



Original Article



Arctigenin Prevents Metabolic Dysfunction-associated Steatohepatitis by Inhibiting NLRP3/GSDMD-N Axis in Macrophages

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Abstract

Background and Aims: Metabolic dysfunction-associated steatohepatitis (MASH) represents a critical step in the progression from simple fatty liver disease to more severe conditions such as cirrhosis and hepatocellular carcinoma, and it remains difficult to treat. Arctigenin (ATG), a monomer of *Fructus Arctii*, exhibits anti-inflammatory activity. Therefore, we aimed to examine its potential protective role against MASH and explore the underlying mechanisms. **Methods:** Male C57BL/6 mice were divided into four groups: control, MASH, low-dose ATG (30 mg/kg/day), and high-dose ATG (120 mg/kg/day). MASH was induced through a choline-deficient, L-amino acid-defined high-fat diet for eight weeks, with concurrent preventive ATG administration. Liver injury, lipid metabolism, inflammation, oxidative stress, and fibrosis were assessed. Network pharmacology was employed to identify the potential protective mechanisms of ATG. Key factors were evaluated *in vitro* to verify the ATG targets. **Results:** ATG administration prevented the progression of MASH in a dose-dependent manner. High-dose ATG significantly reduced hepatic macrophage and neutrophil infiltration, serum enzyme levels, and lipid peroxidation, while enhancing antioxidant enzyme activity. Mechanistic network pharmacology identified modulation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome as the central pathway underlying ATG's bioactivity. Functional analyses in lipopolysaccharide-stimulated RAW264.7 cells confirmed that ATG inhibited NLRP3 expression, pyroptosis-related protein cleavage (hereinafter referred to as GSDMD-N), and pro-inflammatory chemokine production in a concentration-dependent manner. Notably, ATG disrupted NLRP3/GSDMD-

N axis activity in macrophages without causing cellular toxicity. **Conclusions:** ATG may inhibit the inflammatory cascade primarily by targeting macrophage NLRP3 inflammasomes, thereby preventing the progression of MASH.

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Introduction

Metabolic dysfunction-associated fatty liver disease (MAFLD), previously referred to as nonalcoholic fatty liver disease (NAFLD), accounts for the largest proportion of chronic liver disease worldwide and affects diverse populations across all age groups.¹ MAFLD comprises a spectrum of liver disorders, including metabolic dysfunction-associated fatty liver (MAFL) and metabolic dysfunction-associated steatohepatitis (MASH). MASH can progress to liver fibrosis or even hepatocellular carcinoma.² Although recent reports suggest that resmetirom, a thyroid receptor β agonist, may improve MASH, options for clinical intervention remain limited, and further research is necessary.

Recent studies have highlighted the pivotal role of hepatic macrophages in the progression of MAFLD,³ as their interactions with other hepatic cells can amplify inflammation and promote fibrosis. Macrophages, which are derived from resident Kupffer cells and circulating monocytes, can acquire either anti-inflammatory (M2) or pro-inflammatory (M1) phenotypes upon exposure to environmental stimuli.⁴ M1 macrophages exacerbate liver inflammation by producing inflammation-inducing cytokines that facilitate immune responses.^{5–8} Conversely, reducing macrophage infiltration or inducing macrophage polarization toward the M2 phenotype can improve MAFLD.^{6,9–12} These findings underscore macrophages as key therapeutic targets for MASH.

Traditional Chinese medicine and various monomeric compounds extracted from Chinese herbal medicines have garnered attention in recent decades for the prevention

Keywords: Metabolic dysfunction-associated steatohepatitis; Arctigenin; Inflammation; Macrophage; NLR family pyrin domain containing 3; c-FOS.

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and treatment of MAFLD.¹³ Arctigenin (ATG), a glycoside compound derived from *Arctium lappa*, has demonstrated pharmacological effects, including improving glucose tolerance and lipid metabolism, as well as mitigating inflammation.^{14–17} However, its effects on MASH remain largely unexplored. Previous studies suggest that ATG can suppress inflammation and lipid peroxidation in hepatocytes, providing evidence of its ability to alleviate liver injury.¹⁷ Furthermore, ATG reportedly inhibits the assembly of the NLR family pyrin domain containing 3 (NLRP3) inflammasome in colonic macrophages, possibly by downregulating carnitine palmitoyltransferase-1 expression.¹⁸ Thus, ATG may regulate inflammatory and oxidative stress pathways, particularly in macrophages, which play a central role in the progression of MAFL to MASH.

Therefore, we established a rodent model using a choline-deficient, L-amino acid-defined high-fat diet (CDAHFD) and conducted an *in vivo* study to explore whether ATG plays a preventive role in MASH. Mechanistic analyses of ATG treatment were performed, focusing on the regulation of the NLRP3 inflammasome, which mediates hepatic inflammation and fibrosis.

Methods

Animal study

Four groups ($n = 10$ per group) were randomly assigned from 40 male C57BL/6 mice, aged eight weeks and weighing 20–25 g. The animals were housed in an environment with a specific circadian rhythm (12 h of light and 12 h of darkness) and a controlled temperature (21–23°C). They had access to food and water *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committee of Xinhua Hospital affiliated to Shanghai Jiao Tong University School of Medicine.

Following a week of adaptive feeding, the 40 mice were randomized into the following groups: control, model, low-dose ATG treatment (30 mg/kg per day), and high-dose ATG treatment (120 mg/kg per day) (Cat: HY-N0035, Med Chem Express®, Shanghai, China). The control group was exposed to a standard chow diet and vehicle treatment (10% dimethyl sulfoxide (DMSO)) (Cat: 67-68-5, Sigma-Aldrich®, Missouri, USA) for eight weeks. The model group was fed a CDAHFD (Cat: TP36225MCD, Trophic Co., Nantong, China) during the same period, with vehicle therapy (10% DMSO). The two treatment groups were also fed CDAHFD *ad libitum*, with intragastric administration of 30 or 120 mg/kg ATG. Both vehicle and ATG treatments were administered once daily *via* intragastric injection for eight weeks.

After 12 h of fasting and under isoflurane anesthesia, all animals were sacrificed at the end of the 8th week. Blood samples were collected from the retroorbital sinus. Cervical dislocation was performed, and the livers were immediately dissected on ice and weighed. Before analysis, liver samples were snap-frozen in liquid nitrogen and stored at –80°C. Additional liver samples were fixed in 4% formalin for subsequent experiments.

Biochemical analyses

Serum was extracted from blood samples by centrifugation for 15 min at 4°C and 4,000 rpm. The activities of alanine aminotransferase (Cat: 20152400366, ShenSuoYoufu Medical Diagnostic Products, Shanghai, China) and aspartate transaminase (Cat: 20152400367, ShenSuoYoufu Medical Diagnostic Products, Shanghai, China)¹⁹ were measured using kits according to the manufacturer's instructions. The

serum levels of free fatty acid (FFA) (Cat: 633-52001, FUJIFILM Wako Pure Chemical Corporation, Japan)²⁰ and very low density lipoprotein (VLDL) (Cat: ml037709, mlbio, Shanghai, China)²¹ were measured using the respective kits. Hepatic levels of triglyceride (TG) (Cat: E1015) and total cholesterol (Cat: E1015) were quantified using assay kits (Applygen Technologies Inc., Beijing, China).²² Malondialdehyde (MDA) (Cat: A003-1-2), catalase (CAT) (Cat: A007-1-1), and superoxide dismutase (SOD) (Cat: A001-1-1) levels in the liver were analyzed using commercial kits (Nanjing Jiancheng Bio-engineering Institute, Nanjing, China).²³ Briefly, liver samples were homogenized on ice, and supernatants were collected after centrifugation for subsequent analysis. The total protein concentration in the samples was used as a reference for final concentrations.

Histological determination

Following paraformaldehyde fixation, each liver tissue sample was embedded in paraffin and sliced.²⁴ Hematoxylin & eosin staining, Masson's trichrome staining, and Sirius red staining were performed using commercial kits (Servicebio®, Wuhan, China). Sections frozen in optimum cutting temperature compound were snap-frozen in liquid nitrogen and subjected to Oil Red O staining using a commercial kit (Servicebio®, Wuhan, China) according to the manufacturer's guidelines. The NAFLD activity score (NAS), which evaluates lobular inflammation, hepatocyte ballooning, and steatosis, was used for histological analysis.²⁵

Immunohistochemical assays

Paraffin sections were deparaffinized and rehydrated, and antigen retrieval was performed using citrate buffer (0.01 M, pH 6.0).²⁶ The sections were incubated overnight with antibodies specific to F4/80 (GB11027, Servicebio®, 1:500), myeloperoxidase (GB150006, Servicebio®, 1:500), and alpha-smooth muscle actin (α -SMA) (GB111364, Servicebio®, 1:300), respectively. After incubation with the secondary antibody conjugated with HRP for 60 min at room temperature, the sections were washed three times for 5 min each using phosphate-buffered saline (PBS). The DAB chromogen reaction was stopped by washing with running water. Hematoxylin counterstaining was applied for 30 s, followed by rinsing with water, dehydration, and mounting with resin. The slides were viewed under a digital microscope camera (Eclipse E100, Nikon®, Japan). The acquired images were analyzed using ImageJ 1.46r software (National Institutes of Health, Bethesda, MD, USA).

Real-time quantitative polymerase chain reaction

Total RNA was extracted from liver tissues or cells using an RNA purification kit (Cat: B0004D, Ezbioscience®, Roseville, USA) and quantified using the QuantStudio Dx system (Applied Biosystems®, Waltham, Massachusetts, USA).²² DNA templates were obtained by reverse transcription of RNA using a color reverse transcription kit (Cat: A0010CGQ, Ezbioscience®, Roseville, USA).²² Relative mRNA expression was quantified using the SYBR GREEN Master Mix reagent kit (Cat: A0012-R2, Ezbioscience®, Roseville, USA). Gene expression levels were normalized against 18S rRNA expression. The $2^{-\Delta\Delta Ct}$ method was used for data analysis.²⁷ Primer sequences are listed in Table 1.

Immunofluorescence assays

Liver tissue sections were prepared by deparaffinization, rehydration, and antigen retrieval with EDTA buffer (0.01 M, pH 9.0), followed by PBS washes. Endogenous peroxidase

Table 1. Primers for real-time quantitative polymerase chain reaction (mus)

Gene name	Forward primer	Reverse primer
α-SMA	5'-TGCTTCCTCCTCCTCTTG-3'	5'-GAAGTACTGCCGTTTTCCCC-3'
TGF-β	5'-CTGAACCAAGGAGACGGAATA-3'	5'-GGAAGGGCCGGTTCATGT-3'
VIMENTIN	5'-CGGAAAGTGGAATCCTTGCAGG-3'	5'-AGCAGTGAGGTCAGGCTTGGA-3'
COL1A1	5'-CCAGCAAACAAAGGCAATGC-3'	5'-GGTGCTGGGTAGGGAAGTAG-3'
COL2A1	5'-CAACTCAGCTCGCCTTCATG-3'	5'-CTCATCCAGGTACGCAATGC-3'
COL3A1	5'-TGACTGTCCACGTAAGCAC-3'	5'-GAGGGCCATAGCTGAAGTGA-3'
NLRP3	5'-TCACAACGCGCCAAAGGAGGAA-3'	5'-AAGAGACCACGGCAGAAGCTAG-3'
IL-1β	5'-TCCAGGATGAGGACATGAGCAC-3'	5'-GAACGTCACACACCAGCAGGTTA-3'
iNOS	5'-GAGACAGGGAAGTCTGAAGCAC-3'	5'-CCAGCAGTAGTTGCTCCTCTTC-3'
TNF-α	5'-CAGGAGGGGAGAACAGAACTCCA-3'	5'-CCTGGTTGGCTGCTTGCTT-3'
CYBA	5'-GCTCATCTGTCTGCTGGAGTATC-3'	5'-GGACGTAGTAATTCCTGGTGAG-3'
NOX2	5'-TGGCGATCTCAGCAAAAGGTGG-3'	5'-GTACTGTCCACCTCCATCTTG-3'
NRF2	5'-AGAACGAGAGGACACCTGGTCA-3'	5'-GCTTCTGGGATGCTGGAAACG-3'
CCL2	5'-TTAAAAACCTGGATCGGAACCAA-3'	5'-TTAAAAACCTGGATCGGAACCAA-3'
CXCL1	5'-TCCAGAGCTTGAAGGTGTTGCC-3'	5'-AACCAAGGGAGCTTCAGGGTCA-3'
CXCL3	5'-TGAGACCATCCAGAGCTTGACG-3'	5'-CCTTGGGGGTTGAGGCAAACTT-3'
CXCL5	5'-CCGCTGGCATTCTGTTGCTGT-3'	5'-CAGGGATCACCTCCAAATTAGCG-3'
CXCL15	5'-GGTGATATTCGAGACCATTACTG-3'	5'-GCCAACAGTAGCCTTCACCCAT-3'
SREBF1	5'-CGACTACATCCGCTTCTTGACG-3'	5'-CCTCCATAGACACATCTGTGCC-3'
FASN	5'-CACAGTGCTCAAAGGACATGCC-3'	5'-CACCAGGTGTAGTGCCTTCCTC-3'
PPARα	5'-ACCACTACGGAGTTCACGCATG-3'	5'-GAATCTTGACGCTCCGATCACAC-3'
CPT1α	5'-GGCATAAACGCAGAGCATTCTG-3'	5'-CAGTGTCCATCCTCTGAGTAGC-3'
CD206	5'-GTTACCTGGAGTGATGGTTCTC-3'	5'-AGGACATGCCAGGGTCACCTTT-3'
ARG1	5'-CATTGGCTTGCGAGACGTAGAC-3'	5'-GCTGAAGGTCTCTCCATCACC-3'
CD36	5'-GGACATTGAGATTCTTTCTCTG-3'	5'-GCAAAGGCATTGGCTGGAAGAAC-3'
ACSL1	5'-ATCAGGCTGCTTATGGACGACC-3'	5'-CCAACAGCCATCGCTTCAAGGA-3'
FATP1	5'-TGCCACAGATCGGCGAGTTCTA-3'	5'-AGTGGCTCCATCGTGTCTCAT-3'
ACOX1	5'-GCCATTGATACAGTGCTGTGAG-3'	5'-CCGAGAAAGTGGAAGGCATAGG-3'
SLC27A5	5'-CTGCGGTACTTGTGTAACGTCC-3'	5'-TCCGAATGGGACCAAGCGTTG-3'
ACC1	5'-GTTCTGTTGGACAACGCCTTCAC-3'	5'-GGAGTCACAGAAGCAGCCCAT-3'
APOB	5'-GCATGAGTATGCCAATGGTCTCC-3'	5'-CTGGTTGCCATCTGAAGCCATG-3'
APOE	5'-GAACCGCTTCTGGGATTACCTG-3'	5'-GCCTTTACTTCCGTATAGTGTC-3'
MTTP	5'-CCAGGAAAGGTTCTCTATGCC-3'	5'-GACTCTCTGATGTCGTTGCTTGC-3'
TM6SF2	5'-GGTATTTGCTGGAGCCATTGGC-3'	5'-CCAGTGCCAATAGCAGGTTGCT-3'
PNPLA3	5'-AGACAAGGTGCCAGTCAGCCTA-3'	5'-GAGGTTGCAGACTTTGCTCAGG-3'
SPTLC2	5'-CCAGACTGTCAGGAGCAACCAT-3'	5'-CTTCTGTCCGAGGCTGACCAT-3'
ACER2	5'-GAGGACAACTACACTATCGTGCC-3'	5'-TAGATGCCGCTGTTGAAGCACG-3'
ASAHI	5'-GGATGTTGGAAGGAAAGATGCC-3'	5'-AACCTCTCCAGACTTCTTGCC-3'
CERS2	5'-CCTTCTACTGGTCCCTGCTCTT-3'	5'-TGGCAAACCAGGAGAAGCAGAG-3'
CYP7A1	5'-CACCATTCTGCAACCTTCTGG-3'	5'-ATGGCATTCCCTCCAGAGCTGA-3'

(continued)

Table 1. (continued)

Gene name	Forward primer	Reverse primer
HMGR	5' -GCTCGTCTACAGAACTCCACG-3'	5' -GCTTCAGCAGTGCTTTCTCCGT-3'
SGMS1	5' -GCATAGTTGGCAGCTGTACCT-3'	5' -TAAGCCACCTCCAGCAATGAGC-3'
18S	5'-ACGGAAGGGCACCACCAGGA-3'	5'-CACCACCACCCACGGAATCG-3'

α -SMA, alpha-smooth muscle actin; TGF- β , transforming growth factor-beta; COL1A1, collagen type I alpha 1 chain; COL2A1, collagen type II alpha 1 chain; COL3A1, collagen type III alpha 1 chain; IL-1 β , interleukin-1 β ; iNOS, inducible nitric oxide synthase; TNF- α , tumor necrosis factor- α ; CYBA, cytochrome B-245 alpha chain; NOX2, NADPH oxidase 2; NRF2, nuclear factor erythroid 2-related factor 2; CCL2, C-C motif chemokine 2; CXCL1, C-X-C motif chemokine ligand 1; CXCL3, C-X-C motif chemokine ligand 3; CXCL5, C-X-C motif chemokine ligand 5; CXCL15, C-X-C motif chemokine ligand 15; SREBF1, sterol regulatory element binding transcription factor 1; FASN, fatty acid synthase; PPAR α , peroxisome proliferator activated receptor alpha; CPT1 α , carnitine palmitoyltransferase 1 alpha; ARG1, arginase 1; CD36, cluster of differentiation 36; ACSL, acyl-CoA synthetase long chain family member 1; FATP1, fatty acid transport protein 1; ACOX1, acyl-CoA oxidase 1; SLC27A5, solute carrier family 27 member 5; ACC1, acetyl-CoA carboxylase 1; APOB, apolipoprotein B; APOE, apolipoprotein E; MTP, microsomal triglyceride transfer protein; TM6SF2, transmembrane 6 superfamily member 2; PNPLA3, patatin like domain 3, 1-acylglycerol-3-phosphate o-acyltransferase; SPTLC2, serine palmitoyltransferase long chain base subunit 2; ACER2, alkaline ceramidase 2; ASAH1, N-acylsphingosine amidohydrolase 1; CERS2, ceramide synthase 2; CYP7A1, cytochrome P450 family 7 subfamily A member 1; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; SGMS1, sphingomyelin synthase 1.

activity was suppressed with 3% hydrogen peroxide, and the sections were blocked with 3% BSA. Primary antibodies against CD86 (19589, Cell Signaling Technology, CST®; 1:400 in 3% BSA) and α -SMA (ab124964, Abcam®; 1:2,000 in 3% BSA) were incubated overnight. After PBS washes, secondary antibody (ab205718, Abcam®; 1:4,000 in PBS) was applied at 37°C for 45 m, followed by CY5 tyramide staining (11066, AAT Bio®; 1:400 in 0.003% H₂O₂) for 10 m. The slides were washed, stripped in 42°C stripping buffer for 20 m, and re-blocked. The sections were then incubated overnight with primary antibodies against NLRP3 (PA5-79740, ThermoFisher®; 1:100 in 3% BSA). After PBS washes, the secondary antibody (Alexa Fluor™ 488-conjugated donkey anti-rabbit IgG, A21206, ThermoFisher®; 1:400 in PBS) was applied at 37°C for 45 m. Each sample was stained with DAPI, followed by washing with water to terminate the reaction. Fluorescence images were acquired using a BX53 microscope (Olympus®, Japan) and analyzed using ImageJ 1.46r (National Institutes of Health, Bethesda, MD, USA).²⁸

Target analysis of ATG

PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) provides the chemical structure and canonical SMILES of ATG. These data were subsequently entered into multiple target prediction tools, including the PharmMapper server (<https://www.lilab-ecust.cn/pharmmapper/>),²⁹ Similarity Ensemble Approach (<https://sea.bkslab.org/>),³⁰ and SwissTargetPrediction (<http://www.swisstargetprediction.ch/>),³¹ to identify potential molecular targets associated with ATG. Additional targets were obtained from relevant scientific literature. Overlapping targets between ATG and MAFLD were identified using the Venny online tool (<https://cloud.oebiotech.com/#/bio/tools>). Protein-protein interaction networks for the intersecting targets were analyzed using the STRING database (<https://string-db.org/>) and subjected to visualization and topological analyses using Cytoscape 3.7.2.³² Further investigation of the molecular mechanisms underlying ATG's therapeutic effects on MAFLD was conducted through pathway enrichment based on the Kyoto Encyclopedia of Genes and Genomes database, Reactome pathway enrichment analysis, and Gene Ontology analysis. These analyses were performed using the Oebiotech bioinformatics platform (<https://cloud.oebiotech.com/#/bio/tools>), focusing on molecular functions, cellular components, biological processes, and pathways relevant to MAFLD-associated targets. The Liver Cell Atlas (<https://livercellatlas.org/>) was used to analyze NLRP3 expression in different liver cells based on data provided by the Liver Cell Atlas (Mouse NAFLD Atlas).³³ Molecular docking was carried out via CB-Dock2 (<https://cadd.labshare.cn/cb-dock2/index>).

php).³⁴ Transcription factors of the NLRP3 gene were predicted using the UCSC-JASPAR (<https://genome.ucsc.edu/index.html>),³⁵ Chip-Atlas (<https://chip-atlas.org/>),³⁶ Cistrome DB (<http://cistrome.org/db/>),³⁷ hTFtarget (<https://guolab.wch-sc.cn/hTFtarget/#/>),³⁸ and NetworkAnalyst (<https://www.networkanalyst.ca/>)³⁹ databases.

Western blotting

Phosphatase inhibitors (Cat: 20109ES05, Yeasen®, Shanghai, China) along with a protease inhibitor cocktail (Cat: ST505, Beyotime®, Shanghai, China) were added to RIPA buffer before the liver tissues were lysed. The BCA assay (Cat: P0012, Beyotime®, Shanghai, China) was employed to determine the total protein content. Proteins separated by SDS-PAGE were transferred onto polyvinylidene difluoride (PVDF) membranes using a small vertical gel electrophoresis machine (Tanon®, Shanghai, China).⁴⁰ After blocking with 5% fat-free milk, the membranes were incubated with primary antibodies overnight at 4°C. Horseradish peroxidase-labeled secondary antibodies were used for detection. Chemiluminescent signals were collected and assessed using the ChemiDOC XRS+ imaging system (Bio-Rad Laboratories®, California, USA) and Image Lab 6.1.0 software. The following antibodies were used for Western blot analysis: NLRP3 (ab263899, Abcam®), GAPDH (2118, CST®), interleukin (IL)-1 β (12242, CST®), Gasdermin D (GSDMD) (39754, CST®), c-Fos (66590-1-Ig, Proteintech®), α -Tubulin (66031-1-Ig, Proteintech®), and Lamin B1 (AF1408, Beyotime®). GAPDH, α -Tubulin, and Lamin B1 served as controls.

Cell culture, cell viability assay, and treatment

Mouse monocytic macrophage RAW264.7 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM with 10% fetal bovine serum (Cat: 16000-044, Gibco®, Waltham, Massachusetts, USA) and penicillin/streptomycin (Cat: 60162ES76, YEASEN®, Shanghai, China).

Cell viability was evaluated using the Cell Counting Kit-8 assay (Cat: C0037, Beyotime®, Shanghai, China). Briefly, RAW264.7 cells were seeded at 1.5×10^4 cells/well in 96-well plates, and dose ranges from 0–100 μ M ATG were added to the plates for 24 h. Following treatment, each well received 10 μ L of the Cell Counting Kit-8 reagent and was incubated at 37°C for an additional hour. Absorbance was measured at 450 nm.⁴¹ Cells were exposed to 100 ng/mL lipopolysaccharide (LPS) (Cat: 297-473-0, Sigma-Aldrich®, Missouri, USA) for 24 h, either with or without varying doses of ATG, to induce inflammation.

Cellular thermal shift assay (CETSA)

RAW264.7 pellets were collected and washed twice with ice-cold PBS, followed by resuspension in protein lysis buffer (containing 1× protease inhibitor cocktail) and incubation on ice for 15 m. Cell lysis was achieved through sonication, and the lysates were clarified by centrifugation (20,000 × g, 10 m, 4°C) to collect the supernatant. After quantifying the protein concentration using the BCA assay, samples were diluted with distilled water to achieve uniform protein concentrations. The normalized lysates were equally divided into two aliquots: one treated with ATG (100 μM final concentration) and the other with an equal volume of DMSO. After 1 h of incubation at room temperature, each aliquot was subdivided into five equal portions and subjected to 10 m incubations at distinct temperatures (49°C, 52°C, 55°C, 58°C, or 61°C), then immediately chilled on ice. Post-heating clarification was performed by centrifugation (20,000 × g, 10 m, 4°C), and the soluble protein supernatants were mixed with equal volumes of 2× loading buffer, denatured at 99°C for 10 m, and stored at −80°C prior to Western blot analysis of NLRP3 expression.⁴²

Nuclear and cytoplasmic protein extraction

RAW264.7 cells were seeded into 6-well plates at 2×10⁵ cells/well and allowed to attach for 24 h. The cells were then divided into three groups: Control group, LPS-treated group (100 ng/mL LPS), and ATG-treated group (100 μM) for 4 h, followed by washing twice with ice-cold PBS. The Nuclear and Cytosolic Protein Extraction Kit (Cat: P0027, Beyotime®, Shanghai, China) was used to isolate cytosolic and nuclear proteins.⁴³ First, the cells were collected into 1.5 mL micro-centrifuge tubes. Cytosolic protein extraction reagent A, containing protease inhibitor cocktail and phosphatase inhibitor cocktail, was added to each tube. Vortex mixing was performed for 5 s at the highest speed, followed by incubation on ice for 10–15 m. Cytoplasmic protein extraction reagent B was added, followed by vortex mixing at maximum speed for 5 s, then an ice bath for 1 m. After vortex mixing for 5 s, centrifugation was performed at 12,000–16,000 g at 4°C for 5 m. The supernatant containing the cytoplasmic proteins was aspirated into a pre-cooled tube. For the pellet, residual supernatant was aspirated, and 50 μL of nuclear protein extraction reagent, containing protease inhibitor cocktail and phosphatase inhibitor cocktail, was added. The mixture was vortexed for 15–30 s and placed in an ice bath. Every 1–2 m, vortex mixing was repeated vigorously at high speed for 30 m. Finally, centrifugation was performed at 12,000–16,000 g for 10 m at 4°C, and the supernatant containing the nuclear protein was aspirated into a pre-cooled tube. Cytosolic and nuclear fractions were stored at −80°C until required.

Statistical analysis

Data are presented as mean ± standard error. The statistical significance of the parametric variables was investigated using the unpaired Student's t-test between two groups, and one-way ANOVA for comparisons among multiple groups. Multiple comparisons among multiple groups were performed using Tukey's post hoc test. Statistical analyses were conducted using GraphPad Prism (version 9.0). Statistical significance was indicated by a *p*-value < 0.05.

Results

ATG prevented rodent MASH induced by CDAHFD

Compared with the control group, the body mass of the other

groups that were fed the CDAHFD was reduced during the initial two weeks and remained at a significantly lower level until the end of the 8th week (Fig. 1A and B). However, their liver index and TG content were significantly higher than those of the control group (Fig. 1C and D). No significant difference in liver total cholesterol content was observed among the four groups (Fig. 1E). In mice with or without ATG administration, similar alterations in body weight, liver index, and chemical indices were observed after the CDAHFD (Fig. 1A–E, Supplementary Fig. 1A and B). No significant differences were observed in the expression of key genes related to lipid metabolism, including the metabolism of FFA, cholesterol, VLDL, and ceramide (Supplementary Fig. 1C–F).

The significant increase in serum alanine aminotransferase and aspartate transaminase levels indicated that the eight-week CDAHFD treatment caused liver injury (Fig. 1F and G). After ATG prophylaxis, there was a significant improvement in liver enzymes in both the low- and high-dose groups (Fig. 1F and G). Histopathological staining revealed that the model group exhibited bullous steatosis, lobular inflammation, and limited hepatocyte ballooning (Fig. 1H). An NAS score > 5 in most mice (eight out of nine) met the diagnostic criteria for MASH (Fig. 1I). After ATG prophylaxis, both the low-dose (average NAS score = 4.978 ± 0.168) and high-dose groups (average NAS score = 4.125 ± 0.165) showed a decrease (Fig. 1I), mainly due to the attenuation of lobular inflammation. Moreover, the decrease in the NAS score was more significant in the high-dose group than in the low-dose group (Fig. 1I).

ATG reduced macrophage and neutrophil infiltration and ameliorated oxidative stress in MASH

Immunohistochemical staining was performed to determine the effects of ATG on the immune microenvironment in the liver. There were almost no neutrophils in the control group, while macrophages were mainly distributed along the hepatic sinusoids (Fig. 2A–C). In contrast, the hepatic lobules of the model group exhibited obvious infiltration of macrophages and neutrophils (Fig. 2A). Macrophages were enriched around hepatocytes with lipid droplets, forming a coronal structure (Fig. 2A). Neutrophils clumped together to form foci of inflammation (Fig. 2A). Compared to the model group, ATG prophylaxis led to much less infiltration of macrophages and neutrophils, with the most significant effect observed in the high-dose group (Fig. 2A–C).

Furthermore, the expression levels of inflammatory chemokines in the liver tissue were studied. The expressions of C-C motif chemokine 2 (Fig. 2D), C-X-C motif chemokine ligand (CXCL) 1 (Fig. 2E), CXCL15 (Fig. 2F), CXCL5 (Fig. 2G), and CXCL3 (Fig. 2H) were significantly upregulated after CDAHFD feeding, but this upregulation of pro-inflammatory genes did not occur in the low-dose and high-dose ATG prophylaxis groups (Fig. 2D–H).

Oxidative stress plays an important role in the progression of MASH. We assessed the level of oxidative stress in the liver by measuring the content or activity of oxidative products, MDA, and antioxidant enzymes (SOD and CAT). In contrast to the control group, the MDA concentration was significantly elevated in the liver homogenate of the model group (Fig. 2I). However, there was a dose-dependent decrease in MDA levels in both the low- and high-dose groups (Fig. 2I). The SOD and CAT tests showed that only the levels of antioxidant enzymes were significantly higher in the high-dose group than in the model group, whereas these indices displayed a tendency toward improvement in the low-dose group (Fig. 2J and K). By detecting the expression of oxidative stress-related genes, we found significantly upregulated levels of the

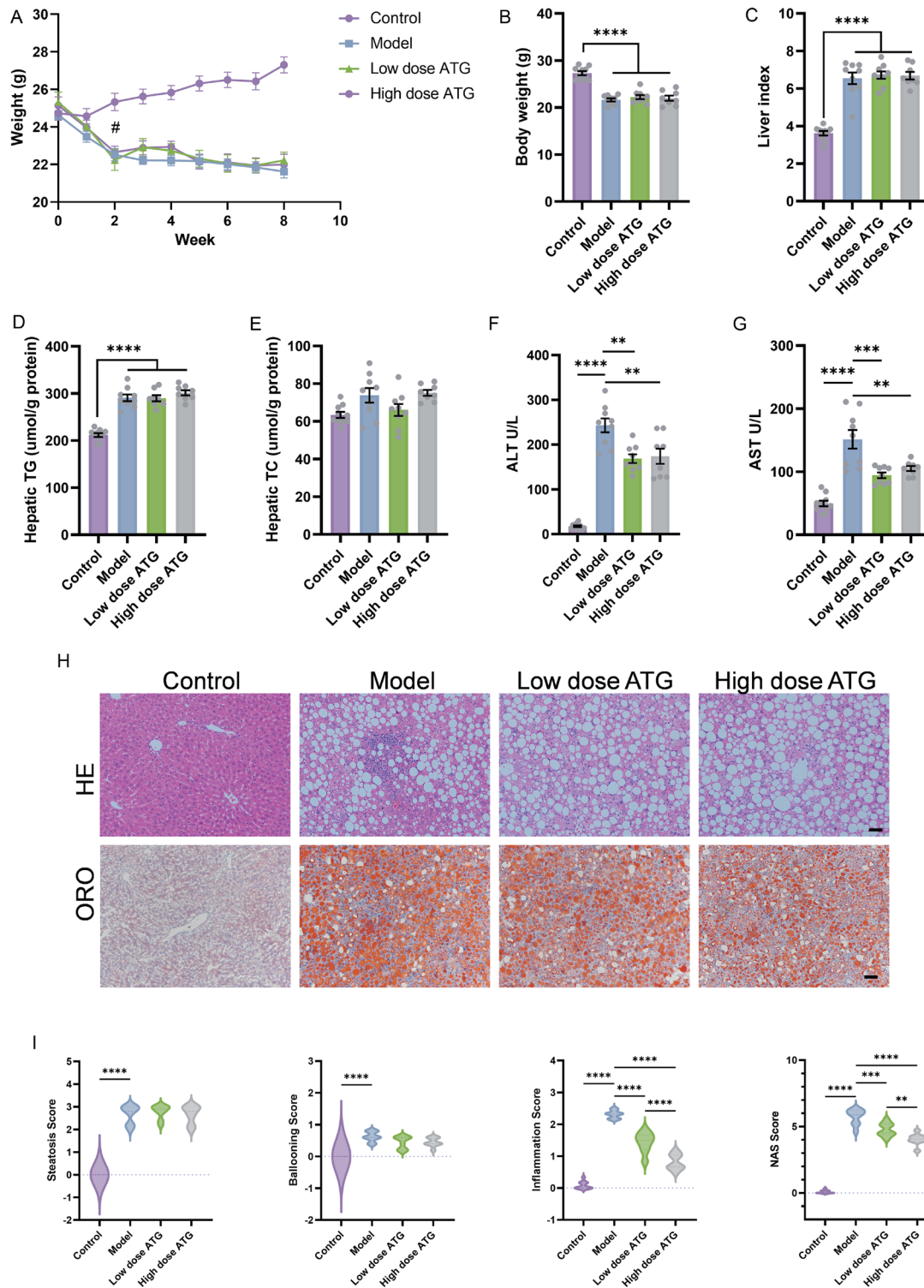


Fig. 1. Effects of ATG on MASH induced by choline-deficient, L-amino acid-defined, high-fat diet. (A) The dynamic changes in body weight were measured in the control, model, low-dose ATG-treatment, and high-dose ATG-treatment groups. #: Model, Low-dose ATG, and High-dose ATG compared to Control, $p < 0.001$. (B–C) Body weight (B) and liver index (C) at the end of eight weeks in the control, model, low-dose ATG-treatment, and high-dose ATG-treatment groups. (D–G) Liver contents of TG (D) and TC (E) and serum levels of ALT (F) and AST (G) of mice after eight weeks of intervention in four groups. (H–I) HE staining (scale bar: 50 μ m) (H, upper panel), ORO staining (scale bar: 100 μ m) (H, lower panel), and NAS score (I) of liver sections from each group after eight weeks of intervention. $n = 8$ –10 per group. Data are presented as mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ATG, arctigenin; MASH, metabolic dysfunction-associated steatohepatitis; CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet; TG, triglyceride; TC, total cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HE staining, hematoxylin & eosin staining; ORO staining, Oil Red O staining; NAS score, nonalcoholic fatty liver disease activity score; SEM, standard error of the mean.

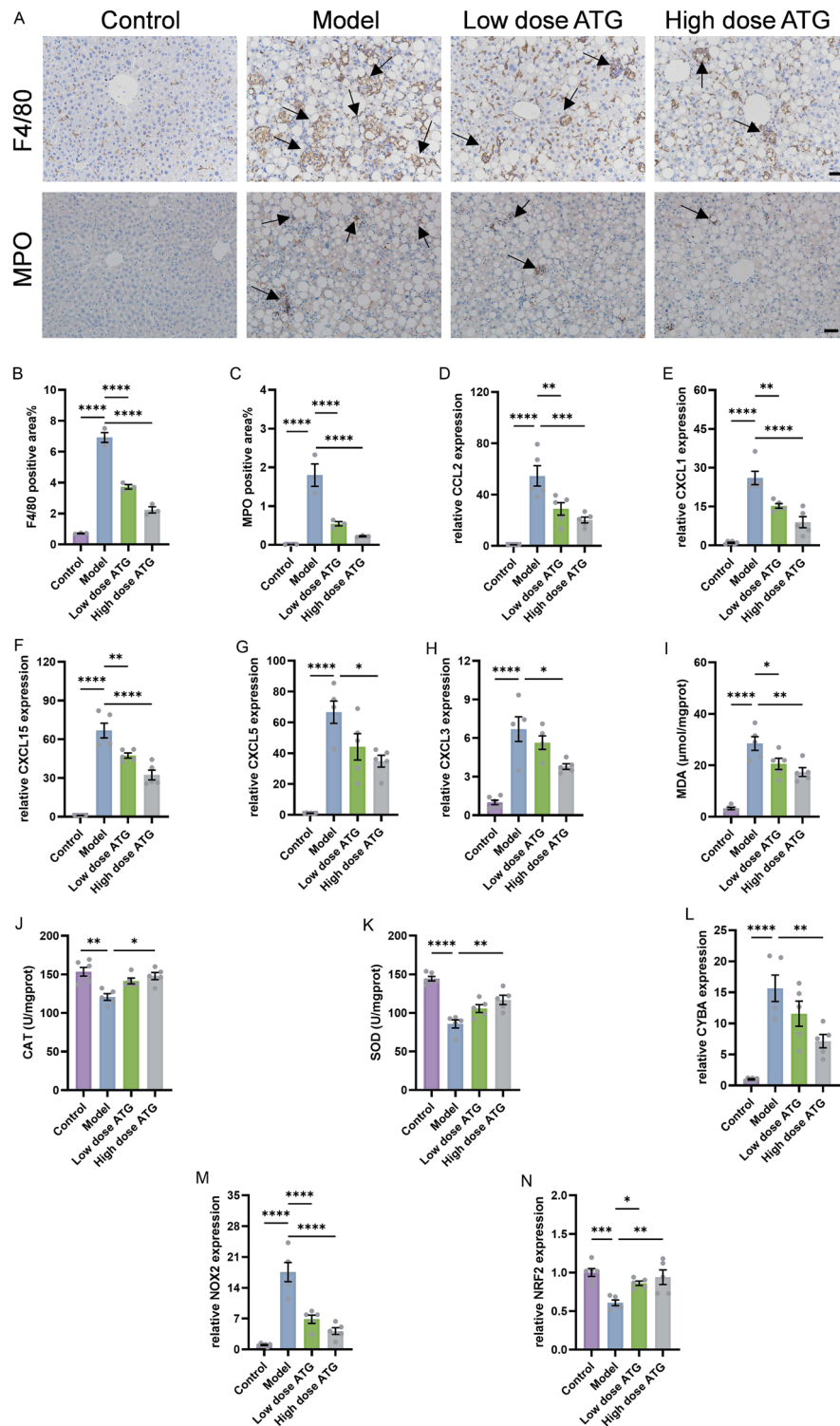


Fig. 2. Effects of ATG on neutrophil and macrophage accumulation and oxidative stress in MASH. (A–C) Immunohistochemical staining for F4/80 (A, upper panel) and MPO (A, lower panel) in liver tissue from the control, model, low-dose ATG-treatment, and high-dose ATG-treatment groups (scale bar: 50 μm), along with the quantification of F4/80 (B) and MPO (C) in liver tissue. (D–H) Relative mRNA expressions of cytokines and chemokines, including CCL2 (D), CXCL1 (E), CXCL15 (F), CXCL5 (G), and CXCL3 (H) in liver tissue from four groups were measured using RT-qPCR. Normalized against 18S. (I–K) Levels of MDA (I), CAT (J), and SOD (K) of liver lysates in the control, model, low-dose ATG-treatment, and high-dose ATG-treatment groups. (L–N) Relative mRNA expressions of CYBA (L), NOX2 (M), and NRF2 (N) in liver tissue from four groups were measured using RT-qPCR. Normalized against 18S. Data are presented as mean ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. ATG, arctigenin; CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet; MPO, myeloperoxidase; MDA, malondialdehyde; CAT, catalase; SOD, superoxide dismutase; MASH, metabolic dysfunction-associated steatohepatitis; SEM, standard error of the mean; RT-qPCR, real-time quantitative polymerase chain reaction.

antioxidant gene (nuclear factor erythroid 2-related factor 2) and downregulated levels of pro-oxidant genes (cytochrome B-245 alpha chain and NADPH oxidase 2) (Fig. 2L–N), further confirming the ameliorating effect of ATG on liver oxidative stress. This may be a downstream mechanism of reduced inflammation. These results indicated that ATG improved MASH progression by reducing inflammatory cell infiltration and preventing inflammation-induced oxidative stress.

ATG ameliorated liver fibrogenesis in MASH

Fibrosis is an indicator of disease severity. First, we performed Sirius red and Masson's trichrome staining to evaluate collagen deposition (Fig. 3A–C). We found that eight-week CDAHFD dietary administration resulted in the formation of fibrous cords with some bridging areas. In contrast, ATG prophylaxis reduced collagen accumulation in a dose-dependent manner (Fig. 3A–C). Immunohistochemical analysis of α -SMA, a marker of activated hepatic stellate cells (HSCs) and fibrosis progression, provided additional confirmation. The α -SMA expression was markedly elevated in the model group but significantly attenuated in the ATG-treated groups (Fig. 3A and D). To further explore the anti-fibrotic effects of ATG, we assessed the expression levels of fibrosis-related genes, including α -SMA, transforming growth factor- β , vimentin, collagen I, collagen II, and collagen III. The expression of these genes was considerably elevated in the model group compared to the control group, whereas ATG treatment markedly suppressed their expression (Fig. 3E–J). Collectively, these results suggested that ATG mitigated fibrosis in the CDAHFD-induced MASH model, potentially by reducing inflammation.

ATG targeted the NLRP3 inflammasome pathway in macrophages

The chemical structure of ATG is shown in Figure 4A. Using Swiss Target Prediction, the PharmMapper database, the Similarity Ensemble Approach, and relevant literature, we identified 177 potential targets for ATG (Fig. 4B). Among these, 49 therapeutic targets were identified by intersecting the predicted ATG targets with 1,391 MAFLD-related targets obtained from the Genetic Association Database (Fig. 4C and D). To investigate the relationships between these targets, we created a protein-protein interaction network (Fig. 4E). Key nodes in the network include AKT1, PPARG, HSP90AA1, PTGS2, NFkB1, MTOR, and NLRP3, highlighting their potential roles in mediating ATG's effects on MAFLD. Gene Ontology enrichment analysis revealed that ATG-associated targets were involved in critical biological functions, including cellular components, molecular functions, and biological processes (Fig. 4F). Kyoto Encyclopedia of Genes and Genomes pathway analysis identified 201 enriched pathways. The most significant pathways suggested that ATG modulates MAFLD through multiple signaling cascades, including the PI3K-Akt, toll-like receptor, and C-type lectin receptor signaling pathways (Fig. 4G). Reactome pathway enrichment analysis revealed 631 enriched pathways, with the top 20 pathways implicating NLRP3 inflammasome activation as a critical mechanism underlying ATG's therapeutic effects (Fig. 4H).

To further explore the distribution of liver NLRP3 expression in cells and determine the cell types targeted by ATG, we searched the online Liver Cell Atlas and found that NLRP3 was mainly expressed in liver macrophages, monocytes, monocyte-derived cells, and neutrophils (Supplementary Fig. 2A). This finding was further supported by immunofluorescence staining, wherein NLRP3 and CD86 (macrophage

activation markers) co-localized in the liver tissue of CDAHFD-fed mice, but not in HSCs (Fig. 4I and J and Supplementary Fig. 2B). Importantly, the number of CD86⁺/NLRP3⁺ macrophages markedly increased in the model group and significantly decreased following ATG treatment (Fig. 4I and J). There were no alterations in CD206 and ARG1 levels after ATG administration *in vivo* or *in vitro* (Supplementary Fig. 2C–F). These results indicate that ATG exerts its preventive effects on MASH through complex molecular interactions and signaling pathways, with the NLRP3 inflammasome playing a central role.

Molecular docking analysis performed on the CB-Dock2 server identified potential ligand binding sites and poses for ATG on the NLRP3 receptor (Table 2, Fig. 4K). The optimal Vina score of -7.3 indicates a strong binding affinity between ATG and NLRP3. CETSA coupled with Western blotting in cell lysates (Fig. 4L; Supplementary Fig. 3A) demonstrated significantly enhanced thermal stability of NLRP3 following ATG treatment. Collectively, these findings demonstrate that ATG may exert its biological effects through direct binding to NLRP3.

ATG reduced NLRP3-dependent inflammatory response

To determine the mechanisms by which ATG prevents MASH, we examined the hepatic levels of major inflammatory cytokines and chemokines. mRNA levels of NLRP3, IL-1 β , and tumor necrosis factor- α (TNF- α) were elevated in the model group, but suppressed in both ATG groups (Fig. 5A–C). We investigated the level of activation of the NLRP3 inflammasome complex in liver tissue, as mature IL-1 β indicates inflammasome activation. Western blotting showed that the levels of NLRP3, mature IL-1 β , and GSDMD-N proteins were significantly increased in the model group, indicating strong inflammasome activation (Fig. 5D–G; Supplementary Fig. 3B). ATG significantly reduced the levels of these proteins in a dose-dependent manner (Fig. 5D–G). These findings suggest that ATG effectively inhibits macrophage infiltration and pro-inflammatory cytokine expression in CDAHFD-fed mice, primarily by inhibiting the NLRP3 inflammasomes. IL-1 β is a central mediator of inflammasome-induced inflammation, and its potent reduction highlights the potential of ATG to modulate the inflammatory environment of MASH.

ATG inhibited the NLRP3-dependent inflammatory response of macrophages *in vitro*

To determine the anti-inflammatory mechanisms of ATG, we investigated its effects on the NLRP3 inflammasome pathway in macrophages using an *in vitro* model. RAW264.7 cells were stimulated with LPS to mimic an inflammatory state. ATG treatment (50 and 100 μ M) significantly reduced the mRNA expression of key inflammatory markers, including NLRP3, IL-1 β , TNF- α , and inducible nitric oxide synthase, in a concentration-dependent manner (Fig. 6B–E). Importantly, ATG had no cytotoxic effects on macrophage viability, even at higher concentrations (Fig. 6A). To investigate the mechanisms underlying NLRP3 transcriptional regulation, we performed cross-database prediction of potential transcription factors for NLRP3. Intersectional analysis of the predicted results identified three candidate transcription factors (Fig. 6F). Further intersection with ATG-associated targets revealed only FOS, a gene encoding the c-Fos protein that acts as a positive regulator of NLRP3 (Fig. 6F). *In vitro* experiments demonstrated that ATG treatment reduced the nuclear localization of c-Fos while increasing its cytoplasmic accumulation (Fig. 6G–I; Supplementary Fig. 3C).

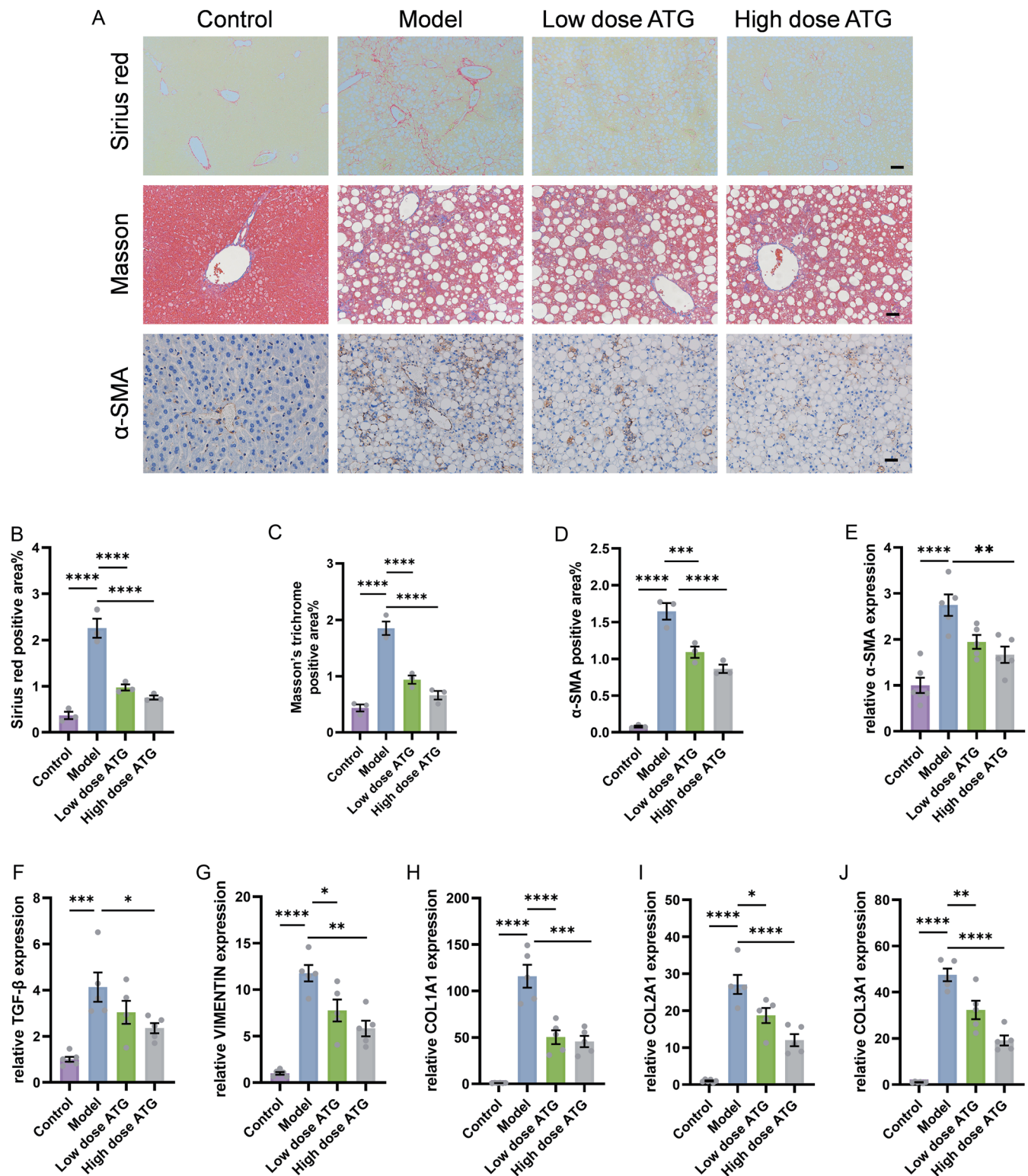


Fig. 3. Effects of ATG on liver fibrosis induced by CDAHFD. (A–D) Sirius red staining (A, upper panel), Masson's trichrome staining (A, middle panel), and immunohistochemical staining for α-SMA expression (A, lower panel) in liver tissue from the control, model, low-dose ATG-treatment, and high-dose ATG-treatment groups (scale bar: 50 μm), along with quantification of each indicator (B–D). (E–J) Relative mRNA levels of fibrosis-related genes, including α-SMA (E), TGF-β (F), VIMENTIN (G), COL1A1 (H), COL2A1 (I), and COL3A1 (J) in the liver tissue from four groups were measured using real-time quantitative polymerase chain reaction. Normalized against 18S. Data are presented as mean ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. ATG, arctigenin; MASH, metabolic dysfunction-associated steatohepatitis; CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet; SEM, standard error of the mean.

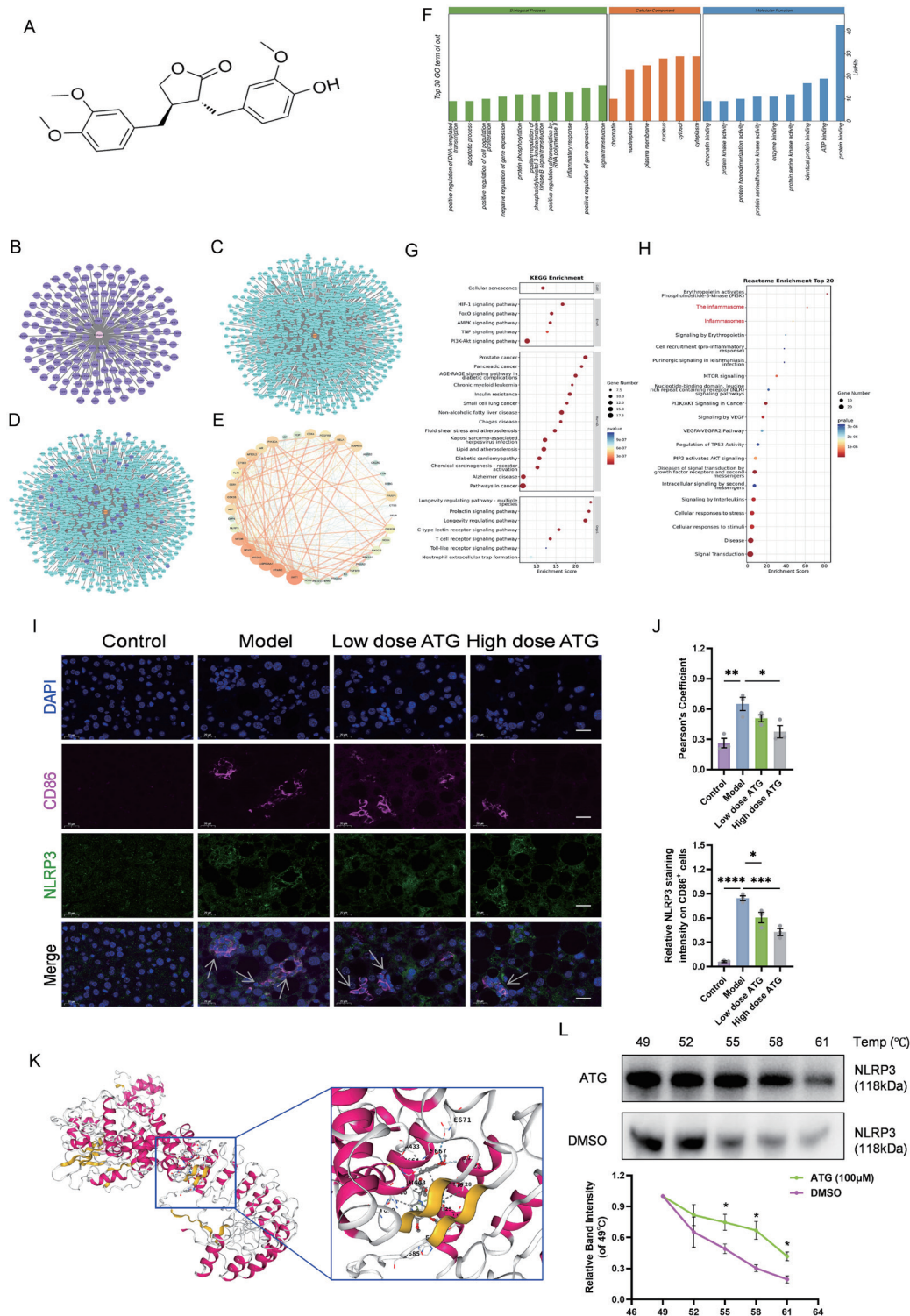


Fig. 4. Target analysis of ATG in MAFLD. (A) Chemical structure of ATG. (B) Target prediction of ATG. (C) Molecular targets associated with MAFLD. (D) Intersection of ATG-specific and MAFLD-related targets. (E) The PPI network of intersection targets between ATG and MAFLD-related targets. (F) GO enrichment analysis of the intersection targets. (G–H) Enrichment of KEGG pathway (G) and Reactome pathway (H) of ATG's targets. (I–J) Immunofluorescence analysis of the colocalization of NLRP3 and CD86 in mice liver from four groups (scale bar: 20 μ m), staining intensity was quantified, and colocalization analysis was performed. (K) NLRP3 with ATG bound to the cavity. (L) Western blot of CETSA experiment to further confirm the interaction between NLRP3 and ATG in RAW264.7 cells, the temperature ranges from 49°C to 61°C, with relative band intensity normalized to 49°C. Data are presented as mean $\pm$ SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. ATG, arctigenin; MAFLD, metabolic dysfunction-associated fatty liver disease; PPI, protein-protein interaction; KEGG, Kyoto Encyclopedia of Genes and Genomes; NLRP3, NLR family pyrin domain containing 3; SEM, standard error of the mean.

Table 2. Molecular docking results of ATG on NLRP3 obtained from the CB-Dock2 server

Curpocket ID	Vina score	Cavity volume (Å ³)	Center (x, y, z)	Docking size (x, y, z)	Contact residues
C4	-7.3	1,847	-4, 6, -1	22, 28, 33	Chain A: ARG393 ALA394 ALA395 SER397 LEU398 GLU401 LYS430 SER431 LEU432 ALA433 GLN434 THR659 ARG660 HIS663 MET664 SER667 GLU671 PHE683 HIS685 ASN722 LEU725 THR726 SER728 PHE729 GLY732 LEU733
C1	-7.1	13,545	21, -10, -26	35, 35, 22	Chain A: ILE151 GLU152 ASP153 ARG154 ARG167 ALA227 ALA228 GLY229 ILE230 GLY231 LYS232 THR233 ILE234 ARG237 GLN308 ARG351 TYR381 PRO412 LEU413 TRP416 LEU450 LEU451 GLN452 PRO453 ARG454 GLY455 GLY456 ASP498 VAL499 SER500 ALA501 PHE502
C2	-6.0	3,535	22, -4, -6	30, 30, 22	Chain A: GLU184 LEU188 ALA189 LYS192 THR193 LYS194 THR195 GLU375 ASN402 GLU403 PHE406 THR407 GLY476 ILE477 ASN479 GLN480 LYS481 ILE482 LEU483 PHE484 MET523 GLN526 GLU527 TYR572
C3	-6.0	1,895	-2, -11, 7	22, 22, 22	Chain A: GLU401 ASN402 GLU403 VAL404 TYR565 GLU569 ILE574 PHE575 ARG578 GLN624 PRO625 SER626 GLN627 LEU628 GLU629 TYR632 ASN656 LEU657 SER658 THR659 MET661 ASP662 LEU684 ASN686
C5	-5.9	825	37, -8, -13	22, 22, 22	Chain A: LYS175 GLU176 HIS177 ARG178 SER179 GLN180 ARG183 ASP212 GLN225 PRO352 LYS355 GLN359 PRO365 ARG366 HIS367 GLU369 ARG504 MET505 ASN506 LEU507 PHE508 GLN509 CYS514

ATG, arctigenin; NLRP3, NLR family pyrin domain containing 3.

At the protein level, ATG suppressed the activation of the NLRP3 inflammasome pathway. ATG treatment (20, 50, and 100 μ M) markedly inhibited the expression of NLRP3, mature IL-1 β , and GSDMD-N, key components of the inflammasome activation cascade (Fig. 6J-M; Supplementary Fig. 3D). In addition, we tested a series of chemokines *in vitro* and found that the release of chemokines CXCL1, CXCL3, CXCL5, and CXCL15 by RAW264.7 cells decreased after ATG treatment (Fig. 6N-Q), which indirectly suggests that ATG attenuates immune cell infiltration by reducing the release of chemokines by macrophages in mouse models. These results suggest that ATG attenuates macrophage-mediated inflammation by interfering with the NLRP3 inflammasome-dependent signaling pathway. Collectively, these findings demonstrate that ATG suppresses NLRP3-driven inflammatory responses, providing a possible molecular explanation for the anti-inflammatory effects observed in CDAHFD-induced MASH.

Discussion

MASH is a key step in the progression of liver steatosis, and its prominent heterogeneity poses a major challenge in identifying therapeutic targets. Interventional approaches for MASH progression are limited. However, traditional herbal medicines and their multiple pharmacological monomers may provide a potential solution for MASH intervention according to recent studies.¹³ The present study revealed, for the first time, that ATG has a preventive effect on CDAHFD-induced MASH by attenuating liver inflammation, oxidative stress, and fibrosis. Based on previous reports and our experiments, hepatic macrophages have been recognized as key drivers of MASH progression.⁴⁴⁻⁵⁰ NLRP3 expression underlies liver inflammation and fibrosis.^{51,52} The present study aimed to explore the MASH-preventive actions of the herbal extract ATG, with a dramatic cascade of NLRP3 and inflammasome activation in hepatic macrophages as the underlying molecular mechanisms. The dose-dependent reduction in the NAS score, particularly through amelioration of lobular inflam-

mation, revealed the anti-inflammatory properties of ATG rather than normalization of lipid dysregulation. This finding is supported by the decreased expression of pro-inflammatory cytokines (IL-1 β , TNF- α) and chemokines (C-C motif chemokine 2, CXCL1/3/5/15), which are essential players in recruiting immune cells to impair hepatocytes.⁵³⁻⁵⁵ Notably, the reduction in CD86⁺/NLRP3⁺ macrophages in ATG-treated mice suggested targeted normalization of macrophage polarization, one of the crucial mechanisms related to the development of steatohepatitis.⁵⁶

Inflammation and oxidative stress reinforce each other through a vicious cycle that drives the progression of MAFL to MASH. Activated inflammatory responses in the liver not only impair hepatocytes but also cause ROS overproduction by damaging the mitochondrial respiratory chain. However, oxidation in both steatotic hepatocytes and immune cells (*i.e.*, macrophages and activated neutrophils) can decrease endogenous antioxidants and recruit circulating immune cells to exacerbate hepatic inflammation. Previous studies have demonstrated the safety and tolerability of ATG and its anti-inflammatory and antioxidant properties through multiple targets to improve a variety of inflammatory diseases.⁵⁷⁻⁵⁹ Our group used a CDAHFD to construct a mouse model of MASH, which effectively simulated the characteristics of inflammatory infiltration and oxidative stress in patients with MASH.⁶⁰ In the current study, we found that the prophylactic use of ATG reduced the expression of multiple hepatic chemokines, attenuated the hepatic infiltration of macrophages and neutrophils, and relieved hepatic oxidative stress. Prophylactic use of ATG has been shown to have an ameliorating effect on MASH through its anti-inflammatory and antioxidant effects.

Mechanistically, ATG significantly reduced the levels of NLRP3, mature IL-1 β , and GSDMD-N in macrophages, indicating that ATG interferes with inflammasome activation to mitigate the macrophage-driven inflammatory response. Unlike its role in inhibiting NLRP3 inflammasome assembly in colon macrophages by downregulating carnitine palmitoyl-transferase-1 expression,¹⁸ ATG employed in our experiments

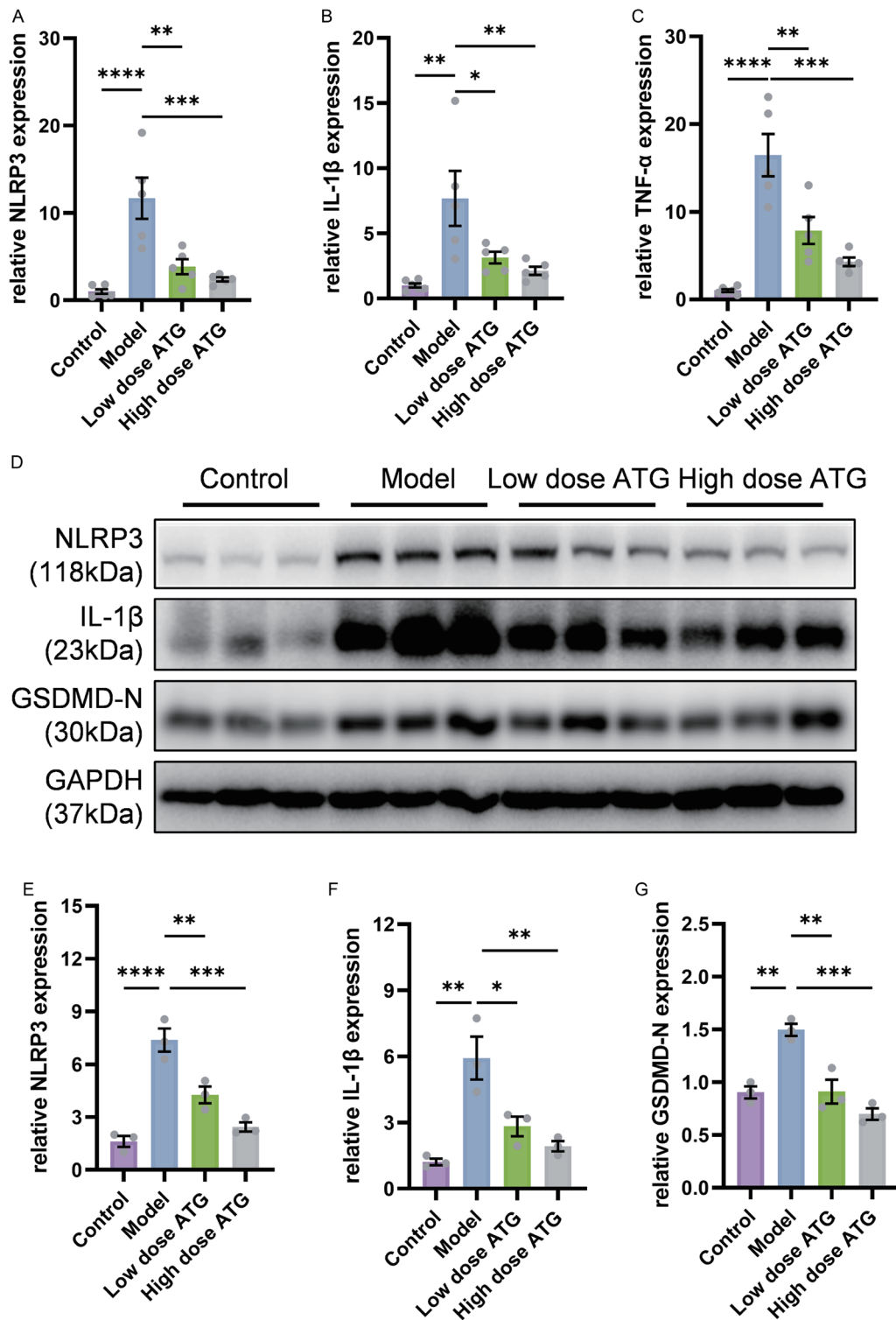


Fig. 5. Effect of ATG on inflammatory response through NLRP3 inflammasome pathway. (A–C) Relative mRNA levels of NLRP3 (A), IL-1 β (B), and TNF- α (C) in liver tissue from four groups were measured using real-time quantitative polymerase chain reaction. Normalized against 18S. (D–G) Western blot (D) and relative protein levels of NLRP3 (E), mature IL-1 β (F), and GSDMD-N (G) in liver tissue from four groups, normalized against GAPDH. Data are presented as the mean $\pm$ SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. ATG, arctigenin; NLRP3, NLR family pyrin domain containing 3; SEM, standard error of the mean; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

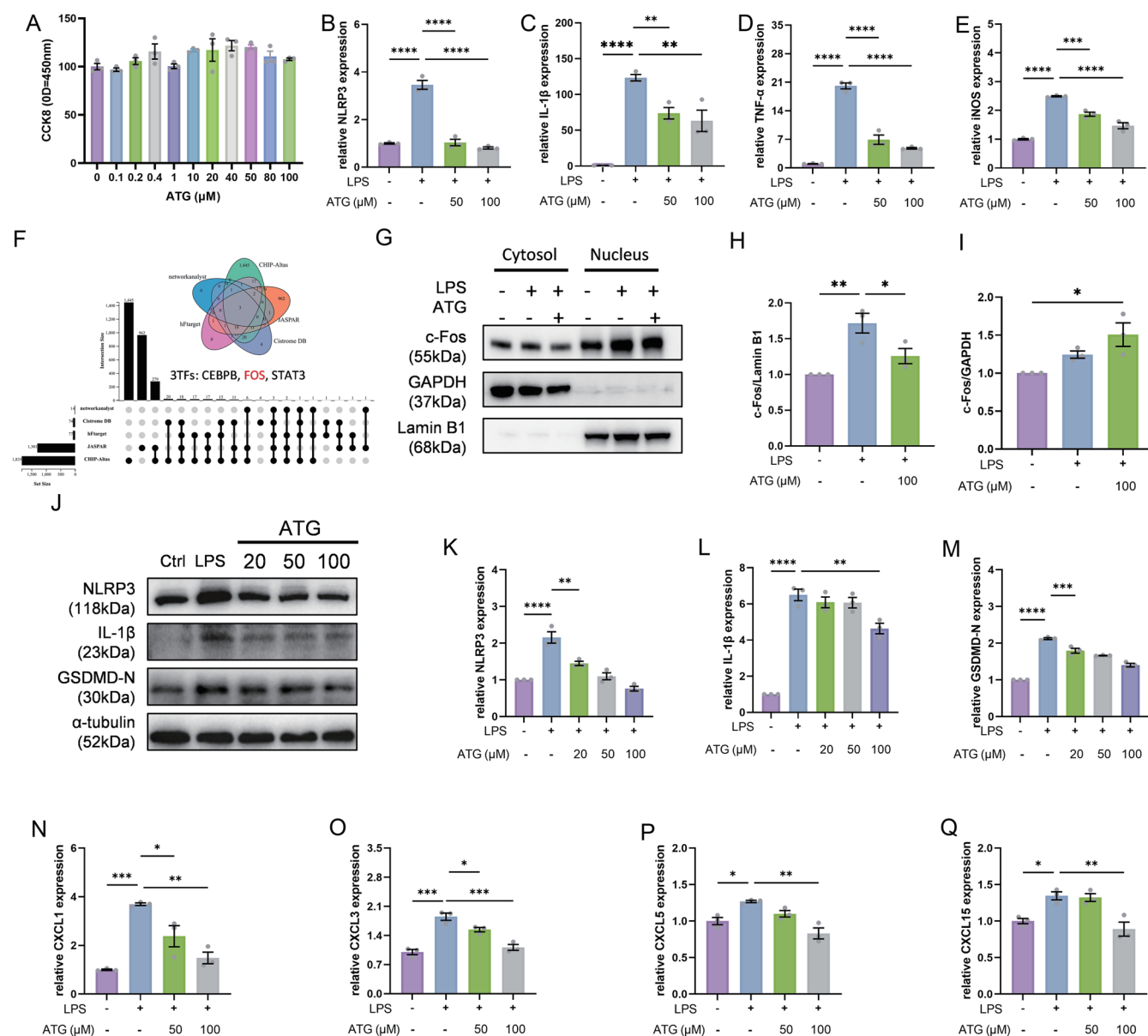


Fig. 6. Effect of ATG on RAW264.7 by regulating NLRP3 inflammasome pathway. (A) Cell viability of RAW264.7 cells after treatment with different concentrations of ATG (0, 0.1, 0.2, 0.4, 1, 10, 20, 40, 50, 80, and 100 μM) for 24 h. (B–E) RT-qPCR was utilized to assess the mRNA expression of NLRP3 (B), IL-1β (C), TNF-α (D), and iNOS (E) of RAW264.7 cells following treatment with LPS in the absence or presence of two concentrations of ATG, along with the Control group. Normalized against 18S. (F) Venn diagram and Upset plot showing the potential transcription factors of NLRP3 predicted by different databases. (G–I) Western blot was utilized to assess the cytosol and nucleus protein level of c-Fos in RAW264.7 cells following treatment with LPS in the absence or presence of ATG, along with the Control group. Normalized against GAPDH and Lamin B1, separately. (J–M) Western blot was utilized to assess the protein level of NLRP3 (K), mature IL-1β (L), and GSDMD-N (M) of RAW264.7 cells following treatment with LPS in the absence or presence of three concentrations of ATG, along with the Control group. Normalized against α-tubulin. (N–Q) RT-qPCR was utilized to assess the mRNA expression of CXCL1 (N), CXCL3 (O), CXCL5 (P), and CXCL15 (Q) of RAW264.7 cells following treatment with LPS in the absence or presence of two concentrations of ATG, along with the Control group. Normalized against 18S. Data are presented as mean ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. ATG, arctigenin; NLRP3, NLR family pyrin domain containing 3; SEM, standard error of the mean; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR, real-time quantitative polymerase chain reaction; LPS, lipopolysaccharide; +, with; -, without.

demonstrated that it inhibited the transcription of NLRP3 in macrophages by decreasing the nuclear translocation of c-Fos. Molecular docking (−7.3 Vina score) and cellular CET-SA analyses confirmed that ATG directly bound to NLRP3. Collectively, ATG may inhibit the expression and function of NLRP3, thus attenuating the secretion of NLRP3-dependent inflammatory cytokines and chemokines, thereby disrupting the positive feedback loop of immune cell recruitment. This

is consistent with evidence that activation of NLRP3 in macrophages exacerbates hepatic inflammation.^{45,61}

Studies have demonstrated that excessive lipid species (e.g., cholesterol, ceramides) activate NLRP3 by inducing organelle stress, thus accelerating the inflammatory response.^{62,63} As for the effect of NLRP3 on lipid metabolism during diabetic nephropathy, MCC950, a NLRP3-specific inhibitor, ameliorates podocyte lipid accumulation by promot-

ing ATP-binding cassette A1 expression and inhibiting both sterol regulatory element-binding protein 1 and 2.⁶⁴ There are also experiments focusing on MCC950-based NLRP3 inhibition in MAFLD. With little impact on hepatic lipid metabolism, MCC950 inhibits inflammatory cell infiltration, thereby improving hepatocyte injury, lobular inflammation, and liver fibrosis.⁵⁶ This inconsistency indicates the diverse effects of NLRP3 on lipid metabolism across different diseases and models. In our experiments, serum levels of FFA and VLDL, together with hepatic content of TG and hepatocyte steatosis, demonstrated a significant increase during CDAHFD modeling. However, these indices experienced limited alteration after ATG intervention. Similarly, there were no statistical differences in the expression of key genes related to FFA, VLDL, cholesterol, and ceramide metabolism upon ATG administration. These results were consistent with findings reported by AR *et al.*,⁵⁶ suggesting an inflammation-rather than metabolism-based pharmacological action of ATG.

The anti-fibrotic effect of ATG has been reflected by reduced collagen deposition and α -SMA expression, possibly due to the aforementioned inhibition of inflammatory pathways. In MASH, the resolution of fibrosis is usually due to a decrease in hepatocyte damage and inflammatory signaling.⁶⁵ This premise is further supported by the down-regulation of transforming growth factor-beta and collagen-related genes, as persistent inflammation directly activates HSCs, promoting extracellular matrix remodeling.⁶⁶ Although our study did not directly assess HSC activation, the simultaneous reduction in NLRP3-driven markers of inflammation and fibrosis suggested an indirect mechanism, consistent with reports linking NLRP3 inhibition to HSC deactivation.⁶⁷

Given the critical actions of macrophages in MASH and related liver fibrosis/cirrhosis, macrophage NLRP3 also serves as a potential target for therapy. Studies of selective NLRP3 inhibitors have shown that NLRP3 inactivation has therapeutic effects on MASH. For instance, *in vivo* administration of the selective NLRP3 inhibitor MCC950 significantly attenuated hepatocyte injury, lobular inflammation, and liver fibrosis in mice with both MASH and chronic ethanol abuse.⁶⁵ In another study of rodent MASH induced by an atherogenic diet, a four-week intervention of MCC950 improved liver enzymes. After eight weeks of treatment, reductions were observed in the NAS score, infiltration of macrophages and neutrophils, as well as liver inflammation and fibrosis.⁵⁶ Therefore, we speculate that ATG is likely to possess therapeutic potential in MASH through transcriptional and functional inactivation of NLRP3. When compared to ATG, MCC950 specifically targets inflammasome assembly without suppressing transcriptional activation.⁶⁸ Moreover, clinical trials have revealed elevated levels of liver enzymes associated with MCC950 administration, indicating drug-induced hepatotoxicity.⁶⁹ Another selective inhibitor, CY-09, directly binds to the ATP-binding domain of NLRP3, thereby inhibiting ATPase activity and subsequent inflammasome activation, with no effect on other pro-inflammatory signaling pathways.⁷⁰ In contrast, ATG exhibits multitarget anti-inflammatory mechanisms without reported drug toxicity,⁷¹ even at doses of 30–120 mg/kg in the present experiments.

Intertwined metabolic and inflammatory abnormalities have been well established as underlying factors in MASH. In our experiments, it is worth noting that the pharmacological effect of ATG on MASH primarily occurs through dose-dependent reduction in inflammation. The mild effect of ATG on MASH-related dysregulation of lipid metabolism reflects the limitations of this study, and its safety needs to be verified in a long-term model. Recent studies have shown that

PPAR α improves lipid metabolism, partially by accelerating fatty acid oxidation, to attenuate hepatic lipid accumulation.⁷² Administration of PPAR α agonists, such as fenofibrate and pemafibrate, demonstrates reliable improvements in the hepatic lipid profile and, consequently, liver function.⁷³ Thus, a combination of ATG and PPAR α agonists could be a better choice for the prevention and/or treatment of MASH through their synergistic effects on both metabolic and inflammatory abnormalities.

Conclusions

ATG may prevent CDAHFD-induced MASH primarily by suppressing the NLRP3/GSDMD-N axis in macrophages, thereby attenuating inflammation, oxidative stress, and fibrosis, with a mild effect on lipid dysmetabolism. These findings suggest that ATG is a promising candidate for MASH treatment, particularly in the context of the unmet therapeutic needs for inflammation-driven fibrosis. However, further studies are needed to explore the synergistic effects of ATG and lipid-lowering therapy to fully exploit their metabolic and inflammatory regulatory potential.

Funding

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Conflict of interest

JGF has been an Associate Editor of the *Journal of Clinical and Translational Hepatology* since 2013. The other authors have no conflicts of interest related to this publication.

Author contributions

Mouse keeping and husbandry (RX, TYR, QJW, QRZ), carrying out of experiments and data analysis (RX, LJ, QJW, QRZ), conceptualization and design of the study (QP, JGF), manuscript preparation (RX, TYR, RXY, QP, JGF), and supervision of the study (QP, JGF). All authors read and approved the final version and publication of the manuscript.

Ethical statement

All animal handling and experimental procedures were approved by the Animal Care and Use Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (Approval No. XHEC-NSFC-2023-273). All animals received humane care.

Data sharing statement

No additional data are available.

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