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Azelaic Acid Regulates the Renin−Angiotensin System and Improves Colitis Based on Network Pharmacology and Experimentation

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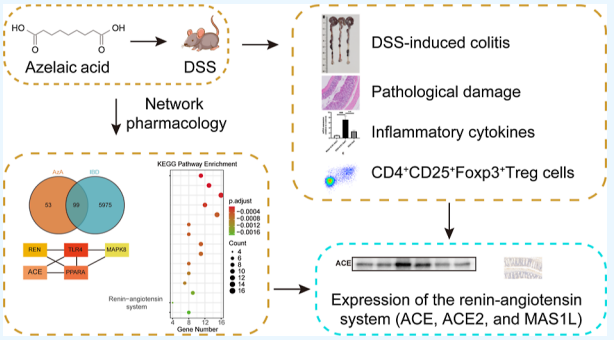
ABSTRACT: Inflammatory bowel disease (IBD), which encompasses Crohn's disease and ulcerative colitis, has a complicated etiology that might be brought on by metabolic dysbiosis. Previous metabonomic studies have found a correlation between decreased azelaic acid (AzA) and IBD. Herein, data from the Metabolomics Workbench showed that the content of AzA decreased in IBD patients (PR000639) and dextran sulfate sodium (DSS)-induced mice (PR000837). The effects of AzA on IBD were then examined using a DSS-induced mouse model, and the results demonstrated that AzA alleviated clinical activity, decreased pro-inflammatory cytokine production, and reduced $CD4^+CD25^+Foxp3^+$Treg percentages in mesenteric lymph nodes. Through network pharmacology analysis, we discovered 99 candidate IBD-associated genes that are potentially regulated by AzA. After the enrichment analysis of the candidate genes, the renin−angiotensin system (RAS) pathway was one of the most substantially enriched pathways. Additionally, AzA reversed the increased expression of important RAS components (ACE, ACE2, and MAS1L) following DSS induction, suggesting that AzA exerts therapeutic effects possibly via the RAS pathway. This study suggests that AzA may be a promising drug for treating IBD.



1. INTRODUCTION

Inflammatory bowel disease (IBD), which consists of Crohn's disease (CD) and ulcerative colitis (UC), is a long-lasting, recurring, and idiopathic condition marked by intestinal epithelium injury and intestinal homeostasis dysregulation.[1,2] This chronic, uncontrolled inflammation can result in life-threatening side effects such as fibrotic stenosis, intestinal fistulas, and intestinal tumors.[1,3] According to epidemiological research, the prevalence and incidence of IBD are rising globally.[4,5] The detailed cause of IBD, however, remains largely unclear, making its diagnosis and treatment difficult.

Accumulating evidence suggests that the intestinal metabolite disorder is associated with the pathogenesis of IBD.[6] Intestinal metabolites are crucial for the healthy development of the intestinal immune system and intestinal homeostasis.[6,7] For example, short-, medium-, and long-chain fatty acids can affect host metabolism and intestinal mucosal immune function, thereby regulating IBD.[8−10]

Several studies have found that azelaic acid (AzA) is reduced in the systems of individuals with IBD.[11,12] Among the fecal differential metabolites of pediatric CD, AzA was substantially linked with the disease severity and responsiveness to infliximab treatment in pediatric CD compared to controls.[12] AzA is an aliphatic dicarboxylic acid that occurs naturally in plants, including cereal grains like wheat, rye, and barley.[13,14] The Malassezia genus is also capable of producing and secreting AzA.[14] Furthermore, under ketogenic conditions of hunger and diabetes mellitus, AzA can be produced endogenously via hepatic oxidation using linoleic acid as a precursor.[14] Currently, AzA has been widely utilized as a topical treatment for skin conditions such as acne and rosacea because of its antibacterial, antioxidant, and anti-inflammatory effects.[15−18] AzA has also demonstrated anticancer effects on a variety of tumors, including acute myeloid leukemia, lymphoma, and malignant melanoma.[19−21] Besides, AzA has been reported to be a ligand of ectopic olfactory receptor 544 (Olfr544) in mice, while activation of Olfr544 encourages glucagon-like peptide-1 (GLP-1) production and controls intestinal inflammation.[22] However, its role in IBD has not been established. We analyzed the metabolites from the

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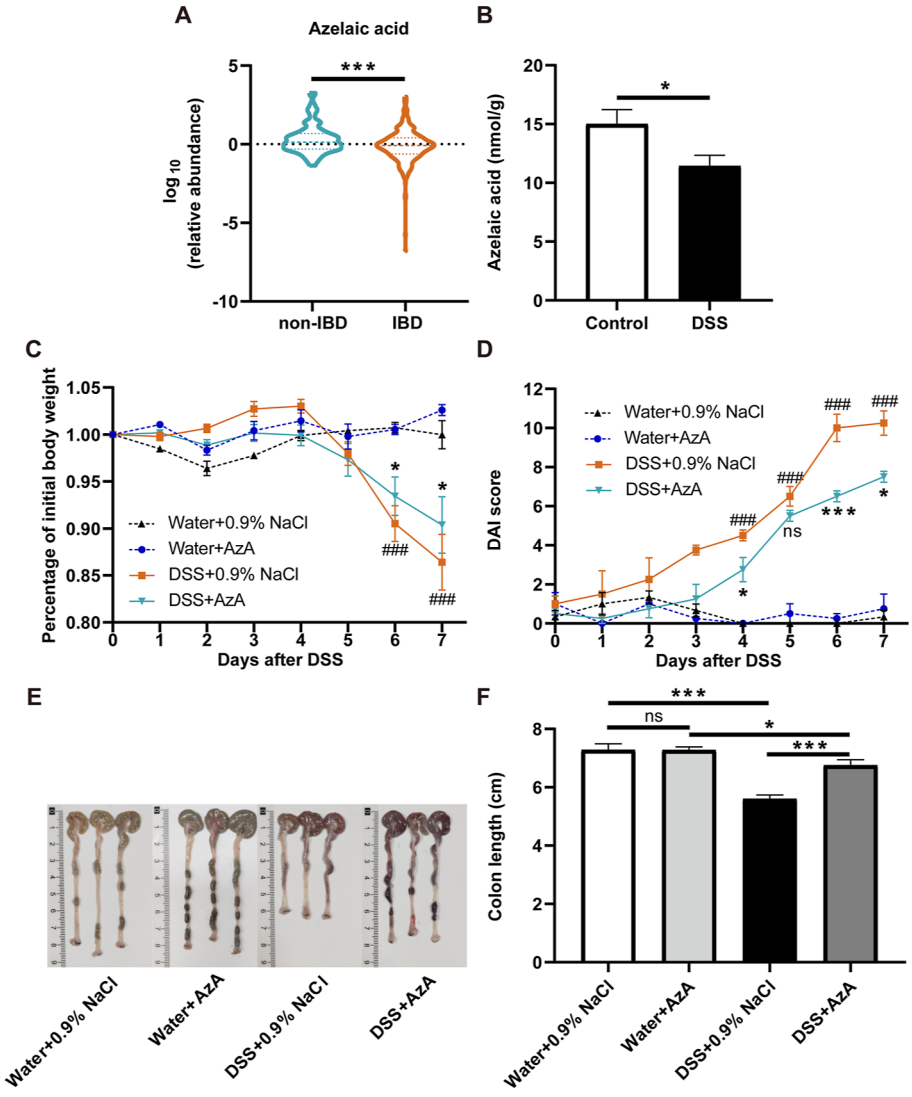

Figure 1. AzA supplementation aids in the prevention of colitis. (A) AzA relative abundance distributions in IBD patients' feces, expressed as a ratio to the mean relative abundance in individuals without IBD (***$P < 0.001$). (B) AzA concentration in mouse feces (*$P < 0.05$). (C) Changes in daily body weight from days 1 to 7. (D) DAI score. (E) Macrophotographs of colon tissues. (F) Colon's length (*$P < 0.05$ and ***$P < 0.001$). (B–F) Data is presented as the mean ± SEM ($n = 6$). Statistical analysis was performed with Student's t-test (A,B) or one-way ANOVA followed by the LSD test (C, D, F). (C,D) ###$P < 0.001$ vs the water + 0.9% NaCl group; *$P < 0.05$; and ***$P < 0.001$ vs the DSS + 0.9% NaCl group.

Metabolomics Workbench and found that AzA levels in the feces of dextran sulfate sodium (DSS)-induced mice and IBD patients are dramatically decreased. Experimental data suggests that AzA could alleviate DSS-induced colitis.

The network pharmacology analysis is used to further investigate how AzA affects IBD. Network pharmacology is a new subject based on the theory of systems biology, which offers deeper insights into the molecular mechanisms underlying drug actions. It is particularly well suited for the study of complex diseases affecting multiple organs, such as IBD.[23] Results show that the renin–angiotensin system (RAS) pathway is one of the most significantly enriched pathways affected by AzA. Nevertheless, whether AzA can regulate the RAS pathway has not been reported. The RAS is a complicated system involving various angiotensin peptides mediated by angiotensin converting enzyme (ACE) homologues, which act on various receptors.[24] The classical RAS pathway produces pro-inflammatory and profibrotic effects mainly via ACE and angiotensin II (Ang II), whereas the alternative RAS pathway, primarily through ACE2 and Ang (1–7), is in contrast with the role of the classical RAS pathway. The balance of these RAS components may play a critical role in the pathophysiology of IBD.[24]

In this paper, the possible mechanism of AzA in treating IBD has been explored based on network pharmacology and animal experiments. Our results suggest that AzA is a potential therapy for IBD.

2. RESULTS

2.1. AzA Is Reduced in IBD Patients and DSS-Induced Mice and Attenuates the Symptoms of DSS-Induced Mice. We analyzed metabolic data from the Metabolomics Workbench and found that AzA levels were considerably lower in the feces of IBD patients and mice induced by DSS (Figure 1A,B).

The signs of DSS-induced mouse colitis, which resembles IBD in humans and includes body weight loss, diarrhea, and bloody stools, are depicted in Figure 1C–F. To define the

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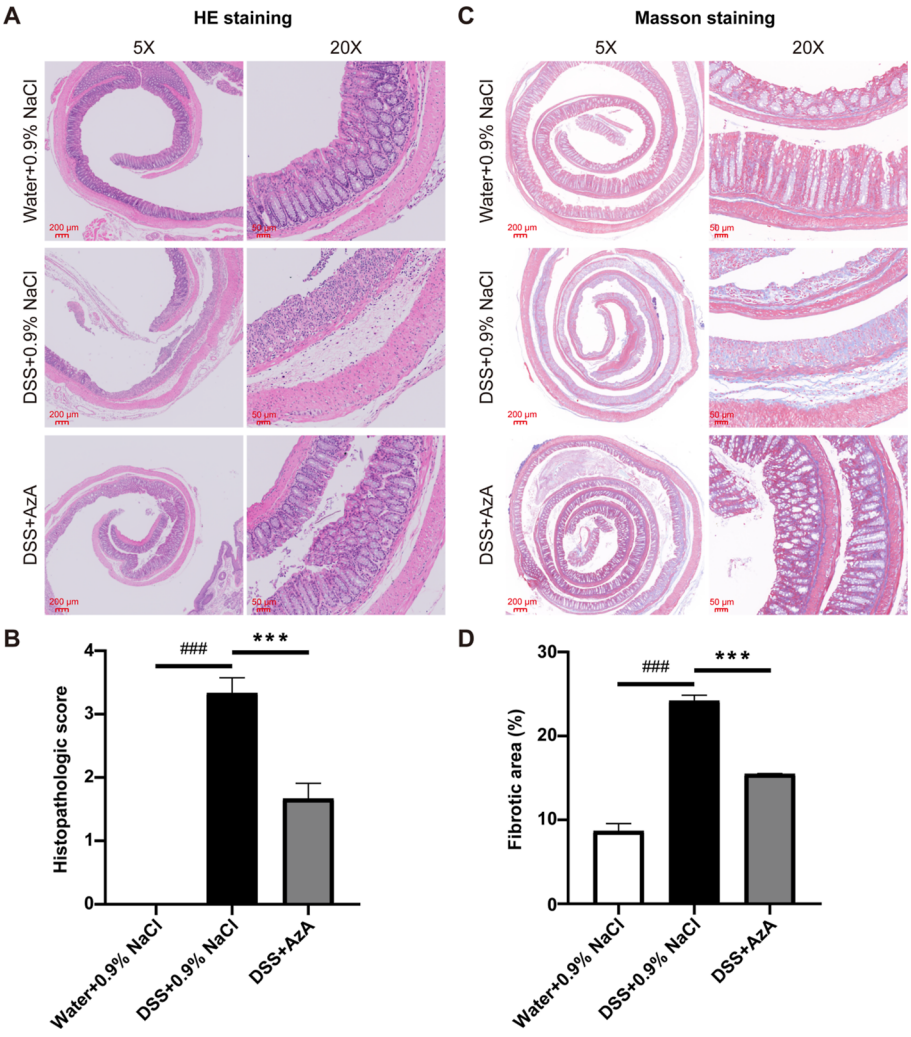

Figure 2. Protective effect of AzA on colon pathological damage in mice induced by DSS. (A) Representative H&E staining of colon tissue. (B) Histological score. (C) Masson staining for collagen deposition (blue). (D) Quantitative expressions of collagen fibers. Data is presented as the mean ± SEM (n = 6). Statistical analysis was performed with one-way ANOVA followed by the LSD test. ###P < 0.001 vs the water + 0.9% NaCl group and ***P < 0.001 vs the DSS + 0.9% NaCl group.

appropriate treatment dosages, we referred to previous studies on mice given oral AzA.[25,26] Preliminary tests with three separate doses of AzA revealed that AzA-treated mice at a dose of 150 mg/kg had considerably alleviated symptoms of DSS-induced colitis, including the weight loss, disease activity index (DAI), and colonic shortening (Figure S1). Thus, AzA at 150 mg/kg was used for all subsequent experiments. In addition, control mice exposed to AzA had no discernible side effects on their body weight, DAI score, or colon length (Figure 1C−F). The colon length of the DSS + AzA group differed from that of the AzA-exposed control mice (Figure 1E,F). These findings imply that AzA therapy offers significant protection against experimental colitis brought on by DSS.

2.2. AzA Inhibits Colon Tissue Pathological Injury. Mice from different groups were tested for colonic pathological alterations using Hematoxylin−Eosin (H&E) and Masson staining. As shown in Figure 2A, in the colon of DSS-induced mice, H&E staining demonstrated inflammatory cell infiltration, epithelial cell loss, and submucosal edema compared to the control group, leading to a higher microscopic score (Figure 2B). Meanwhile, Masson staining showed that the DSS group had more collagen deposited in the colon's mucosa and submucosa (Figure 2C). AzA therapy groups were shown to effectively repair the crypt architectural epithelia, relieve severe histologic inflammation, and decrease collagen deposition. The results of quantitative Masson staining analysis revealed that the region of collagen deposition was substantially reduced in the colon of AzA group mice compared to DSS mice (Figure 2D). These findings showed that AzA might greatly alleviate the colon's pathological damage in DSS-induced mice.

2.3. AzA's Impact on the Immune-Inflammation Status in DSS-Induced Colitis Mice. Pro-inflammatory cytokines are linked to the development of IBD in humans and are elevated in colitis brought on by DSS.[27,28] AzA dramatically downregulated the gene expression levels of main inflammatory cytokines, such as IL-1β, IL-6, TNF-α, IL-17A, and IL-22, which were elevated in the DSS model group (Figure 3A−E). The level of IL-6 in serum increased significantly after DSS induction but decreased after AzA treatment (Figure 3F).

Neutrophil infiltration was measured using myeloperoxidase (MPO) activity. As shown in Figure 3G, the colitis group showed much higher levels of MPO activity than the control group. However, AzA therapy substantially reduced the elevated MPO activity.

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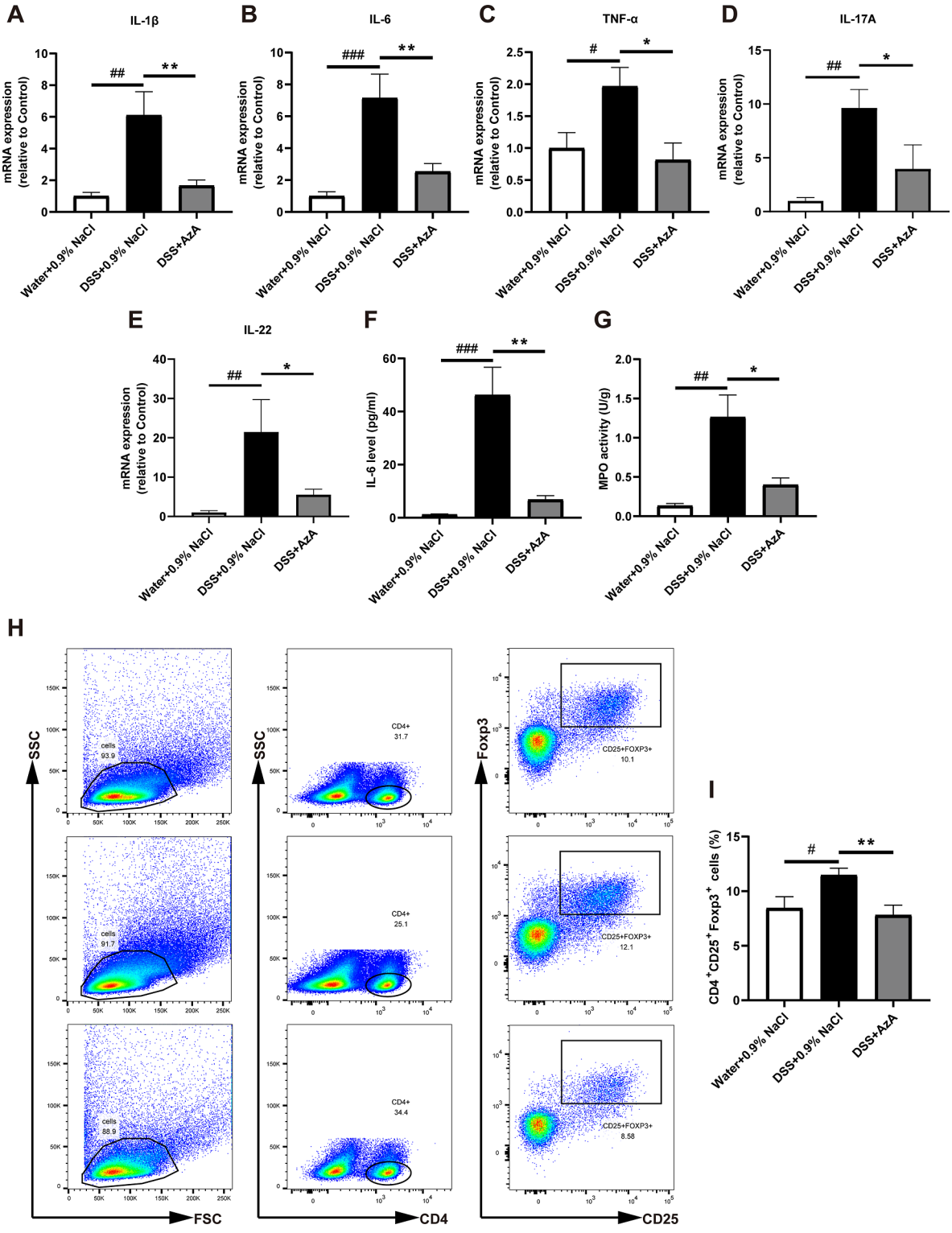

Figure 3. AzA's impact on the immune-inflammation status in DSS-induced colitis mice. The relative mRNA expression levels of the inflammatory cytokines IL-1β (A), IL-6 (B), TNF-α (C), IL-17A (D), and IL-22 (E) were assessed using RT-PCR. (F) Serum IL-6 levels in each group. (G) MPO activity in the colon. (H) The cells of the MLNs were stained with mAbs against mouse CD4, CD25, and Foxp3. Flow cytometry was used to analyze the stained cells. (I) The number of $CD4^+CD25^+Foxp3^+$Treg cells was calculated. Data is presented as the mean ± SEM ($n = 6$). Statistical analysis was performed with one-way ANOVA followed by the LSD test. #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$ vs the water + 0.9% NaCl group and *$P < 0.05$ and **$P < 0.01$ vs the DSS + 0.9% NaCl group.

Treg function has been related to the development of inflammatory diseases, such as IBD, and the $CD4^+CD25^+Foxp3^+$Treg subsets play an important role in regulating immune tolerance and homeostasis. Mesentery lymph node (MLN) cells treated with AzA were stained with fluorescence-conjugated monoclonal antibodies (mAbs) against CD4, CD25, and Foxp3 to examine $CD4^+CD25^+Foxp3^+$Treg subset levels using flow cytometry

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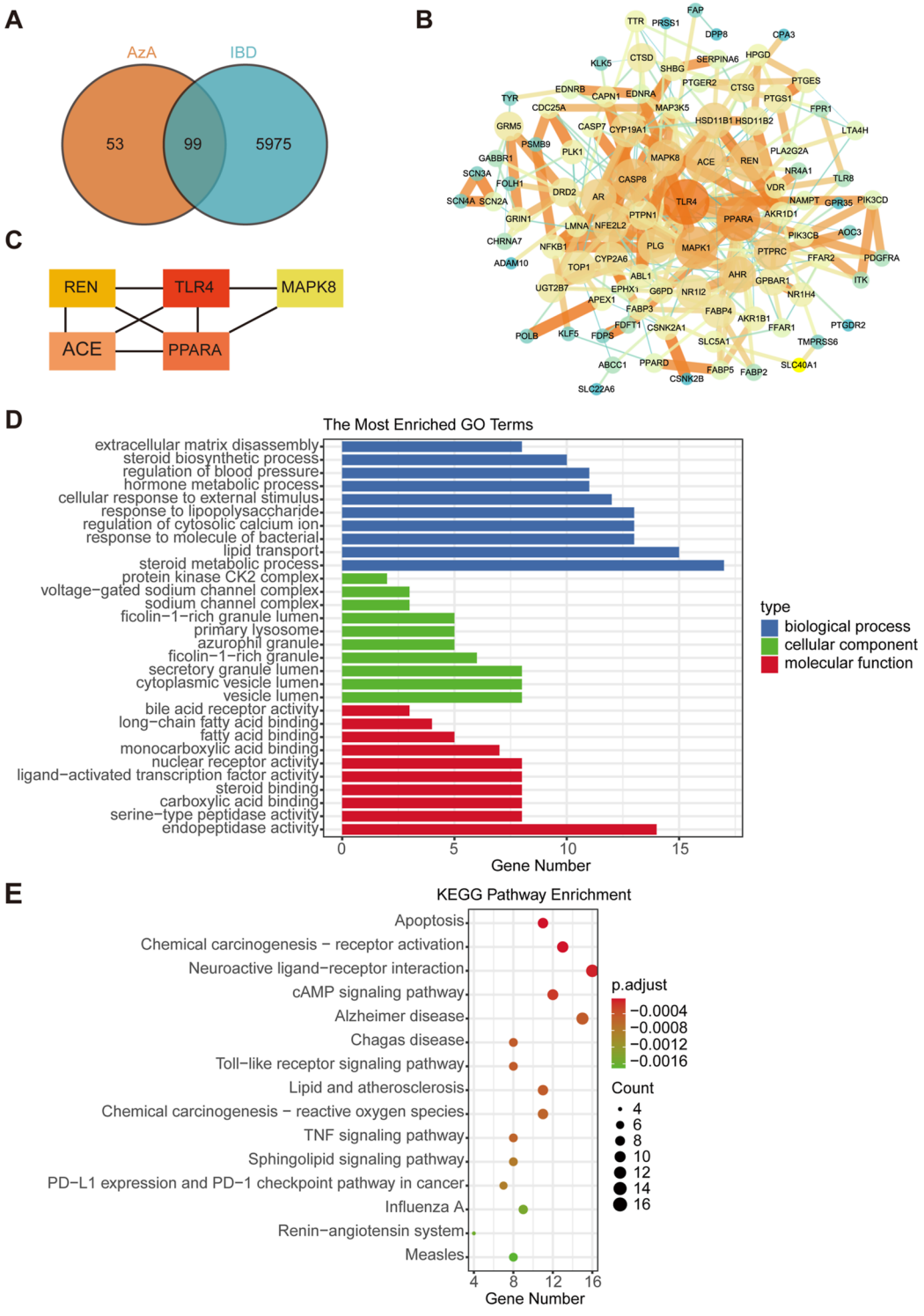

Figure 4. Analyses of the network pharmacology of AzA. (A) Venn diagram of targets of AzA against IBD. (B) The PPI network includes disease- and drug-related overlapping targets. The area of the circle was greater, and the color was darker when the connection value (degree) of a node relating to the gene was higher. (C) The top 5 targets of PPI network analysis were TLR4, PPARA, ACE, REN, and MAPK8. (D) Bar diagram of GO analysis function annotation. (E) Bubble diagram of KEGG pathway enrichment analysis.

(Figure 3H). MLNs from DSS-induced colitis mice treated with AzA had significantly fewer $CD4^+CD25^+Foxp3^+$ cells than those treated with vehicles (Figure 3I).

2.4. AzA Regulates Multiple Genes Related to IBD. Network pharmacology predicted the likely targeted proteins and pathways of AzA in order to study AzA's pharmacological mechanisms for treating IBD. A total of 152 targets were projected to interact with AzA, whereas 6074 targets were expected to relate to IBD. According to the Venn analysis diagram, 99 common AzA targets for the treatment of IBD were identified (Figure 4A).

The STRING database's protein−protein interaction (PPI) network revealed that these 99 common target genes' proteins have intricate connections (Figure 4B). For additional

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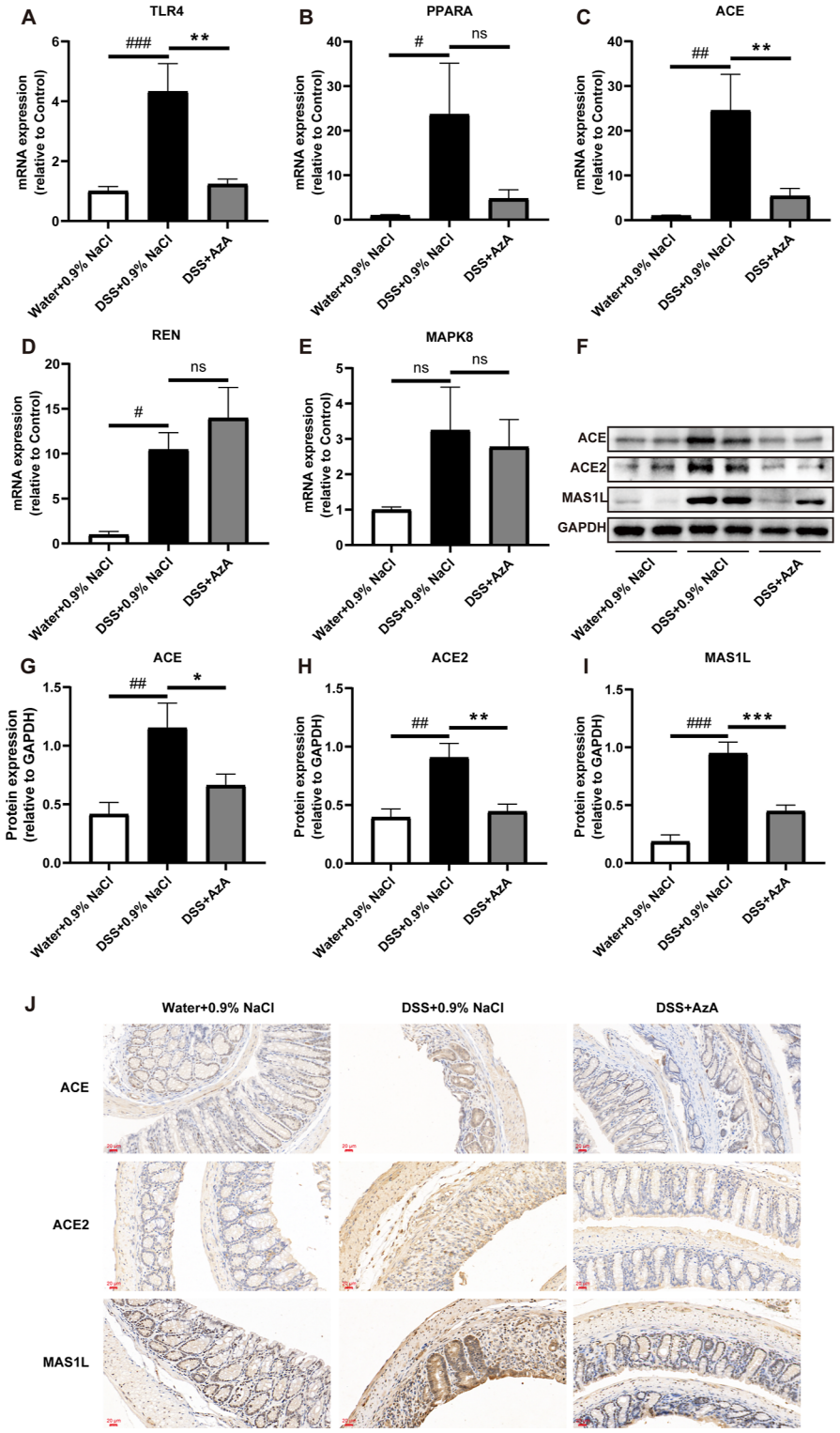

Figure 5. Effects of AzA on the RAS in DSS-induced colonic tissues. The mRNA expression of five hub genes, TLR4 (A), PPARA (B), ACE (C), REN (D), and MAPK8 (E), was identified by RT-PCR in mouse colon tissues. (F) ACE, ACE2, and MAS1L western blotting images. Changes in the relative protein expression levels of ACE (G), ACE (H), and MAS1L (I) were measured. (J) Representative pictures of ACE, ACE2, and MAS1L immunohistochemical staining are displayed (original magnification ×400, scale bar = 20 μm). Data is presented as the mean ± SEM (n = 6). Statistical analysis was performed with one-way ANOVA followed by the LSD test. #P < 0.05, ##P < 0.01, and ###P < 0.001 vs the water + 0.9% NaCl group and *P < 0.05, **P < 0.01, and ***P < 0.001 vs the DSS + 0.9% NaCl group.

investigation, the PPI network was imported into Cytoscape. We selected the top 5 genes as the hub genes based on their degree. These hub genes could serve as the primary molecular targets for AzA's anti-IBD actions. Consequently, the five key target proteins, TLR4, PPARA, ACE, REN, and MAPK8, were discovered (Figure 4C).

To determine the function of the potential targets, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and

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ACS Omega 2023, 8, 15217−15228

Genomes (KEGG) pathway enrichment analyses were performed. The GO study (Figure 4D) contains biological processes (BPs), cell components (CCs), and molecular functions (MFs). The top 15 signaling pathways for the AzA therapy of IBD were displayed (Figure 4E). Among these potential pathways, the RAS was investigated in terms of the potential pharmacological qualities of AzA. This was done in conjunction with the findings of the enrichment analysis and core targets.

2.5. AzA Regulates the RAS. The hub gene expression levels in the mouse colon were detected by quantitative real-time polymerase chain reaction (RT-PCR) (Figure 5A−E). In DSS-induced colitis mice, mRNA expression of TLR4, PPARA, ACE, and REN increased significantly in comparison to the control group, while MAPK8 increased slightly but not significantly. Compared with DSS induction, TLR4 and ACE levels were markedly reduced by the administration of AzA, while other hub genes were unaffected.

As depicted in Figure 5F−J, the protein expression of ACE, ACE2, and the Mas receptor (MAS1L) in colonic tissue was determined using immunohistochemistry and western blotting. ACE, ACE2, and MAS1L were found throughout the colon's layers, mostly in the enterocytes, according to immunohistochemical tests. The DSS-induced colitis group substantially increased the protein expression of ACE, ACE2, and MAS1L when compared to the control group. Nevertheless, AzA therapy markedly reduced the expression of these three molecules. Together, our findings show that AzA prevents DSS-induced colitis, probably by regulating the RAS.

3. DISCUSSION

AzA is an aliphatic dicarboxylic acid with antibacterial, antioxidant, and anti-inflammatory properties that are commonly used to treat various skin diseases.[15−18] Research has shown that individuals with IBD have lower levels of AzA in their stool.[11,12] AzA has been found to increase GLP-1 secretion and regulate intestinal inflammation,[22] but its effects on IBD have not yet been studied. In the current study, we provided data that suggest that AzA has an anti-inflammatory, immune-regulatory, and anti-fibrosis effect, providing evidence for AzA as a potential therapy for IBD.

We analyzed the fecal metabolomics data obtained from the Metabolomics Workbench and found that AzA was significantly reduced in the feces of IBD patients and DSS-induced mice, and our mouse model confirmed that AzA reversed the intestinal inflammation induced by DSS in mice. In our study, oral AzA treatment significantly reduced weight loss, colon shortening, and DAI scores caused by DSS and restored mucosal and crypt structures. AzA also dramatically decreased the overproduction of pro-inflammatory cytokines in DSS mice, including TNF-α, IL-1β, IL-6, IL-17A, and IL-22. These data suggest that oral AzA can reduce intestinal inflammation.

The current study also demonstrated the immune-regulatory ability of AzA. In the colons of the DSS mice treated with AzA, there was less neutrophil infiltration. Moreover, in autoimmune and chronic inflammatory diseases, such as IBD, $CD4^+CD25^+Foxp3^+$Treg subsets play an important role in regulating immune homeostasis and tolerance.[29−31] We observed fewer $CD4^+CD25^+Foxp3^+$ cells in the MLNs of DSS-induced colitis mice treated with AzA, suggesting that AzA appears to have protective benefits against the onset of colitis by lowering $CD4^+CD25^+Foxp3^+$Treg percentages.

Interestingly, we also showed that AzA significantly reduced DSS-caused collagen deposition, implying its potential as a treatment for intestinal fibrosis, which is a severe complication of IBD which severely impairs the patients' quality of life. We concluded from network pharmacology that AzA may be a novel regulator of the RAS. The experiment further shows that the main components of the RAS (ACE, ACE2, and MAS1L) are expressed in all layers of DSS mouse colon tissue, mainly in the epithelial layer, which is consistent with the results of intestinal tissue from IBD patients. The expression of ACE, ACE2, and MAS1L increased significantly after DSS induction and decreased significantly after AzA treatment. In our experiment, the change of MAS1L protein is inconsistent with that of IBD patients but consistent with that of DSS-induced colitis previously reported.[32,33] The relative involvement of two complementing RAS arms, which have conflicting roles in inflammation, fibrosis, cell proliferation, and angiogenesis, determines the final tissue homeostatic effect.[34−37] Several findings imply that the RAS is dysregulated in IBD and plays a role in the development of fibrosis.[32,38−40] Compared to IBD patients not receiving angiotensin receptor blockers (ARBs), individuals with IBD treated with ARBs have reduced mucosal pro-inflammatory cytokines.[41] In a retrospective cohort analysis, individuals on RAS blockers had lower disease activity indices and tended to need fewer hospital stays and surgical procedures for IBD and CD, respectively.[32] In a subsequent prospective trial, single factor analysis revealed that the likelihood of intestinal resection in individuals on RAS blockers for IBD had considerably decreased.[38] On the other hand, several animal models of IBD have shown an association between colitis and imbalances in RAS pathway components. Blocking the classical RAS may reduce the production of pro-inflammatory cytokines via ERK1/2, MAPK, Akt, and NF-κB[42] while also triggering regulatory T cells and modifying TH1- and TH17-mediated autoimmunity.[43] In acute and chronic animal models, blocking the classical RAS has been shown to decrease colon fibrosis by lowering TGF-β.[44−46] Additionally, classical RAS blockade may potentially diminish leukocyte recruitment by inhibiting the expression of mucosal addressing cell adhesion molecule-1 (MAdCAM-1), vascular cell adhesion molecule-1, and intracellular adhesion molecule-1.[47] Furthermore, inhibiting the classical RAS can improve oxidative stress by decreasing MPO activity and malondialdehyde levels while increasing glutathione levels.[48,49] The alternative pathway of ACE2/Ang (1−7)/MAS1L is generally considered to have anti-inflammatory and anti-fibrotic effects.[33,50] However, paradoxically, Byrnes et al. demonstrated that the ACE2 inhibitor GL1001 could improve DSS-induced colitis in mice.[51] In summary, RAS regulation may mitigate the harmful effects of colitis through various mechanisms, including anti-inflammatory and anti-fibrotic effects, inhibition of leukocyte recruitment, and reduction of oxidative stress. Our study shows that AzA could improve DSS-induced colitis in mice, probably by inhibiting the classical RAS and normalizing the alternative RAS.

AzA can also reduce the increase of TLR4 caused by DSS. TLR4 is a well-known inflammatory mediator that functions as a signaling molecule between innate and adaptive immunities and between inflammation and infection.[52] TLR4 has been recognized as the primary pattern recognition receptor and the canonical receptor for Gram-negative bacteria's lipopolysaccharide and has been implicated in the development of IBD.[53,54] Therefore, inhibiting TLR4 may be a potential

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strategy for treating IBD.[55,56] In cardiovascular and renal diseases, the RAS and TLR4 have been found to have cross-talk. Ang II can stimulate AT1R to release high-mobility group protein 1,[57] a TLR4 ligand that causes inflammatory responses.[58] Ang II also binds to the myeloid differentiator protein 2/TLR4 complex, triggering MyD88-dependent activation of mitogen-activated protein kinases (MAPKs), which promote local ACE/AT1R gene expression and Ang II production.[59−61] In recent years, parallel changes in the TLR4 and RAS have also been observed in intestinal diseases,[62,63] and Jaworska et al. linked the RAS and TLR4 in gut microbiota.[64] However, the specific mechanism is still unclear.

4. CONCLUSIONS

Our results indicate that AzA has protective effects on DSS-induced colitis as it reduces clinical disease activity, collagen deposition, pro-inflammatory cytokine production, and $CD4^+CD25^+Foxp3^+$Treg proportions in MLNs. We also found that AzA modulates the RAS pathway. These results provide evidence that AzA may be a potential therapy for IBD.

5. MATERIALS AND METHODS

5.1. Difference Analysis of AzA. Data and sample information from IBD patients and DSS-induced mice were downloaded from the Metabolomics Workbench (https://www.metabolomicsworkbench.org), where they are designated as PR000639 (https://doi.org/10.21228/M82T15) and PR000837 (https://doi.org/10.21228/M8H118). The human study population includes 546 stool samples (276 males and 270 females). Among them, 134 samples are from non-IBD, 266 samples are from CD, and 146 samples are from UC. For the mouse study, five samples each were in the DSS and control groups. The difference analysis of AzA was performed using MetaboAnalyst (https://www.metaboanalyst.ca/).

5.2. Animals. The experimental animal department at Central South University (Changsha, China) provided C57BL/6J male mice that weighed 20−22 g and were 7−8 week old. Mice were raised in specified pathogen-free environments with unrestricted access to food and water at 22 ± 2 °C on 12 h light/dark cycles. Euthanasia was evaluated based on the following humane endpoints: (1) weight loss of more than 20%; (2) loss of appetite; (3) inability to stand for 24 h; (4) body temperature below 37 °C; and (5) infection. The Medical Research Ethics Committee of Central South University gave its approval to all animal studies (no. CSU-2022-0385), which followed the guidelines of the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

5.3. DSS-Induced Colitis and AzA Treatment. After a week of acclimatization, mice were distributed at random into the following groups: water + 0.9% NaCl, DSS + 0.9% NaCl, and DSS + AzA (50 mg, 100 mg, and 150 mg/kg, respectively). The statistical significance of the experiment was ensured by having six animals per group. Colitis developed after 7 days of drinking water with 2.5% DSS (36−50 kD, MP Biomedicals, USA). Every 2 days, the DSS solution was changed. AzA (cat. no. V900595; Sigma-Aldrich, USA) was gavaged once per day during the early hours (between 9:00 and 10:00 am) in a saline suspension for 7 days (day 0 to day 6). Control mice were gavaged with AzA alone (i.e., the water + AzA group) to assess AzA toxicity.

Under anesthesia, blood samples were taken using a glass capillary from the retro-orbital venous plexus for 24 h following the last dose of drug administration. Then, mice were sacrificed via cervical dislocation, and their colons were removed and measured for length (between the ileocecal junction and the anus). Additionally, for histological analysis, 1−1.5 cm of the distal colon tissues was opened longitudinally and rolled into so-called Swiss rolls. For quantitative RT-PCR, the proximal 0.5 cm colon segment was used. The colonic tissues that were left were kept at −80 °C for further examination.

5.4. Evaluation of Clinical Scoring. Each group of mice was inspected every morning throughout therapy, and variations in body weight, diarrhea, and rectal bleeding were noted. A Hemoccult test kit was used to detect blood in the feces (Nanjing Jiancheng Bioengineering Institute, China). Changes in body weight were measured relative to the initial. The DAI was calculated using weight loss, stool consistency, and fecal blood.[65]

5.5. Hematoxylin−Eosin (H&E) and Masson Staining. The colons were handled as usual before being paraffin-embedded after being fixed in 4% formaldehyde. Following this, 5 μm thick colon slices were created and stained with Masson and H&E. The previously described criteria were used to calculate the histopathological scores.[66] In brief, the histological scores were assessed based on the level of inflammation, lymphocyte infiltration, crypt damage, surface epithelium loss, and the abnormal colon wall. Regarding Masson staining, the semi-quantitative expression of the collagen fibers presented in blue was calculated using ImageJ software.

5.6. Cell Isolation and Flow Cytometry. Cells from the MLNs were extracted as previously mentioned.[65] The mice's MLNs were gently broken up and run through a cell strainer with a 40 micron pore size. The cells were obtained and resuspended in cell staining buffer (Elabscience, China) for further staining. A Fixable Viability Kit (423102; BioLegend, USA) was used to stain live cells, and cell surfaces were stained with fluorescein isothiocyanate anti-mouse CD4 antibody (E-AB-F1097C; Elabscience, China) and allophycocyanin anti-mouse CD25 antibody (E-AB-F1102E; Elabscience, China). Fixation/permeabilization buffer (Thermo Scientific, USA) was used to permeabilize the cells, and the intracellular Foxp3 was stained using the phycoerythrin anti-mouse Foxp3 antibody (E-AB-F1238D; Elabscience, China). According to the manufacturer's instructions, the fluorescent antibodies were applied. A FACS Arial II flow cytometer (BD Biosciences, USA) was used to detect cells, and FlowJo software was used to conduct the analysis.

5.7. Measurement of Colonic MPO Activity. Homogenized colonic tissues in phosphate buffered saline (pH 7.4) were centrifuged for 15 min at 4 °C at 14,000×*g*. After this, the supernatants were applied to measure MPO activity using an assay kit (Nanjing Jiancheng Bioengineering Institute, China).

5.8. Enzyme-Linked Immunosorbent Assay. Mouse peripheral blood was centrifuged for 20 min at 2000×*g* at room temperature for the subsequent experiments. The ELISA kits (4A Biotech, China) were used to measure the amount of IL-6 in mouse serum.

5.9. RNA Extraction and RT-PCR. The Trizol reagent (TransGen Biotech, China) was used to lyse the 50 mg sample of mouse colon tissue to extract the RNA. The ratio of the absorbance at 260 nm to that at 280 nm was then used to

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ACS Omega 2023, 8, 15217−15228

calculate the quantity of extracted RNA. 1.0 g of total RNA was applied to synthesize complementary DNA (cDNA) using a Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The SYBR Green QPCR Master Mix (TransGen Biotech, China) and a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA) were applied to amplify cDNA. The target gene's mRNA expression was determined using the $2-\Delta\Delta Ct$ method, with GAPDH serving as the reference gene. Table 1 includes a list of the RT-PCR primers.

Table 1. Primers Used for RT-PCR Analysis

genes	primer	sequence (5′→3′)
IL-1β	forward primer	CTCACAAGCAGAGCACAAGC
	reverse primer	TCCAGCCCATACTTTAGGAAGA
IL-6	forward primer	TAGTCCTTCCTACCCCAATTTCC
	reverse primer	TTGGTCCTTAGCCACTCCTTC
IL-17A	forward primer	GCTCCAGAAGGCCCTCAGACT
	reverse primer	CCAGCTTTCCCTCCGCATTGA
IL-22	forward primer	TCAGTGCTAAGGATCAGTGCT
	reverse primer	TGATTGCTGAGTTTGGTCAGG
TNF-α	forward primer	TCAGCCTCTTCTCATTCCTG
	reverse primer	CAGGCTTGTCACTCGAATTT
TLR4	forward primer	GAGCCGGAAGGTTATTGTGGTAGTG
	reverse primer	AGGACAATGAAGATGATGCCAGAGC
PPARA	forward primer	TATTCGGCTGAAGCTGGTGTAC
	reverse primer	CTGGCATTTGTTCCGGTTCT
ACE	forward primer	CGATGTTAGAGAAGCCCACCGATG
	reverse primer	GATCCTGAAGTCCTTGCCGTTGTAG
REN	forward primer	GCGAGGTGGTGCTAGGAGGTAG
	reverse primer	CTTCATCGTGATCTGCCAGGAGTC
MAPK8	forward primer	TTGAAAACAGGCCTAAATACGC
	reverse primer	GTTTGTTATGCTCTGAGTCAGC
GAPDH	forward primer	TGGTCCTCAGTGTAGCCCAAG
	reverse primer	CTGCCCAGAACATCATCCCT

5.10. Network Pharmacology. Well-reported pharmacological targets of AzA were discovered using the DrugBank,[67] SuperPred,[68] and Swiss Target Prediction databases.[69] The gathered targets were classified as "AzA" after the duplicate targets were removed. "Inflammatory bowel disease" was used as a keyword to filter the results in the GeneCards,[70] OMIM,[71] and DisGeNET databases,[72] and the results were then immediately exported. To compare target data and standardize gene names, UniProt was employed. Utilizing Venn diagrams, all key targets of AzA and IBD were analyzed to determine possible targets for AzA against IBD.

After acquiring the combined targets of AzA and IBD, the PPI network was built using the online STRING database. Cytoscape, a program for the investigation and display of biological networks, was used to build all the networks. The degree, betweenness, proximity, and average shortest path length were the topological criteria employed by Cytoscape's MCODE plugin to evaluate the core network. The nodes in the network stand in for the targets, and the edges indicate the connections between them.

The R packages of "ClusterProfiler" were used to conduct an analysis of the functional enrichment of the GO and KEGG databases. Significant pathway terms were those with $P < 0.05$.

5.11. Western Blotting. The radioimmunoprecipitation assay lysis solution, including protease inhibitors, was used to lyse the colon tissue protein samples. Before being transferred to polyvinylidene fluoride membranes (Millipore, Germany), 50 μg of proteins was loaded into a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Following blocking with 5% non-fat milk, the membranes were then treated with primary antibodies overnight at 4 °C, including GAPDH mouse mAb (1:2000; cat. no. 60004-1-Ig; ProteinTech Group, China), ACE mouse mAb (1:500; cat. no. sc-23908; Santa Cruz Biotechnology, USA), ACE2 mouse mAb (1:100; cat. no. sc-390851; Santa Cruz Biotechnology, USA), and MAS1L rabbit polyclonal antibody (pAb) (1:2000; cat. no. 20080-1-AP; ProteinTech Group, China). After this, membranes were incubated for 1 h at 37 °C with matched secondary antibodies. An enhanced chemiluminescence detection system (Bio-Rad Laboratories, USA) was employed to identify protein bands. Relative protein expression was calculated and normalized to GAPDH expression using ImageJ software.

5.12. Immunohistochemistry. In order to assess the levels of ACE, ACE2, and MAS1L expression in the aforementioned formalin-fixed paraffin-embedded colonic tissue, immunohistochemistry was used. The steps in the process were as follows: The paraffin pieces were deparaffinized via baking and then dried using xylene and ethanol. After 20 min of microwave irradiation in citrate buffer (pH 6.0), the samples were subjected to antigen retrieval. Next, endogenous peroxidase activity was blocked by incubating the sections with 3% H_2O_2 for 15 min at room temperature, followed by blocking with 1% bovine serum albumin. Then, the sections were incubated with ACE rabbit mAb (1:200; cat. no. 24743-1-AP; ProteinTech Group, China), ACE2 rabbit pAb (1:500; cat. no. 21115-1-AP; ProteinTech Group, China), and MAS1L rabbit pAb (1:200; cat. no. 20080-1-AP; ProteinTech Group, China) overnight at 4 °C. Thereafter, the sections were exposed to the corresponding secondary antibody for 30 min at room temperature. 3,3′-Diaminobenzidine was used to measure the horseradish peroxidase activity for 30 s.

5.13. Statistical Analysis. Since every experiment was performed at least three times, the results are displayed as means ± standard error (SEM). Animals used for experiments were not excluded. Student's *t*-test was used to compare two groups. Statistical differences between multiple groups were compared by one-way ANOVA followed by the LSD test. A *P*-value less than 0.05 was considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c00210.

Therapeutic effect of AzA at various doses (50 mg, 100 mg, and 150 mg/kg) on DSS-induced colitis (PDF).

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Notes

The authors declare no competing financial interest.

"The gray mouse images were designed by brgfx/Freepik."

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