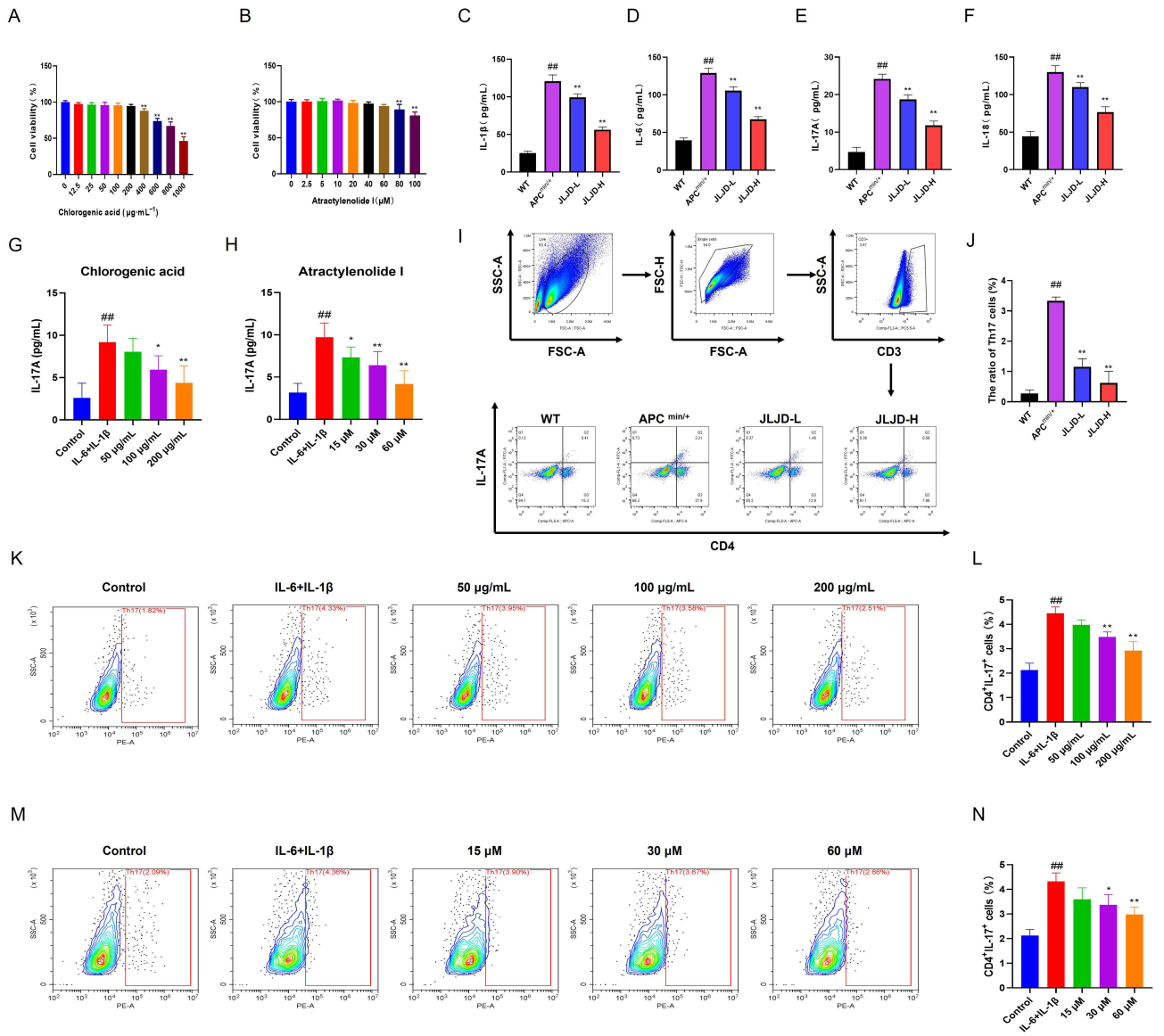

Figure 4 JLJD Inhibits Inflammatory Response in both in vitro and in vivo models. (**A** and **B**) The maximum non-toxic concentrations of chlorogenic acid and atractylenolide I were determined by MTT assay. (**C–F**) The levels of IL-17, IL-6, IL-1β, and IL-18 in the tissues were measured using ELISA. (**G** and **H**) IL-17A concentration in cell supernatant after treatment with chlorogenic acid and atractylenolide I. (**I** and **J**) JLJD corrected the increase of Th17 cells in *Apc*$^{Min/+}$ mice. Mesenteric lymph nodes (MLN) were collected from *Apc*$^{Min/+}$ mice, and the frequency of CD4+IL-17A+ Th17 cells was examined by FACS. (**K–N**) Effects of chlorogenic acid and atractylenolide I on primary Th17 cell differentiation. All data are presented as mean ± SD: ##*P* < 0.01 vs WT group; **P* < 0.05, ***P* < 0.01 vs *Apc*$^{Min/+}$ group (one-way ANOVA).

To investigate the in vivo intervention effect of JLJD on Th17 cells, *Apc*$^{Min/+}$ spontaneous intestinal tumorigenesis model mice were employed as research subjects. The immune phenotypic differences between the drug-treated group and the untreated model group were systematically compared. Our results demonstrated that JLJD treatment effectively reversed the elevated levels of Th17 cells observed in *Apc*$^{Min/+}$ mice. As shown in Figure 4I and J, JLJD treatment reduced the number of Th17 cells in the mesenteric lymph nodes (MLN) of *Apc*$^{Min/+}$ mice.

To further elucidate the pharmacological material basis of JLJD, in vitro cell experiments were conducted. The primary active components, chlorogenic acid and atractylenolide I, were selected to treat primary Th17 cells. The differentiation of Th17 cells was quantitatively assessed by flow cytometry. Results demonstrated that the proportion of CD4+IL-17+ cells in the drug-treated group was significantly reduced compared to the model group (Figure 4K–N). These findings suggest that the spleen-strengthening, dampness-removing, and detoxifying prescription inhibits Th17

differentiation via its active components, chlorogenic acid and atractylenolide I, and exhibits significant Th17 inhibitory activity in both in vitro and in vivo models.

JLJD Effectively Intervenes in the Protein Expression of the Jak/Stat3/RORγt Pathway and Phosphorylation of JAK2

Western blot analysis showed that JLJD treatment downregulated the protein levels of key proteins involved in Th17 cell differentiation pathways (Jak, Stat3, RORγt) (Figure 5A–D). Jak/Stat3 signalling is closely associated with inflammatory responses, and our data indicate that JLJD treatment reduced the phosphorylation of Jak2 (Figure 5E and F).

In vitro experimental results demonstrated that inflammation induction led to an increase in the levels of p-Stat3 and RORγt proteins. Notably, following cellular treatment with chlorogenic acid and atractylenolide I, the levels of p-Stat3 and RORγt proteins were significantly decreased (Figure 5G–L).

JLJD Inhibits Adenoma by Suppressing Jak Protein Phosphorylation

The effects of JLJD depend on the inhibition of Jak protein phosphorylation. To determine whether Jak phosphorylation is a functional target of JLJD-mediated regulation of inflammatory responses, $Apc^{Min/+}$ mice were treated with the JAK2 activator (RO8191) (Figure 6A). As shown in Figure 6B and C, JLJD exhibited a significant anti-tumour effect in $Apc^{Min/+}$ mice, but when Jak protein phosphorylation was activated, the number of adenomas in $Apc^{Min/+}$ mice significantly increased. The adenoma count in the RO8191+H-JLJD group was between that of the high-dose group and the RO8191 group.

As shown in Figure 6D–F, treatment of $Apc^{Min/+}$ mice with the JAK2 activator (RO8191) significantly upregulated p-Stat3 and RoRyt proteins. The data suggest that activation of Jak phosphorylation can eliminate the anti-tumor effects of JLJD. Therefore, we believe that the effects of JLJD depend on the inhibition of Jak protein phosphorylation.

As shown in Figure 6G and H, treatment of $Apc^{Min/+}$ mice with the JAK2 activator (RO8191) resulted in a significant increase in Th17 cells in the MLN of $Apc^{Min/+}$ mice. The data indicate that activation of Jak2 phosphorylation can attenuate the inhibitory effect of JLJD on Th17 cell differentiation.

Discussion

Recent advances in the study of colorectal adenomas have highlighted their role as precursors to colorectal cancer (CRC). The endoscopic removal of precancerous polyps identified during colonoscopic screening plays a pivotal role in CRC prevention.[31] However, endoscopic removal carries significant risks of complications and mortality. The high recurrence rate of colorectal adenomatous polyps after removal remains a major obstacle in the prevention and treatment of CRC.[32] However, to date, there is no effective pharmaceutical method to control this issue. Therefore, finding treatments that can intervene in the growth and recurrence of colorectal adenomatous polyps is crucial. Research has found that certain active ingredients in traditional Chinese medicine can significantly inhibit the inflammatory microenvironment of tumour tissue in patients' intestinal mucosa, suppressing the occurrence, development, and recurrence of adenomas.[33,34] Traditional Chinese medicine believes that syndromic differentiation and treatment can regulate the internal environment of the patient, enhance the body's immune function, and broadly adjust the body's anti-cancer capability.[35] JLJD has achieved good clinical outcomes in clinical use.[24,25] This study further elucidates its mechanism, providing scientific evidence for clinical medication and laying the theoretical foundation for developing new drugs to prevent and treat CRC and colon polyps.

The tumour microenvironment is critical in cancer development and progression. Chronic inflammation fosters an environment that promotes carcinogenesis. Tumor cells secrete various cytokines and chemokines to recruit multiple types of white blood cells, including neutrophils, dendritic cells (DC), macrophages, mast cells, and lymphocytes. These cells, in turn, release cytokines and other cytotoxic mediators that contribute to tumour immunity.[36] IL-17 is a pro-inflammatory cytokine mainly produced by Th17 cells, and recent studies have found a close relationship between IL-17 and tumorigenesis. The expression level of IL-17 is positively correlated with the invasiveness of malignant tumours. Increasing evidence suggests that IL-17 may exert carcinogenic effects by inhibiting tumour cell apoptosis, impairing anti-tumour responses, promoting tumour angiogenesis, and facilitating tumour metastasis and invasion.[37,38] Our study

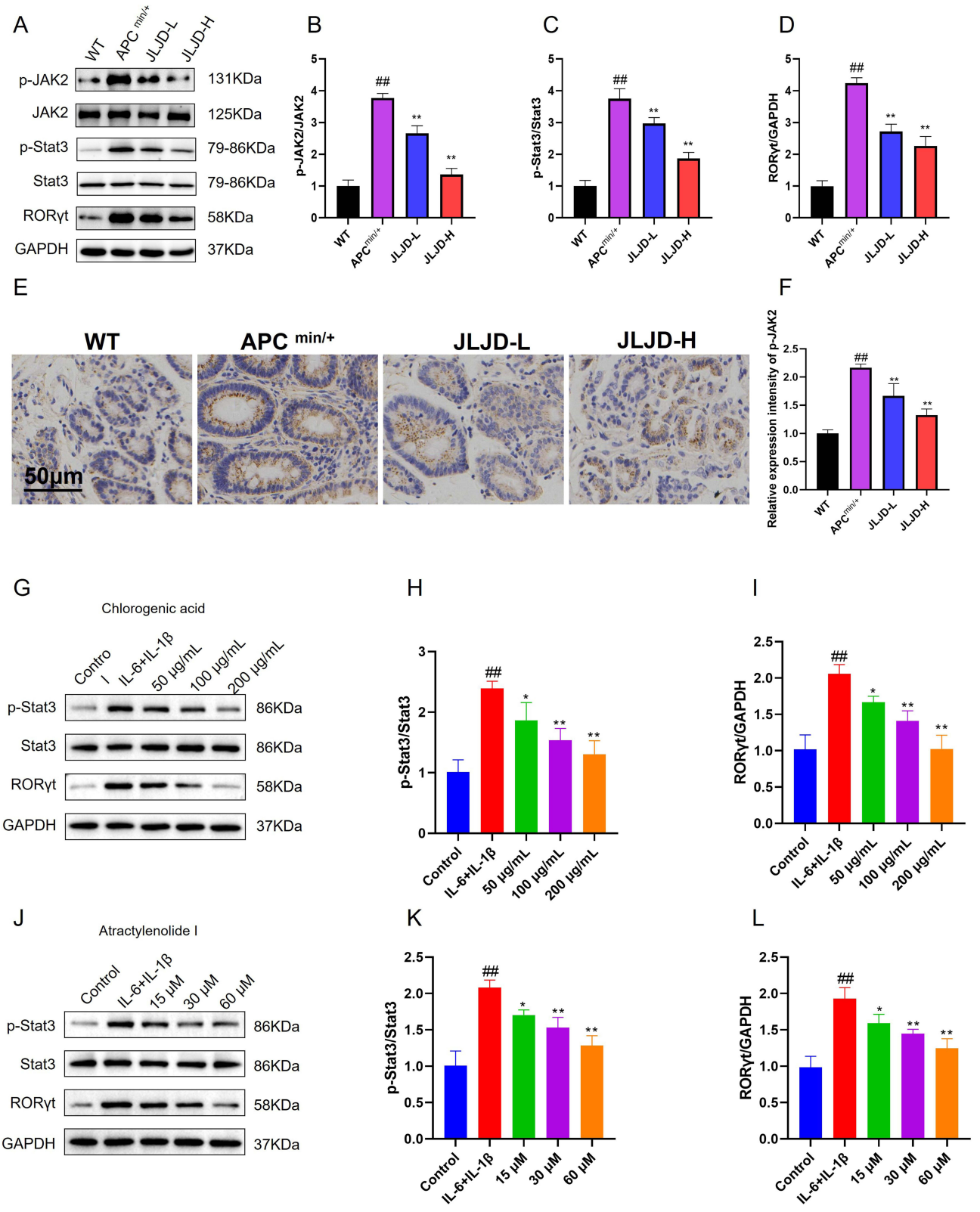

Figure 5 JLJD Effectively Intervenes in the Jak/Stat3/RORγt Pathway. (**A–D**) Immunoblot analysis of Jak, Stat3, and RORγt. (**E** and **F**) The Jak/Stat3 signaling pathway is closely associated with inflammatory responses. Immunohistochemical data revealed that JLJD treatment reduced the phosphorylation of Jak2. (**G–L**) In vitro treatment with chlorogenic acid and atractylenolide I reduced p-Stat3 and RORγt protein expression.All data are presented as mean ± SD: ##$P < 0.01$ vs WT group; *$P < 0.05$, **$P < 0.01$ vs $Apc^{Min/+}$ group.

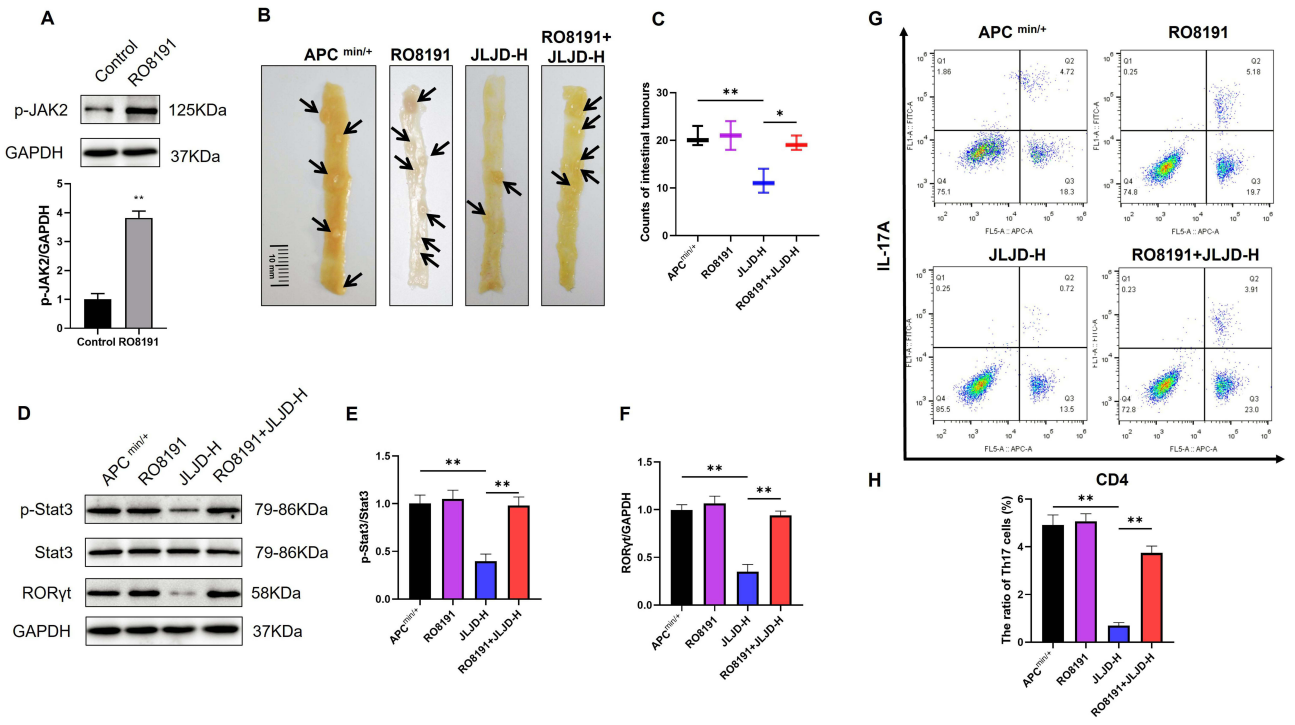

Figure 6 The Effect of JLJD Depends on the Inhibition of Jak Protein Phosphorylation. (**A**) RO8191 significantly activates the phosphorylation of JAK2. (**B** and **C**) After oral administration of the JAK2 activator RO8191 in *Apc*$^{Min/+}$ mice, the number of tumours was significantly increased in the RO8191+High group compared to the high-dose JLJD treatment group. (**D–F**) Proteins related to the Th17 cell differentiation pathway, p-Stat3 and RoRyt, were increased compared to the high-dose JLJD treatment group in *Apc*$^{Min/+}$ mice, and the JAK activator RO8191 significantly reduced the inhibitory effect of JLJD on these proteins. (**G** and **H**) Flow cytometric analysis of mesenteric lymph nodes (MLN) in *Apc*$^{Min/+}$ mice showed that after oral administration of RO8191, the proportion of Th17 cells was upregulated after JLJD treatment, compared to the high-dose JLJD treatment group. All data are presented as mean ± SD: *P < 0.05, **P < 0.01 vs *Apc*$^{Min/+}$ group (one-way ANOVA).

found that by intervening in the APC adenoma model mice, JLJD inhibited the expression of IL-17 and other cytokines and corrected the increase in Th17 cells in APC mice.

Th17 cells are crucial in modulating the tumour microenvironment. These cells secrete cytokines such as IL-17, IL-17F, IL-21, IL-22, and granulocyte-macrophage colony-stimulating factor (GM-CSF).[39] In the context of cancer, the role of Th17 cells is complex. Some studies suggest that Th17 cells may induce angiogenesis through IL-22,[40] promote tumour cell migration and invasion37, and inhibit anti-tumour immunity, thereby promoting tumour growth and progression.[18] The "TH17 signature" of colorectal tumors, including IL-17A, RORγt, and IL-23R, is associated with the prognosis of human stage I/II colorectal cancer.[41]

Th17 cells are differentiated from CD4+ T cells, and their differentiation pathway is influenced by differentiation factors such as TGF-β plus IL-6 or IL-21, growth and stabilising factors (IL-23), and various transcription factors (signal transducer and activator of transcription 3 (STAT3), retinoic acid-related orphan receptor α (RORα), and retinoic acid-related orphan receptor γt (RORγt).[42] Lactate secreted by tumours also promotes Th17 differentiation.[43]

The JAK-STAT signalling pathway is a highly conserved pathway essential for Th cell differentiation.[44] Generally, the JAK-STAT pathway exhibits similarity across various T cell subsets. Cytokines induce receptor tyrosine kinase phosphorylation, which activates JAK and subsequently triggers the phosphorylation of downstream STAT proteins.[45] Recent studies have found that by analysing the dynamic progression of different epithelial cell populations from normal tissue to adenoma and finally to cancer, the JAK-STAT pathway promotes cell proliferation and adenoma initiation.[46] In this study, protein blot analysis showed that JLJD treatment downregulated the expression of proteins associated with Th17 cell differentiation pathways (Jak, Stat3, RORγt), and the use of a JAK activator abolished the anti-adenoma effect mediated by JLJD.

In conclusion, we provide direct evidence of the anti-colorectal adenoma effect of JLJD in mice. Our study also reveals that JLJD inhibits colorectal adenoma by reducing Th17 cell differentiation through suppression of the JAK2/STAT3/RORγt pathway and improving the tumour inflammatory environment.

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Data Sharing Statement

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

All animal experiment was authorized by the Animal Experimentation Ethics Committee of Nanjing University of Chinese Medicine (Ethics Approval No.: 202501A001) and is consistent with the Experiments Guide for the Care and Use of Laboratory Animals.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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