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Clematichinenoside AR ameliorated spontaneous colitis in *Il-10*$^{-/-}$ mice associated with improving the intestinal barrier function and abnormal immune responses

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ABSTRACT

Objectives: Clematichinenoside AR (AR) is a saponin extracted for traditional Chinese medicine with the effects of improving the expression of tight junction (TJ) proteins and mediating anti-inflammatory activities. However, its effect on Crohn's disease (CD) is still unknown. We aimed to investigate the impact of AR on CD-like colitis and determine the mechanism underlying its effects.

Methods: Interleukin-10 gene knockout (*Il-10*$^{-/-}$) mice (male, fifteen weeks old) with spontaneous colitis were allocated to the positive control and AR-treated (32 mg/kg AR administered every other day by gavage for 4 weeks) groups. Wild-type (WT) mice (male, fifteen weeks old) composed the negative control group. The effects of AR on intestinal barrier function and structure and T cell responses as well as the potential mechanisms underlying these effects were investigated.

Results: AR treatment significantly improved spontaneous colitis in *Il-10*$^{-/-}$ mice as demonstrated by reductions in the inflammatory score, disease activity index (DAI) and levels of inflammatory factors. The effects of AR on colitis in *Il-10*$^{-/-}$ mice were related to protecting intestinal barrier function and maintaining immune system homeostasis (regulatory T cell (Treg)/T helper 17 (Th17) cell balance). The anticolitis effect of AR may partly act by downregulating PI3K/Akt signaling.

Conclusions: AR may have therapeutic potential for treating CD in humans.

1. Introduction

Individuals with Crohn's disease (CD) commonly present with mucosal inflammation throughout the entire digestive tract, particularly the distal ileum and colon [1]. Worldwide increases in the incidence and prevalence of CD have led to increases in health-related costs [2]. Regardless of the therapy used, some CD patients remain seriously ill with active disease after all therapeutic options have been exhausted [3]. Medications with few side effects and improved efficacy are needed for the treatment of CD [4].

Intestinal barrier destruction and abnormal mucosal immune responses are thought to be involved in the pathophysiological processes of CD. Destruction of the epithelial barrier in the intestine and increased gut permeability are the basic defects in CD [5,6]. Gut barrier function is achieved by a variety of structures, including surfaces composed of epithelial cells, which are sealed at the cell-side interface by tight junctions (TJs); an adhesive mucous gel layer and antibacterial peptides [7]. There are a large number of microorganisms in the gastrointestinal tract; when the intestinal epithelium is intact and epithelial TJs have low permeability, the epithelial layer forms a barrier that effectively inhibits bacteria from entering the layer of the lamina propria (LP) [8]. It should be noted that epithelial cell apoptosis leads to barrier loss regardless of TJ function [9]. In addition, T helper type 17 (Th17) cells are abundant during intestinal inflammation in

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inflammatory bowel disease (IBD) patients and produce interleukin-17A (IL-17A) and other cytokines that aggravate the inflammatory process [10]. Strong immune responses contribute to intestinal damage in CD; consequently, the induction of intestinal mucosal immune suppression is required to maintain immune system homeostasis [11]. However, it is still inconclusive whether the loss of barrier function is the cause or a clinical manifestation of CD. The interaction between intestinal barrier function loss and abnormal immune responses seems to play an important role in the progression of CD.

There is currently no ideal treatment for CD, and common treatment regimens mainly include drug maintenance (antibiotics, immunosuppressants and biopharmaceuticals), surgical resection and enteral nutrition [12]. In many centers, medical treatment is the first choice to induce and maintain remission [13]. The strategy for treating CD starts with protecting intestinal barrier function and maintaining immune system homeostasis by reducing epithelial cell apoptosis [14], downregulating the responses of T helper type 1 (Th1)/Th17 cells [15], promoting regulatory T cell (Treg) activities [16], attenuating the inflammatory response [17], reducing intestinal permeability and restoring the epithelial barrier structure [18], among other approaches. However, there is still a need to develop more agents, especially those with rapid application potential, to meet the needs of CD patients.

Clematichinenoside AR (AR), a triterpene saponin, has long been used in clinical practice in Asia and is extracted from Clematis medicinal herbs in China [19]. It has been reported that AR has anti-inflammatory activity and is a potential drug for treating arthritic diseases by inhibiting PI3K/Akt signaling [20]. Recent findings have demonstrated that AR has a strong ability to ameliorate blood–brain barrier (BBB) injury by suppressing the levels of tumor necrosis factor (TNF)-α and interleukin-1 beta (IL-1β) and improving the expression of TJ proteins such as ZO-1 and occludin [21]. In addition, AR can decrease the proportion of Th17 cells in the T cell population by promoting Treg activities in the Peyer's patches of rats with adjuvant-induced arthritis [22]. All these findings indicate that AR has potential therapeutic effects on CD that, with its safety profile, making this compound especially promising.

A variety of rodent models have been used to study the pathogenesis of CD and new treatments, and interleukin-10 gene knockout (*Il-10*$^{-/-}$) mice have been recognized as the model with a phenotype most similar to the characteristics of CD [23]. In this study, we explored the therapeutic effect of AR on spontaneous CD-like colitis in *Il-10*$^{-/-}$ mice as well as the underlying mechanism to provide evidence of AR as a drug to treat and suppress CD.

2. Materials and METHODS

2.1. Animals

All animal experimental protocols were approved by the Animal Ethics Committee of Bengbu Medical College (Bengbu, China). Wild-type (WT) C57Bl/6 mice and *Il-10*$^{-/-}$ (C57Bl/6 background) mice were obtained from The Jackson Laboratory (Maine, USA) and housed in the Animal Laboratory Center of Bengbu Medical College under specific pathogen-free (SPF) conditions (Bengbu, China). All experiments involving animals were conducted in full compliance with the guidelines of the National Institutes of Health (NIH) on the care and use of animals. As Bramhall et al. described, *Il-10*$^{-/-}$ mice consistently developed CD-like colitis when raised under normal conditions [24].

2.2. Drug administration

Il-10$^{-/-}$ mice (male, fifteen weeks old) with spontaneous colitis were divided into an AR-treated (AR) group and a positive control (*Il-10*$^{-/-}$) group. WT C57Bl/6 mice (male, fifteen weeks old) composed the negative control (WT) group. Every group in the experiment contained 10 mice. AR with a purity ≥99% (solid) was purchased from Absin Bioscience Inc. (Shanghai, China; Cat #: abs47013581) and was suspended in distilled water to prepare an AR working solution. The final concentration of AR was 4 mg/mL. Based on previous reports, AR (32 mg/kg, gavage) was administered to mice in the AR group every other day for 4 weeks, and mice in the *Il-10*$^{-/-}$ group were orally administered an equal volume of distilled water as a treatment control [25]. After 4 weeks, the mice were humanely sacrificed in a 4 °C room by an expert from the DMU Animal Center. The entire colon of each mouse was collected and carefully rinsed with PBS for subsequent processing.

Table 1
Primer sequences (5′ to 3’).

Gene Name	Forward Primer	Reverse Primer
Il-1β	GCTCATCTGGGATCCTCTCC	CCTGCCTGAAGCTCTTGTTG
Tnf-α	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
Il-17A	TCTCAGGCTCCCTCTTCAG	GACTCTCCACCGCAATGA
p-PI3K	GGCTTGGACCGAATGCT	TTGTTGAAGGCTGTGGC
p-Akt	AGCAAACAGGCTCACAGGTT	TAAGTCCTCCCCATCTCCCT
β-actin	GATTACTGCTCTGGCTCCTAGC	GACTCATCGTACTCCTGCTTGC

2.3. Colitis symptom assessment

The extent of colitis in all the *Il-10*$^{-/-}$ mice was scored once a week with a numerical disease activity index (DAI) as described by Spencer et al. [26]. The index assigns 1 point for each of the following characteristics: occult fecal blood, ruffled fur, soft stool and rectal abscission less than 1 mm, with an additional point for either severe rectal abscission greater than 1 mm or diarrhea. The DAI scores ranged from 0 to 5.

2.4. Histological analysis

Tissue from the colon was fixed in 10% formalin and embedded in paraffin for routine staining with hematoxylin and eosin (H&E) and subsequent pathological analysis. The samples were scored via intestinal inflammation grading as described by Schultz et al. [27]. The intestinal inflammation in mice was calculated on a 0–4 scale: no inflammation (grade 0); a modest number of mononuclear cell infiltrates into the LP (grade 1); mild mucosal hyperplasia or separation of the crypts caused by the infiltration of mononuclear cells (grade 2); moderate inflammation accompanied by epithelial cell hyperplasia and disrupted mucosal architecture (grade 3); and all the above characteristics with additional ulceration or abscesses (grade 4). All mouse colon samples were evaluated by 2 independent pathologists blinded to the treatments.

2.5. Intestinal permeability in vivo

At the end of the AR treatment regimen, mice were fasted for 4 h and then administered fluorescein isothiocyanate (FITC)-dextran (4 kDa; Sigma-Aldrich; Cat #: F-7250) via gavage (600 mg/kg) [28]. Four hours after FITC-dextran administration, the mice were euthanized, and blood was collected by cardiac puncture. The sera were separated by centrifugation, and the serum level of FITC-dextran was measured by evaluating the fluorescence intensity.

2.6. Bacterial translocation

Bacteria were extracted from tissue samples of the mesenteric lymph nodes (MLNs) and liver for culture using sterile techniques. Two samples were taken for each histological type for bacterial culture. The weights of the collected tissue samples were recorded, and 0.1 g of each sample was homogenized in a tissue mill and mixed with 0.9 ml of sterile saline. A total of 100 μL of diluted homogenate was cultured on

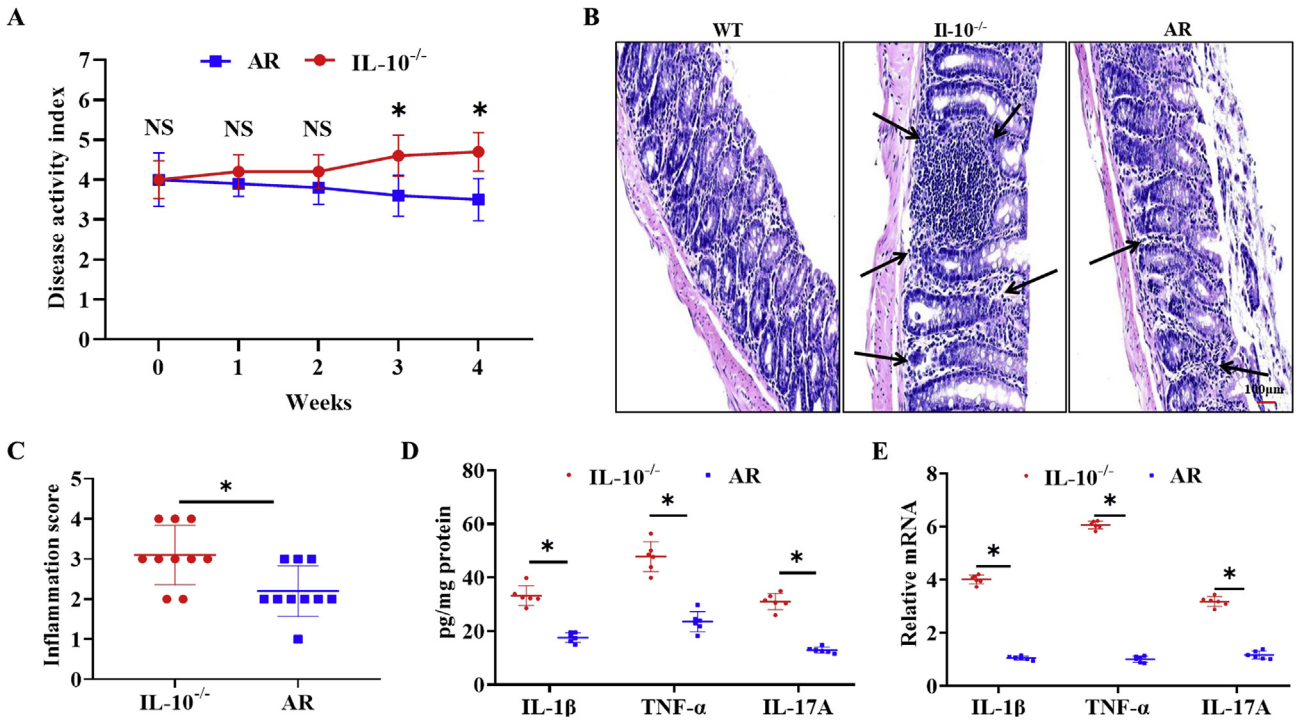

Fig. 1. AR treatment improved colitis in *Il-10*$^{-/-}$ mice. (A) The mean DAI values of the AR group were lower than those of the *Il-10*$^{-/-}$ group during the 2 nd week after AR treatment. (B) AR treatment could alleviate the histological manifestations of experimental colitis, as shown by H&E staining. The arrow indicates an inflamed area in the colitis tissue. (C) Histological inflammation scores show that administration of AR clearly decreased inflammation in tissue samples from AR-treated *Il-10*$^{-/-}$ mice (AR) compared with those from untreated *Il-10*$^{-/-}$ mice. (D) The protein levels of IL-1β, TNF-α and IL-17A were clearly decreased in colon samples from the AR group compared with those from the untreated *Il-10*$^{-/-}$ group. (E) The mRNA levels showed the same trend as the protein levels. AR, clematichinenoside AR; WT, wild-type; IL-17A, interleukin 17A; TNF-α, tumor necrosis factor-α; and IL-1β, interleukin 1β; DAI, disease activity index. The experiments were performed at least 3 times independently with 6 to 8 mice in each group, and one representative result is shown. The results are presented as the means ± SD. *$P < 0.05$.

MacConkey agar (Sigma-Aldrich) at 37 °C for 24 h. Detectable colony-forming units/g of tissue indicate bacterial growth on the plate, and results were identified as positive when more than 102 colony-forming units/g of sample were observed [29].

2.7. TUNEL staining

As previously reported [30], apoptosis in colon sections was measured by TUNEL staining with an In Situ Cell Death Detection kit (Roche, Germany; Cat #: 11684795910) according to the manufacturer's instructions. 4,6′-Diamidino-2-phenylindole (DAPI; Abcam, Cambridge, MA, UK; Cat #: ab104139) was used to stain the nucleus, and TUNEL-positive cells in the crypts were counted (at least 10 crypts with normal morphology per section).

2.8. Flow cytometry

Cells from the MLNs and splenocytes were cultured in 48-well plates (2×10^6 cells/ml) and stimulated with a cell stimulation cocktail (eBioscience) at a volume of 2 μL/well for 2 h. T cell responses were analyzed on a FACSCalibur flow cytometer (BD Biosciences, CA, USA); anti-IL-17A, anti-Foxp3 (intracellular staining), anti-CD25 and anti-CD4 (eBioscience, CA, USA; Cat #: 11-7177-81, 15-4776-41, 12-0257-41, and 50-0048-41, respectively) antibodies were used for analyzing the frequencies of Tregs and Th17 cells in the MLN cell and splenocyte populations. The data were analyzed with FlowJo V10 software.

2.9. Enzyme-linked immunosorbent assay (ELISA)

Total protein lysates were extracted from frozen proximal colon samples, and the levels of TNF-α, IL-1β and IL-17A in the intestine were examined with ELISA kits (R&D Systems, Emeryville, CA, USA; Cat #: MTA00B, MLB00C, and M17AF0) according to the manufacturer's instructions.

2.10. Western blot analysis

Western blotting was performed as previously described to evaluate changes in intestinal protein levels. In brief, protein lysates from intestine samples were separated by SDS-PAGE on a 10% gel and then transferred onto PVDF membranes for enhanced chemiluminescence (ECL) analysis. Rabbit monoclonal antibodies against Bax, Bcl-2, cleaved caspase-3, occludin, ZO-1, phosphatidylinositide 3-kinase (PI3K), p-PI3K, protein kinase b (Akt), p-Akt, and β-actin were used as primary antibodies at a dilution of 1:800 (Abcam; Cat #: ab32503, ab182858, ab214430, ab216327, ab251568, ab151549, ab182651, ab179463, and ab81283, respectively). The membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 2 h (1:5,000; Cell Signaling, USA) followed by ECL detection reagents (Amersham, USA). Protein bands were measured using an ECL detection system and reported as the value of the optical density. The protein levels in each experimental group were evaluated using ImageJ software (NIH, USA) with respect to the optical density value of β-actin [31].

2.11. Immunofluorescence analysis

Immunofluorescence was performed with sections of colonic segments using rabbit polyclonal antibodies against occludin and ZO-1 (1:80; Abcam) to evaluate the expression of epithelial TJ proteins in the intestinal mucosa. DAPI was used to stain nuclei, and the results were analyzed by confocal scanning microscopy (Leica Microsystems,

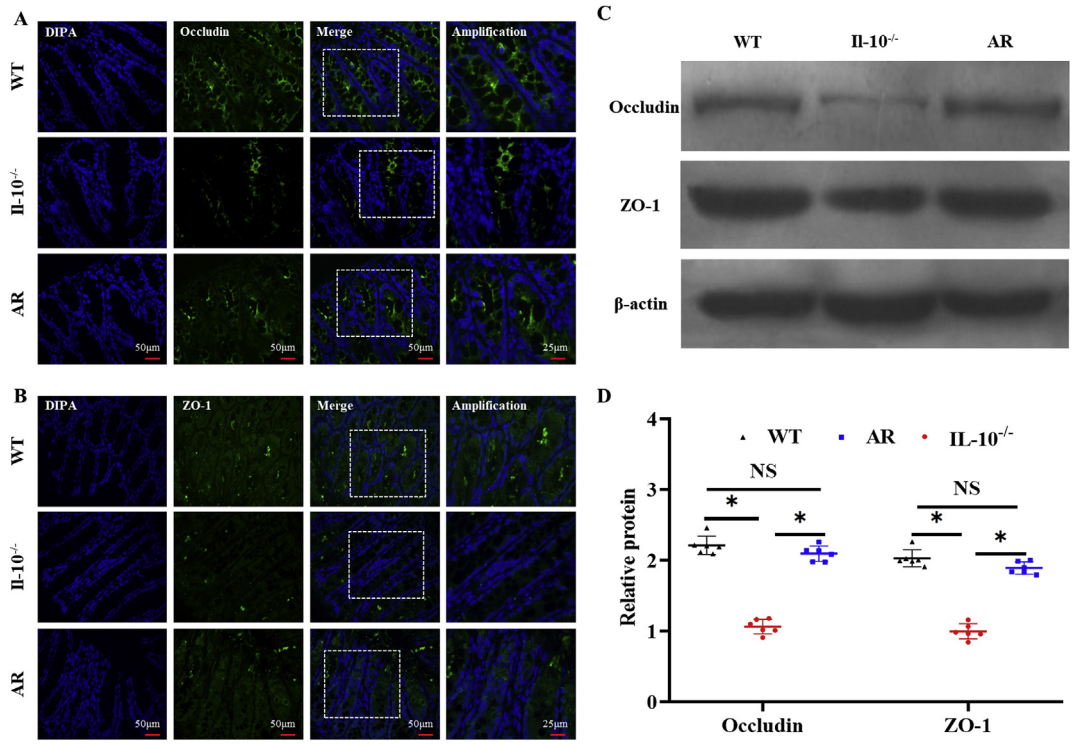

Fig. 2. AR treatment improved the expression of junctional molecules. (A–B) IF showed that the expression of the TJ proteins occludin and ZO-1 (green) was inhibited in the intestinal mucosal epithelium. DAPI was used to stain nuclei (blue), and the merged image shows the overlay of TJ protein and DAPI staining. (C–D) The protein levels of ZO-1 and occludin in the intestinal mucosa of the AR group were clearly lower than those in the intestinal mucosa of the untreated *Il-10*$^{-/-}$ group and was similar to those in the WT group. AR, clematichinenoside AR; WT, wild-type; TJ, tight junction; IF, immunofluorescence; DAPI, 4′,6-diamidino-2-phenylindole; and NS, no significance. The experiments were performed at least 3 times independently with 6 mice in each group, and one representative result is shown. The results are presented as the means ± SD. *$P < 0.05$. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Heidelberg GmbH, Germany).

2.12. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed as previously described to evaluate changes in mRNA levels. In brief, total RNA lysates were extracted from frozen proximal colon samples with TRIzol reagent (Life Technologies, USA), the RNA concentration was calculated, and reverse transcription was performed to obtain complementary DNA. qRT-PCR analysis of intestinal proinflammatory factors, including TNF-α, IL-1β, and IL-17A, as well as of p-PI3K, p-Akt, and β-actin was conducted with Synergy Brands (SYBR) Green detection reagent (Applied Biosystems, USA). The sequences of the mouse gene-specific primers are shown in Table 1. The RNA levels in all experimental groups were evaluated with respect to the level of β-actin to obtain expression values. Each qRT-PCR analysis was run three times to ensure reliability of the data.

2.13. Statistical analysis

The obtained data were analyzed by using Statistical Product and Service Solutions software (SPSS Inc., Chicago, IL) version 17.0. The mean ± standard deviation (SD) is used to represent continuous, normally distributed data. Data from two groups were compared by unpaired t-tests. Chi-squared tests were used to evaluate categorical data. All tests were two-sided. Statistical significance was defined as $P < 0.05$.

3. RESULTS

3.1. AR treatment improved colitis in *Il-10*$^{-/-}$ mice

Colitis severity significantly decreased during the second week after AR administration, and the mean DAI values of the AR group began to decline below those of the *Il-10*$^{-/-}$ group (Fig. 1A). Moreover, the histological inflammation score in the intestines of AR-treated mice was lower than that of positive control (*Il-10*$^{-/-}$) mice (Fig. 1B–C). In addition, compared with positive control *Il-10*$^{-/-}$ mice, AR-treated mice exhibited significantly lower concentrations of inflammatory factors (IL-1β, TNF-α and IL-17A) in the colonic tissue (Fig. 1D). qRT-PCR further demonstrated reductions in IL-1β, TNF-α and IL-17A mRNA levels in the intestines of the *Il-10*$^{-/-}$ mice treated with AR (Fig. 1E).

3.2. AR treatment improved the expression of TJ proteins in *Il-10*$^{-/-}$ mice

Intestinal mucosal barrier damage is a key pathological change in CD, and TJ proteins are an important component of the intestinal barrier structure. To detect whether there were changes in the expression and distribution of key TJ proteins after AR administration, we assessed occludin and ZO-1 levels in colon tissue samples by immunofluorescence and Western blotting. The expression of occludin and ZO-1 in colon tissue samples was significantly increased in the AR group compared with the *Il-10*$^{-/-}$ group. In addition, there was no significant difference in the expression levels between the AR group and WT group (Fig. 2A–B). Western blotting confirmed this trend (Fig. 2C–D). These phenomena indicate that intestinal mucosal barrier damage was improved after administration of AR.

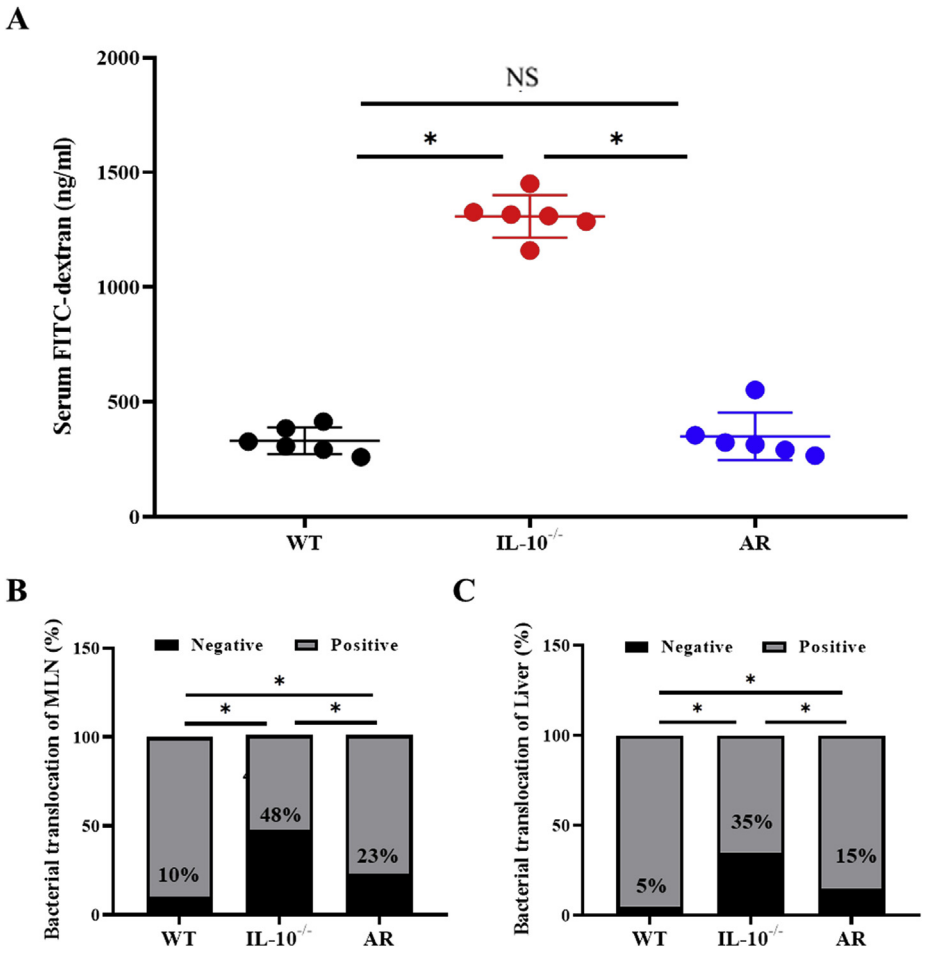

Fig. 3. AR treatment reduced the permeability of the intestines of *Il-10*$^{-/-}$ mice. (A) The intestinal permeability detection results indicated that the serum dextran conjugate levels of the AR group were lower than those of the *Il-10*$^{-/-}$ group and similar to those of the WT group. (B–C) AR-treated mice had lower rates of bacterial translocation in the MLN and liver than did the untreated *Il-10*$^{-/-}$ mice and had similar rates as the WT mice. AR, clematichinenoside AR; WT, wild-type; and MLN, mesenteric lymph node. The experiments were performed at least 3 times independently with 6 mice in each group, and one representative result is shown. The results are presented as the means ± SD. *$P < 0.05$.

3.3. AR treatment reduced the permeability of the intestine in Il-10$^{-/-}$ *mice*

Because bacterial translocation caused by increasing intestinal permeability is a key change in the pathogenesis of CD [18], intestinal permeability in *Il-10*$^{-/-}$ (AR-treated and untreated) and WT mice was detected. Our data indicated that the serum levels of dextran conjugates in the AR group were lower than those in the *Il-10*$^{-/-}$ group and similar to those in the WT group (Fig. 3A). As shown in Fig. 3, bacteria generated from tissue samples of the MLN and liver were cultured. The bacterial translocation rates of the liver and MLN in the AR group were lower than those in the *Il-10*$^{-/-}$ group and similar to those in the WT group (Fig. 3B–C). All the findings partly demonstrate that the structure and function of the intestinal barrier were protected in *Il-10*$^{-/-}$ mice after administration of AR.

3.4. AR treatment reduced the apoptosis rate of epithelial cells in Il-10$^{-/-}$ *mice*

To further investigate the contribution of AR to reducing colitis, TUNEL staining was performed to detect apoptosis in intestinal epithelial cells from *Il-10*$^{-/-}$ mice. The TUNEL assay results demonstrated that the number of TUNEL-positive cells per crypt in the AR group was lower than that in the *Il-10*$^{-/-}$ group and similar to that in the WT group (Fig. 4A–B). The expression of Bcl-2, an antiapoptotic factor, was improved in the AR group compared with the *Il-10*$^{-/-}$ group; however, the Bcl-2 level in the AR group was not as high as that in the WT group (Fig. 4C–D). In contrast, the levels of both cleaved caspase-3 and Bax were attenuated in the AR group compared with the *Il-10*$^{-/-}$ group; however, they were still higher than those in the WT group (Fig. 4C–D). These findings partly demonstrate that epithelial cell apoptosis was reduced by the administration of AR and that AR exerts a protective effect on *Il-10*$^{-/-}$ mice.

3.5. AR treatment reduced Th17 responses and enhanced Treg responses in Il-10$^{-/-}$ *mice*

Because inhibiting Th17-mediated immune responses has long been considered a target for treating CD [10], intracellular cytokine staining was performed to explore the effect of AR on Th17 cell function in *Il-10*$^{-/-}$ mice. The flow cytometry assay showed that there were significant decreases in the percentage of IL-17A$^+$ CD4$^+$ T cells within the CD4$^+$ T cell population in the *Il-10*$^{-/-}$ mice treated with AR compared to that in the untreated *Il-10*$^{-/-}$ mice in samples taken from both the MLN (Fig. 5A–B) and spleen (Fig. 5C–D). However, the percentage of IL-17A$^+$ CD4$^+$ T cells in the treated *Il-10*$^{-/-}$ mice was still not as low as that in the WT mice. These results indicate that AR was able to play a protective role in *Il-10*$^{-/-}$ mice by inhibiting the function of Th17 cells. Because of the inhibitory effect of Tregs on Th17 immune responses, intracellular cytokine staining was performed to explore the effect of AR on Treg function in *Il-10*$^{-/-}$ mice. The flow cytometry assay showed that there were significant increases in the percentage of CD4$^+$ CD25$^+$ Foxp3$^+$ T cells (Tregs) within the CD4$^+$ CD25$^+$ T cell population in the *Il-10*$^{-/-}$ mice treated with AR in both the MLN (Fig. 5E–F) and spleen (Fig. 6G–H) compared to that in the untreated *Il-10*$^{-/-}$ mice. These phenomena indicate that AR could inhibit the function of Th17 cells by promoting Treg responses.

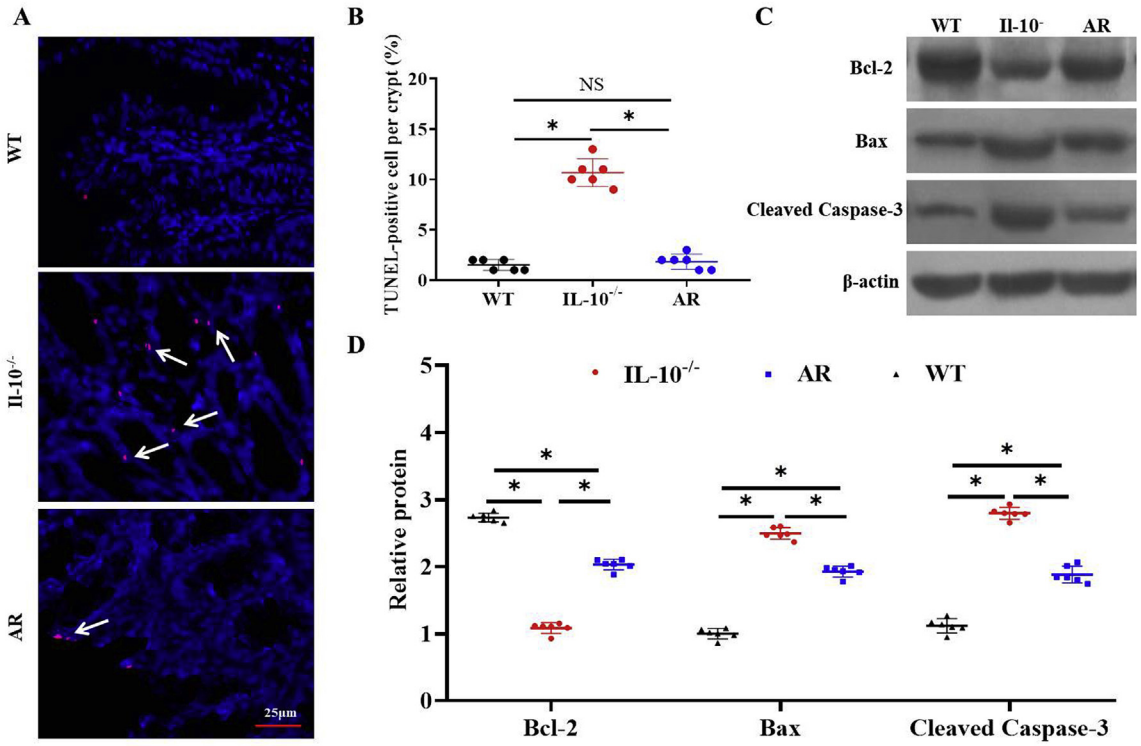

Fig. 4. AR treatment reduced the apoptosis rate of epithelial cells in *Il-10*$^{-/-}$ mice. (A–B) TUNEL staining showed that the AR group had fewer apoptotic cells in the intestinal epithelium than did the *Il-10*$^{-/-}$ group and a similar number as the WT group. The arrow indicates TUNEL-positive cells. (C–D) The expression of the antiapoptotic factor Bcl-2 was higher in the AR group than in the *Il-10*$^{-/-}$ group but was lower than that in the WT group. However, the expression levels of Bax and cleaved caspase-3 were lower in the AR group than in the *Il-10*$^{-/-}$ group, and AR-treated *Il-10*$^{-/-}$ mice showed significant decreases in Bax and cleaved caspase-3 expression; however, the expression levels were still higher than those in the WT group. AR, clematichinenoside AR; WT, wild-type; and NS, no significance. The experiments were performed at least 3 times independently with 6 mice in each group, and one representative result is shown. The results are presented as the means ± SD. *$P < 0.05$.

3.6. The anticolitis effect of AR treatment may be partly mediated by attenuating PI3K/Akt signaling

Western blotting for p-PI3K and p-Akt was performed with intestinal tissue samples extracted from *Il-10*$^{-/-}$ mice (AR-treated and untreated) and WT mice. The results show that p-PI3K and p-Akt were undetectable in the colon tissue samples from the WT mice. In contrast, the levels of p-PI3K and p-Akt were high in the untreated *Il-10*$^{-/-}$ group but were remarkably attenuated in *Il-10*$^{-/-}$ mice administered AR (Fig. 6A–B). To verify the reliability of the data, we further assessed the effect of AR on PI3K/Akt signaling by qRT-PCR. All the results showed that the levels of both p-PI3K and p-Akt were attenuated in the AR group compared with the untreated *Il-10*$^{-/-}$ group; however, the p-PI3K and p-Akt levels in the AR group were still higher than those in the WT group (Fig. 6C). These data could partly explain how AR attenuated the inflammatory response in *Il-10*$^{-/-}$ mice.

4. Discussion

To the best of our knowledge, the present study demonstrated for the first time that AR could ameliorate colitis in *Il-10*$^{-/-}$ mice, with the following specific results: (1) the administration of AR improved signs of spontaneous CD-like colitis in *Il-10*$^{-/-}$ mice by improving the expression of junctional molecules; (2) AR maintained intestinal barrier function in *Il-10*$^{-/-}$ mice partly by reducing the apoptosis rate of epithelial cells; and (3) the inflammatory response in *Il-10*$^{-/-}$ mice was attenuated by inducing immune suppression in the intestinal mucosa and attenuating PI3K/Akt signaling.

This study demonstrated that the administration of AR could provide significant protection against spontaneous CD-like colitis in *Il-10*$^{-/-}$ mice. Activated proinflammatory cytokines, such as IL-1β, TNF-α and IL-17A, that contribute to barrier damage are highly expressed during intestinal inflammation in IBD patients [32]. Our results demonstrated the effect of AR based on the decreases in the inflammatory score, DAI values and proinflammatory factor expression levels. To investigate the anti-inflammatory effect and underlying mechanism of AR, changes in the intestinal barrier associated with pathogenesis in human CD were analyzed [33]. The results indicated that the protection mediated by AR treatment occurred in part through enhancing the levels of TJ proteins (ZO-1 and occludin) in the colons of *Il-10*$^{-/-}$ mice. Disruption of intestinal epithelial barrier function and the consequent increase in intestinal permeability maintain and promote the development of intestinal inflammation in CD [34]. Our data demonstrated that the serum levels of dextran conjugates and bacterial translocation rates of the liver and MLN were significantly decreased in the AR group, further demonstrating that intestinal barrier function was protected by AR. These findings encouraged us to continue investigating, and we found that epithelial cell apoptosis was reduced by AR treatment in *Il-10*$^{-/-}$ mice. Moreover, activated Bcl-2 signaling shows strong antiapoptotic effects, and the Bcl-2/Bax ratio is another important factor that influences the apoptotic balance [35]. We found that Bcl-2 expression was increased in AR-treated *Il-10*$^{-/-}$ mice but that Bax levels were decreased. Among the various caspases, caspase-3 is important and indicates the promotion of cell death [36]. Consistent with these observations, our data showed decreased cleaved caspase-3 expression in the AR group. These findings suggested that AR had a potent protective effect on CD-like colitis.

The protective effects of AR on epithelial barrier function and intestinal inflammation in *Il-10*$^{-/-}$ mice were very inspiring and solidified the notion that AR has a potential therapeutic effect on CD-like

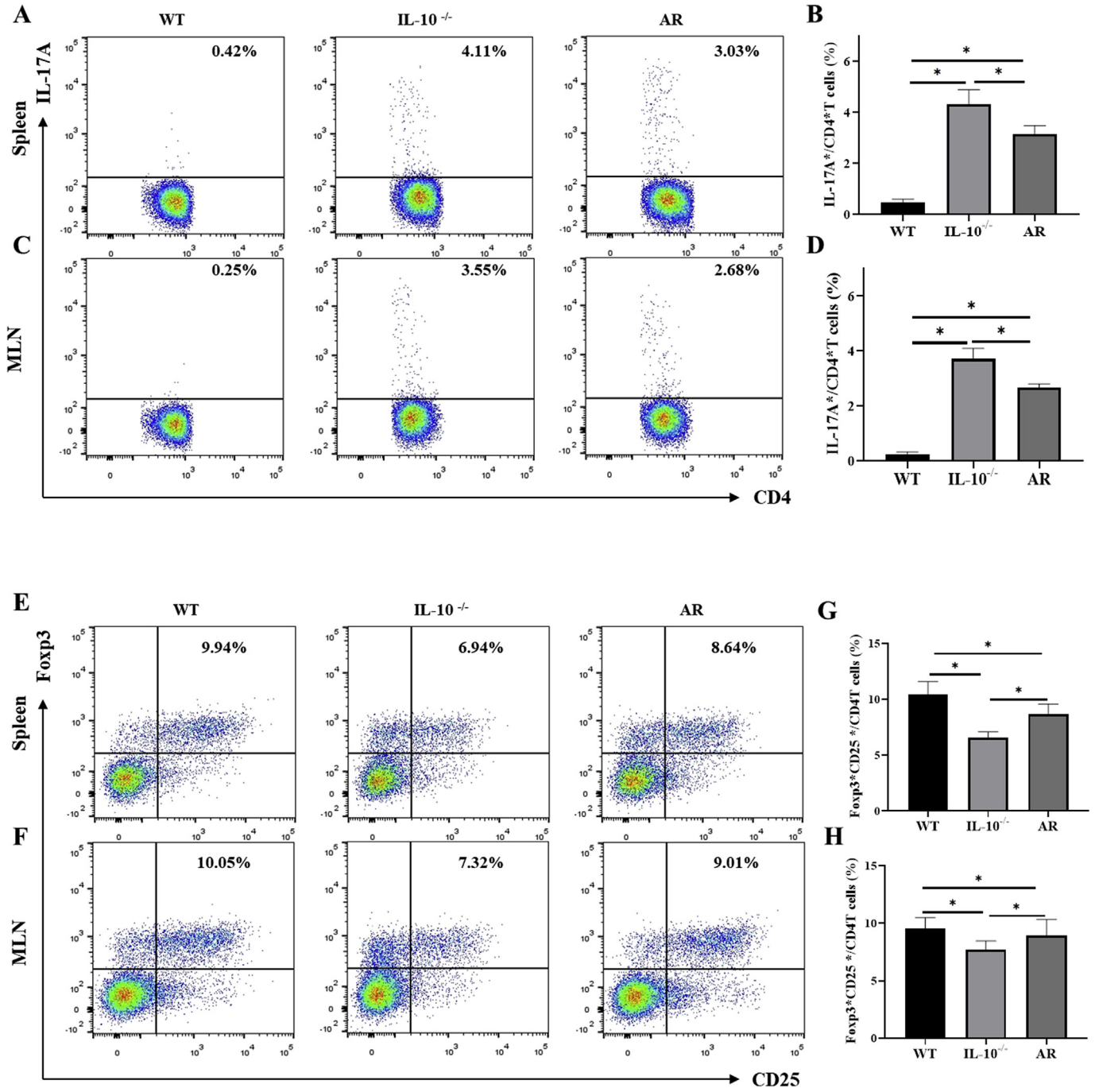

Fig. 5. AR treatment reduced Th17 responses and enhanced Treg responses in *Il-10*$^{-/-}$ mice. (A–B) A significant decrease in the percentage of IL-17A^{+} T cells within the CD4^{+} T cell population in the spleen was observed in the AR group compared with the *Il-10*$^{-/-}$ group, but this T lymphocyte proportion was still higher than the proportion in the WT group. (C–D) A significant decrease in the percentage of IL-17A^{+} T cells within the CD4^{+} T cell population in the MLN was observed in the AR group compared with the *Il-10*$^{-/-}$ group, but this T lymphocyte proportion was still higher than the proportion in the WT group. (E–F) A significant increase in the percentage of Foxp3^{+} T cells (Tregs) within the CD4^{+} CD25^{+} T cell population in the spleen was observed in the AR group compared with the *Il-10*$^{-/-}$ group, but this T lymphocyte proportion was still lower than the proportion in the WT group. (G–H) A significant increase in the percentage of Foxp3^{+} T cells within the CD4^{+} CD25^{+} T cell population in the MLN was observed in the AR group compared with the *Il-10*$^{-/-}$ group, but this T lymphocyte proportion was still lower than the proportion in the WT group. AR, clematichinenoside AR; WT, wild-type; and MLN, mesenteric lymph node. The experiments were performed at least 3 times independently with 6 mice in each group, and one representative result is shown. The results are presented as the means ± SD. *$P < 0.05$.

colitis. These findings led our team to continue investigating the potential mechanisms underlying the anti-inflammatory effect of AR. We evaluated the effects of AR on the regulation of the intestinal mucosal immune reaction and found that administration of AR inhibited Th17 cell functions and promoted Treg responses in *Il-10*$^{-/-}$ mice. The PI3K/Akt signaling pathway has been reported to play important roles in the progression and development of inflammatory reactions in CD [37]. In addition, recent studies have shown that this pathway has an important regulatory effect on the immune response [38]. The results revealed that the levels of both p-PI3K and p-Akt were decreased by AR treatment in *Il-10*$^{-/-}$ mice. Activated PI3K and Akt are important for the differentiation of Th17 cells [39], and inactivation of the PI3K/Akt signaling pathway is involved in the regulation that maintains the immune balance by inducing T cells to differentiate in a way that

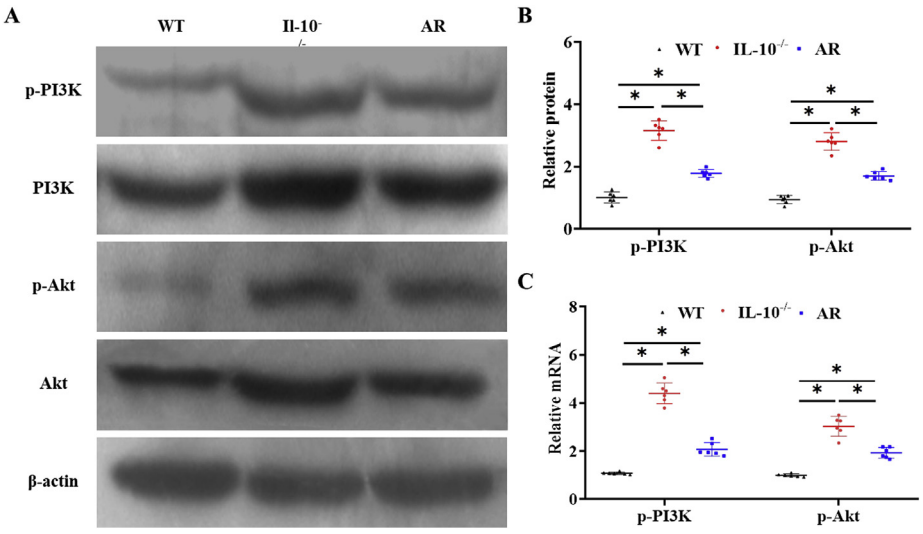

Fig. 6. AR treatment partly ameliorated the inflammatory response in *Il-10*$^{-/-}$ mice by attenuating PI3K/Akt signaling. (A–B) Western blot analysis showed that the levels of both p-PI3K and p-Akt were attenuated in the AR group compared with the *Il-10*$^{-/-}$ group but were still higher than those in the WT group. (C) The mRNA levels showed the same trend as the protein levels. AR, clematichinenoside AR; and WT, wild-type. The experiments were performed at least 3 times independently with 6 mice in each group, and one representative result is shown. The results are presented as the means ± SD. *$P < 0.05$.

counteracts Th17 cell activities [40]. These results may partly explain how AR attenuated the inflammatory response in *Il-10*$^{-/-}$ mice.

The above findings provide direct evidence for the potential of AR to attenuate the inflammatory response in CD. Due to the increasing incidence of CD and the importance of drug maintenance, our research team has been working to find possible alternative therapies to improve the prognosis of patients. However, alternatives that are likely to be clinically available soon are more meaningful than those that still require in-depth study. AR is a triterpene saponin from the antiarthritic herbal formula Wei-Ling-Xian in China, which is a commonly used herb with a long history of clinical use in Asia [19]. Recently, AR has been frequently studied in treating rheumatoid arthritis (RA) [41] and suppressing excessive inflammation to ameliorate BBB injury [21]. Increasing evidence has shown that AR has therapeutic potential for treating systemic inflammatory and immune function disorders, which are similar to the mechanisms that drive CD [22]. We consider AR to be a potential approach for the management of CD in humans.

Of course, our research still has some limitations. For instance, our results suggest that AR can improve CD-like colitis by protecting the structure and function of the intestinal barrier and limiting abnormal intestinal mucosal immune responses; however, AR can also improve colitis by other means. The downregulation of PI3K/Akt signaling may partly explain the treatment effects of the anti-inflammatory properties of AR and the mechanism underlying AR efficacy, but other signaling pathways should also be considered. As mentioned earlier, AR seems to have a variety of biological functions [21].

In conclusion, this study partly demonstrates that the administration of AR ameliorates pathology related to colitis in *Il-10*$^{-/-}$ mice and that this therapeutic effect is associated with improving the expression of junctional molecules and reducing the apoptosis rate of epithelial cells. The positive effects of AR may occur through inducing intestinal mucosal immune suppression and attenuating PI3K/Akt signaling. All of these results indicate that AR has a potent protective effect on CD-like colitis and provides a new choice for drug maintenance therapy in CD.

Author contributions

X. Song and J. Li contributed to the study concept and design, data acquisition, experiments, data analysis and manuscript drafting. J. Hu designed the experiments. M. Shen, C. Zhou, Z. Zhang and Y. Wang contributed to the animal experiments and testing. P. Xiang, X. Zhang, H. Zhao, L. Yu and L. Zuo contributed technical support and scientific advice and helped with manuscript revision. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no financial conflicts of interest.

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