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Original Article

Metformin suppresses inflammation in TNF-α-induced fibroblast-like synoviocytes of rheumatoid arthritis and alleviates adjuvant-induced arthritis in mice by inhibiting Wnt-3a/β-catenin signaling

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Abstract: Objectives: Rheumatoid arthritis (RA) is a disabling autoimmune disease characterized by abnormal fibroblast-like synoviocytes (FLS) proliferation, synovial inflammation, and cartilage erosion, ultimately leading to joint destruction. RA-FLSs are important drivers of synovial inflammation. Metformin (Met), a first-line antidiabetic drug, has shown potential for therapeutic effects in RA; however, its anti-inflammatory activity and underlying mechanisms in RA-FLSs remained unclear. Methods: Immunohistochemistry (IHC) was used to evaluate the expression of IL-1β, IL-6, IL-8, and β-catenin in synovial tissues from patients with RA and controls. Primary TNF-α stimulated RA-FLSs were identified by flow cytometry, and the CCK-8 assay was used to determine non-cytotoxic concentrations of metformin. The expression of inflammatory cytokines and activation of the Wnt-3a/β-catenin signaling pathway were assessed using western blotting, ELISA, and phosphorylation analysis. In an adjuvant-induced arthritis (AIA) mouse model, the therapeutic effects of metformin were compared with those of methotrexate (MTX). Safety was evaluated based on body weight, serum biochemical indices, and liver and kidney histologic changes. Joint inflammation was assessed by paw swelling, and histologic examination. Synovial Wnt-3a and β-catenin expression were examined by IHC. Results: TNF-α stimulation significantly increased the production of IL-1β, IL-6, and IL-8 in RA-FLSs. Metformin (10-40 mM) attenuated these inflammatory responses in a concentration-dependent manner through inhibiting Wnt-3a/β-catenin signaling. In AIA mice, daily intraperitoneal administration of metformin (100 or 200 mg/kg) was well-tolerated and markedly alleviated synovitis, with superior therapeutic efficacy to twice-weekly MTX administration (2 mg/kg). Conclusions: Metformin attenuated inflammatory responses in TNF-α-stimulated RA-FLSs and alleviated synovial inflammation in AIA mice by inhibiting the Wnt-3a/β-catenin signaling. These findings provide support metformin as a potential adjunctive treatment for RA.

Keywords: Metformin, rheumatoid arthritis, inflammation, adjuvant-induced arthritis, fibroblast-like synoviocytes

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease that causes pathologic damage primarily to the intra-articular synovial membrane [1]. It affects approximately 0.5-1% of the global population and occurs more frequently in women and older adults. Persistent synovial inflammation is a hallmark of RA and is accompanied by progressive cartilage destruction and subchondral bone loss [2]. The thin lining membrane of normal joints consists of only two to three layers of fibroblast-like synoviocytes (FLSs) interspersed with synovial macrophages. These cells synthesize and secrete hyaluronic acid and lubricin to maintain joint lubrication and provide nutritional support [3]. In RA, the synovial lining undergoes drastic hyperplasia, expanding to 10-15 cell layers in pathological synovial tissues. As the major stro-

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mal cell subset within inflamed RA synovium, RA-FLSs have been widely recognized as key contributors throughout the pathologic progression of RA. RA-FLSs promote early RA progression by triggering inflammatory reactions and impairing the resolution of inflammation [4, 5]. Therefore, targeting the inflammatory activation of RA-FLSs may represent a promising complementary strategy to existing long-term immunosuppressive therapies [6, 7].

Evidence shows that canonical Wnt/β-catenin signaling contributes to the inflammatory activation of RA-FLSs [8]. Accordingly, inhibition of aberrant pathway activation has been proposed as a therapeutic strategy for RA [9, 10]. Recent studies have shown that suppression of Wnt-3a/β-catenin signaling reduces the migration, invasion, and inflammatory response of RA-FLSs [11, 12]. Collectively, the Wnt-3a/β-catenin signaling pathway represents a promising therapeutic target for alleviating inflammatory responses in RA-FLSs. However, whether metformin regulates RA-FLS-mediated inflammation through this pathway remains unclear.

Current therapeutic interventions for RA include conventional synthetic disease-modifying antirheumatic drugs (DMARDs), glucocorticoids, nonsteroidal anti-inflammatory agents, and biologic agents. However, these regimens do not achieve satisfactory therapeutic responses in all RA patients, and prolonged administration carries risks of organ toxicity and diverse adverse reactions [13]. Metformin, a hypoglycemic agent introduced for the treatment of type 2 diabetes in 1957, has become a first-line oral hypoglycemic medicine due to its low cost, established efficacy, and favorable safety profile. Existing preclinical data demonstrate that metformin inhibits the proliferation and metastatic potential of various malignant tumor cells, suppresses excessive proliferation of RA-FLSs, and regulates bone metabolic homeostasis [14-17]. In other inflammatory disorders, metformin relieves oxidative stress and represses nuclear factor-kappa B (NF-κB)-mediated pro-inflammatory signaling cascades, thereby alleviating tissue injury in inflammatory disorders such as inflammatory bowel disorders [18]. Evidence from cell culture experiments showed that metformin decreases the secretion of IL-1β and TNF-α from primary RA-FLS cultures and reduces circulating pro-inflammatory cytokine levels in rodent models of RA [19, 20]. These findings are further supported by a series of clinical and animal studies demonstrating that metformin may inhibit the progression of RA [21, 22]. Taken together, these observations suggest that metformin may have therapeutic potential for RA management.

However, two important issues remain unresolved: how metformin regulates TNF-α-induced inflammatory responses in RA-FLSs and whether its anti-arthritic effects involve Wnt-3a/β-catenin signaling. In this study, we first investigated the concentration-dependent effects of metformin on cytokine production in TNF-α-stimulated primary RA-FLSs. Then, rescue experiments with lithium chloride (LiCl), a GSK-3β inhibitor that activates canonical Wnt signaling, were performed to determine the involvement of this pathway. Finally, we evaluated the *in vivo* anti-inflammatory effects of intraperitoneally administered metformin in an adjuvant-induced arthritis (AIA) mouse model, with MTX used as a positive control. The findings from cellular and animal experiments provide insight into the molecular mechanism underlying metformin-mediated regulation of pathologic RA-FLS function and support the potential translational application of metformin for RA treatment.

Materials and methods

Reagents and antibodies

Metformin hydrochloride (purity ≥ 99.99%, cat. no. S1950) was supplied by Selleck Chemicals (Shanghai, China). Methotrexate and lithium chloride were procured from domestic commercial suppliers, and recombinant human TNF-α were purchased from PeproTech (NJ, USA). Cell culture reagents, including DMEM medium, trypsin, and penicillin-streptomycin solution, were obtained from Gibco. CCK-8 cell viability assay kit, BCA protein quantification reagent, SDS-PAGE loading buffer and prestained protein markers were purchased from Beyotime Biotechnology (Wuhan, China). The anti-Wnt-3a monoclonal antibody (ab219412) was obtained from Abcam. Antibodies against IL-8, p-GSK-3β, and total GSK-3β, were acquired from Cell Signaling Technology. Anti-IL-1β, anti-β-catenin, anti-α-tubulin, CD90, CD31,

CD45 and HRP-conjugated goat anti-rabbit IgG antibodies were obtained from Proteintech. The anti-IL-6 antibody was supplied by Bioworld. Complete and incomplete Freund's adjuvant (CFA/IFA) were obtained from Sigma-Aldrich. RIPA lysis buffer and PVDF transfer membranes were from Millipore. ELISA kits for inflammatory factors were sourced from Applygen Technologies.

Human articular synovial specimens

Knee joint synovial tissues were harvested from six patients with definite RA who fulfilled the American College of Rheumatology (ACR) updated classification criteria. All subjects had severe knee deformities and underwent total knee arthroplasty. Six control synovial tissue samples were collected from individuals undergoing arthroscopic meniscus repair without inflammatory joint disease (Supplementary Table 1). All samples were collected after obtaining written informed consent from participants, and the study was approved by the Ethics Committee of The Affiliated Huizhou Hospital, Guangzhou Medical University (2025-KY-070-01). Tissues were either immediately processed for primary cell isolation or stored at -80°C for subsequent molecular analyses. No patients had received immunosuppressive agents or biologic therapies within 3 months before surgery.

Isolation, identification, and treatment of primary RA-FLSs

Fresh RA synovial tissues were minced into roughly 1 mm^3 fragments and digested with 0.2% collagenase II solution at 37°C for 1.5 h. After enzymatic digestion, isolated cells were cultured in DMEM/F12 medium supplemented with 10% FBS in a humidified incubator containing 5% CO_2 at 37°C. Culture medium was refreshed every 72 h. When cell confluence exceeded 92%, cells were detached using 0.25% trypsin and passaged. Cells from passages 3-5 were used for all functional experiments. CD90 is a well-established surface marker for RA-FLS identification and is also associated with an inflammatory phenotype [23]. In this study, FITC-conjugated antibodies against human CD90, CD31, and CD35 were used for flow cytometric identification. Only cell preparations with > 92% CD90-positive cells were selected for subsequent experiments.

CCK-8 cell viability assay

RA-FLSs were seeded into 96-well plates at a density of 5×10^3 cells per well and cultured until complete cell adhesion. After serum starvation for 24 hours, cells were treated with different concentrations of metformin and stimulated with TNF-α for 24 or 48 hours. Subsequently, CCK-8 solution was added to each well at a final concentration of 10% (v/v). After incubation in the dark for 2 hours, absorbance was measured at 450 nm using a Bio-Rad microplate reader. Relative cell viability was then calculated.

β-catenin immunofluorescence staining

After indicated drug intervention in each group, RA-FLSs were incubated with primary antibodies for β-catenin overnight at 4°C, followed by incubation with FITC-conjugated secondary antibody at room temperature for 1 h. Nuclei were counterstained with DAPI for 10 min in dark. Fluorescence signals were visualized using a fluorescent microscope equipped with a digital camera.

Western blot analysis

Cellular proteins were extracted using RIPA lysis buffer, quantified using a BCA protein assay and normalized to equal protein concentrations. Proteins were separated on SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% skim milk, membranes were incubated with primary antibodies overnight at 4°C. After washing with TBST, membranes were incubated with HRP-conjugated secondary antibody for 1 h and visualized using a chemiluminescence detection system. α-Tubulin was used as the internal control, and relative protein expression levels were quantified by densitometric analysis normalized to α-tubulin.

ELISA quantification of inflammatory mediators

In vitro detection: Cell culture supernatants collected after drug treatment were stored at -80°C until analysis. The concentrations of inflammatory cytokines were measured using ELISA kit according to the manufacturers' instructions. Standard curves were generated using serial dilutions of standard samples, and

sample concentrations were calculated accordingly. Each sample was analyzed in duplicate, and the mean value was used for statistical analysis.

Construction of AIA mouse model and drug delivery

SPF-grade male C57BL/6 mice aged 6-8 weeks (body weight 20-25 g) were purchased from Guangdong Viton Leverhua Laboratory Animal Co., Ltd. (production license: SCXK (Yue) 2022-0063). All animal feeding and experimental procedures were performed at the Animal Experiment Center of Huizhou Third People's Hospital. Mean value was used for statistical analysis. Procedures were approved by the Institutional Animal Care and Use Committee of Guangzhou Medical University (Approval No. P2025-34014; approval date: October 24, 2025).

To establish the AIA model, 1% CFA was subcutaneously injected into the bilateral axillary regions and hind footpads of mice. Seven days after induction, mice with significant bilateral hind paw swelling were included for subsequent drug intervention. Initially, 30 mice were divided into five experimental groups (n=6 per group): normal control, AIA model, low-dose metformin (100 mg/kg/day), high-dose metformin (200 mg/kg/day), and MTX-treated groups (2 mg/kg, twice weekly). Before grouping, two mice were excluded: one mouse in the AIA model group failed to develop swelling in the left hind paw, and one mouse in the Met (100 mg/kg) group showed insufficient inflammatory responses. Finally, additional eligible model mice were included to maintain six animals per group.

Equivalent IFA was injected intra-articularly into bilateral knee joints for secondary immune stimulation. The MTX dose was converted based on the human equivalent dose according to previous studies [24]. Starting on day 7 after induction, body weight, paw swelling, and arthritis scores were monitored weekly until day 42. Mice were deeply anesthetized by intraperitoneal injection of 1.25% trichloroethanol solution (0.2 mL/10 g body weight). Adequate anesthesia was confirmed by the absence of the toe withdrawal reflex. Subsequently, blood samples were collected by cardiac puncture. After blood collection, mice were euthanized by cervical dislocation, and death was confirmed by the absence of heartbeat and bilateral pupil dilation.

Joint tissue sampling and histopathologic staining

Serum, knee joint specimens, and synovial tissues were collected for ELISA, histologic examination, and immunohistochemistry analyses. Joint specimens were fixed, decalcified, embedded in paraffin, and subjected to serial sectioning. Hematoxylin and eosin (H&E) staining was performed to evaluate synovial hyperplasia, inflammatory cell infiltration, and pannus formation. Liver and kidney tissues were collected and stained to assess systemic organ toxicity.

Statistical analysis

All experimental data were processed using GraphPad Prism 8.0 software. Measured data were presented as mean ± standard error of the mean (SEM). Kruskal-Wallis nonparametric test was adopted for pathologic scoring of data with a non-normal distribution. Inter-group comparisons of two sets were performed using an independent-sample t-test; multi-group comparisons were performed using one-way analysis of variance (ANOVA). $P < 0.05$ was considered significant.

Results

RA synovium and primary RA-FLSs exhibited upregulated expression of inflammatory factors and activated Wnt-3a/β-catenin signaling

We first investigated whether pro-inflammatory cytokine expression and Wnt-3a/β-catenin signaling were simultaneously activated in human RA synovial tissues and isolated primary RA-FLSs. IHC staining and western blot analysis showed significantly increased expression levels of IL-1β, IL-6, and IL-8 in rheumatoid synovial tissues compared to healthy synovial tissues (**Figure 1A**). Consistently, primary RA-FLSs isolated from RA synovium also exhibited markedly increased secretion and intracellular accumulation of these inflammatory mediators compared to healthy fibroblast-like synoviocytes (HC-FLSs) (**Figure 1D**).

Regarding the canonical Wnt pathway, both Wnt-3a expression and intranuclear accumula-

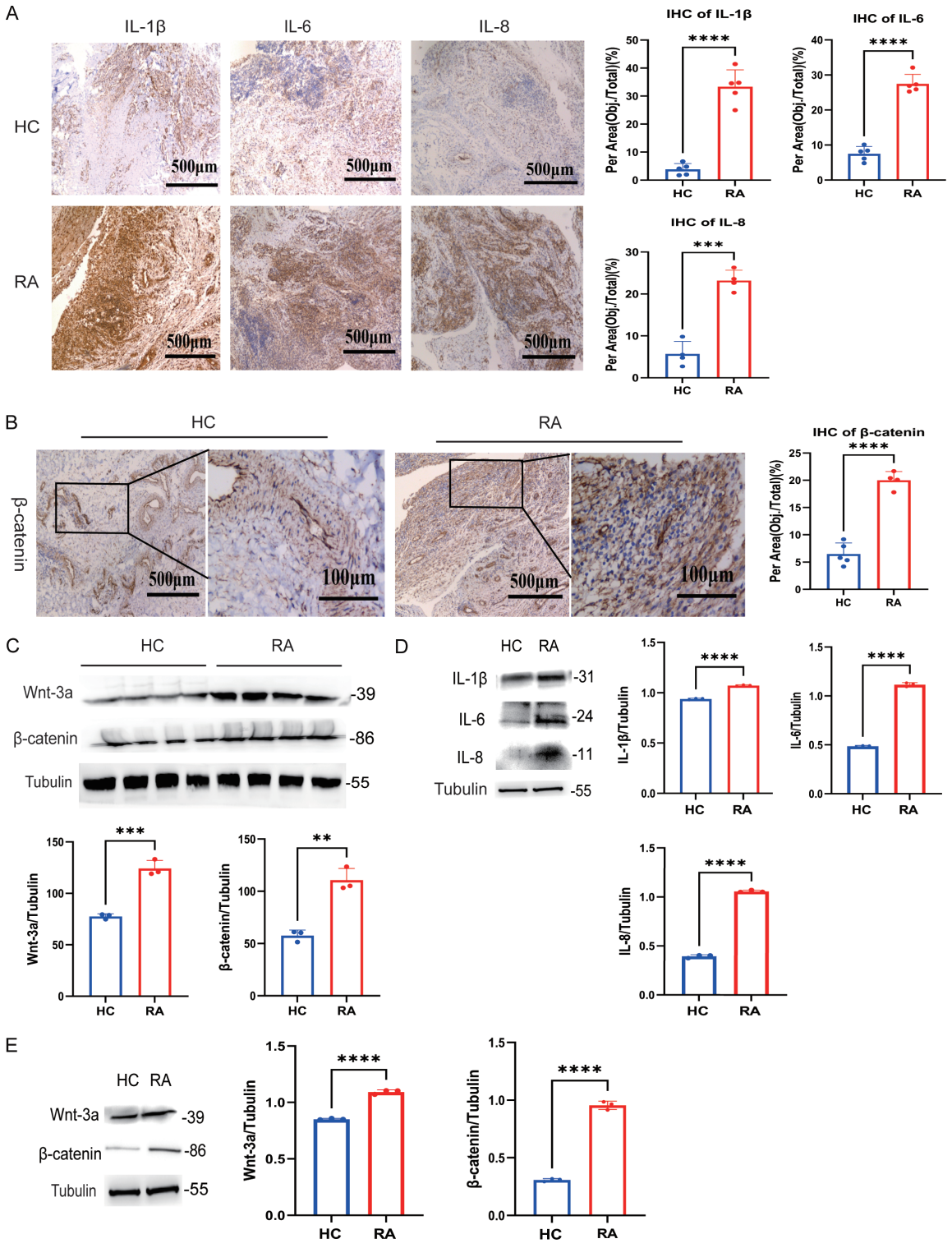


Figure 1. Elevated expression of pro-inflammatory mediators and Wnt-3a/β-catenin pathway components in RA synovium and primary RA-FLSs compared to healthy controls. A: Representative immunohistochemical images and corresponding quantitative analysis of pro-inflammatory mediators in human synovial samples. B: Immunohistochemical staining images and quantitative analysis of β-catenin abundance in synovial tissue sections from RA and health control samples. C: Western blot bands and densitometric quantification of Wnt-3a and β-catenin protein in normal and RA synovial specimens. D: Immunoblot detection and statistical quantification of pro-inflammatory mediators in primary HC-FLSs and RA-FLSs lysates. E: Western blot analysis with quantitative bar charts showing differential Wnt-3a and β-catenin expression between primary HC-FLSs and RA-FLSs populations. All data are presented as mean ± standard deviation (SD). **P < 0.01, ***P < 0.001, ****P < 0.0001.

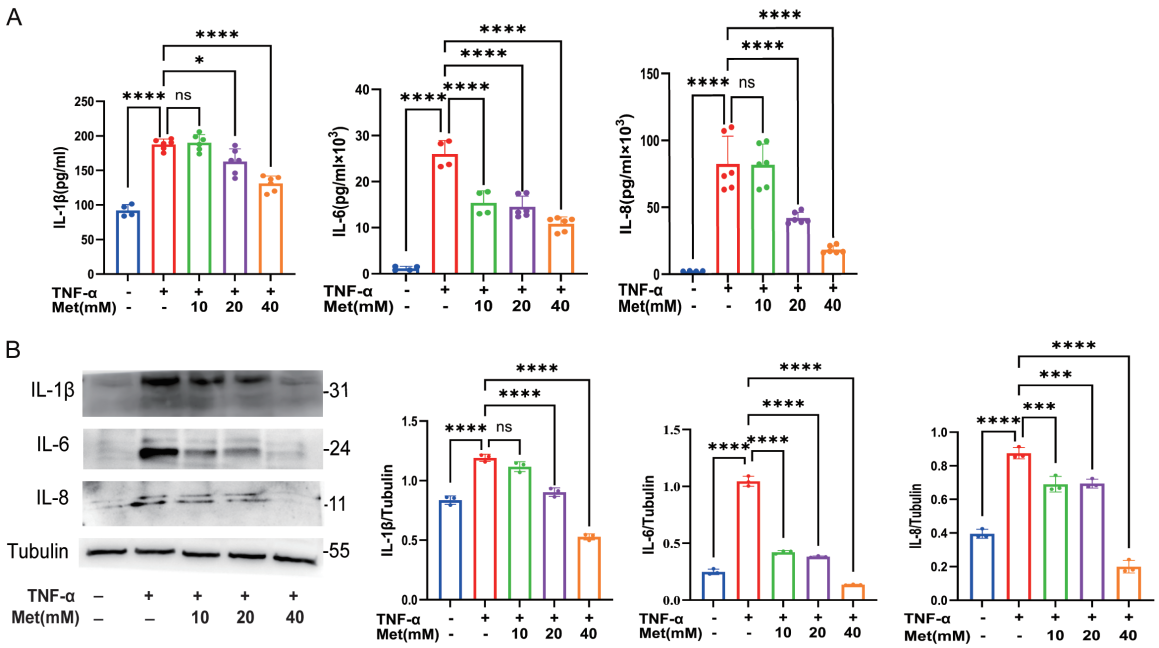


Figure 2. Metformin reduced the secretion of pro-inflammatory cytokines in TNF-α-activated RA-FLSs. A: ELISA-based quantitation of secreted IL-1β, IL-6, and IL-8 protein concentrations in culture supernatants. B: Immunoblotting and densitometric analysis of intracellular levels of the same proteins in whole-cell lysates. All data are presented as mean ± SD. *P < 0.05, ***P < 0.001, ****P < 0.0001; ns, non-significant comparison.

tion of β-catenin were markedly elevated in RA synovial tissues and corresponding primary RA-FLSs compared to controls (**Figure 1B**, **1C** and **1E**). These findings demonstrate persistent inflammatory activation and abnormal activation of Wnt-3a/β-catenin signaling in the rheumatoid synovial microenvironment.

Identification of primary RA-FLSs and determination of non-cytotoxic metformin concentrations

Before evaluating the anti-inflammatory effects of metformin, we first confirmed the purity of isolated RA-FLSs and determined the appropriate non-cytotoxic concentrations of metformin. Flow cytometric analysis showed that isolated synovial fibroblasts exhibited more than 92% positivity for CD90, and negative staining of both CD31 and CD45, confirming the high purity of RA-FLSs used for subsequent functional assays (Supplementary Figure 1A). CCK-8 assays were performed to exclude cytotoxic concentrations of metformin using a concentration gradient (10, 20, for 40, 60, 80, 100 mM). After 24 h of TNF-α stimulation, metformin concentrations up to 40 mM did not significantly affect RA-FLS viability (Supplementary Figure 1B). After 48 h of intervention, concentrations below 60 mM showed no significant cytotoxic effects on RA-FLSs (Supplementary Figure 1C). Therefore, metformin concentrations of 10, 20, 40 mM and a 48-h intervention duration were selected for subsequent *in vitro* experiments.

Metformin reduced the release of proinflammatory mediators

We next investigated whether metformin could suppress TNF-α-induced overproduction of pro-inflammatory cytokines in purified RA-FLSs. ELISA analysis of culture supernatants showed that TNF-α intervention significantly increased the extracellular release of IL-1β, IL-6, and IL-8. Co-treatment with metformin dose-dependently counteracted this increase (**Figure 2A**). Western blot analysis obtained consistent results: metformin treatment decreased the intracellular expression of TNF-α-induced inflammatory proteins at a 48-h intervention duration (**Figure 2B**).

Metformin inhibited TNF-α-induced activation of Wnt-3a/β-catenin signaling

We further investigated whether the anti-inflammatory effects of metformin were correlated with suppression of Wnt-3a/β-catenin

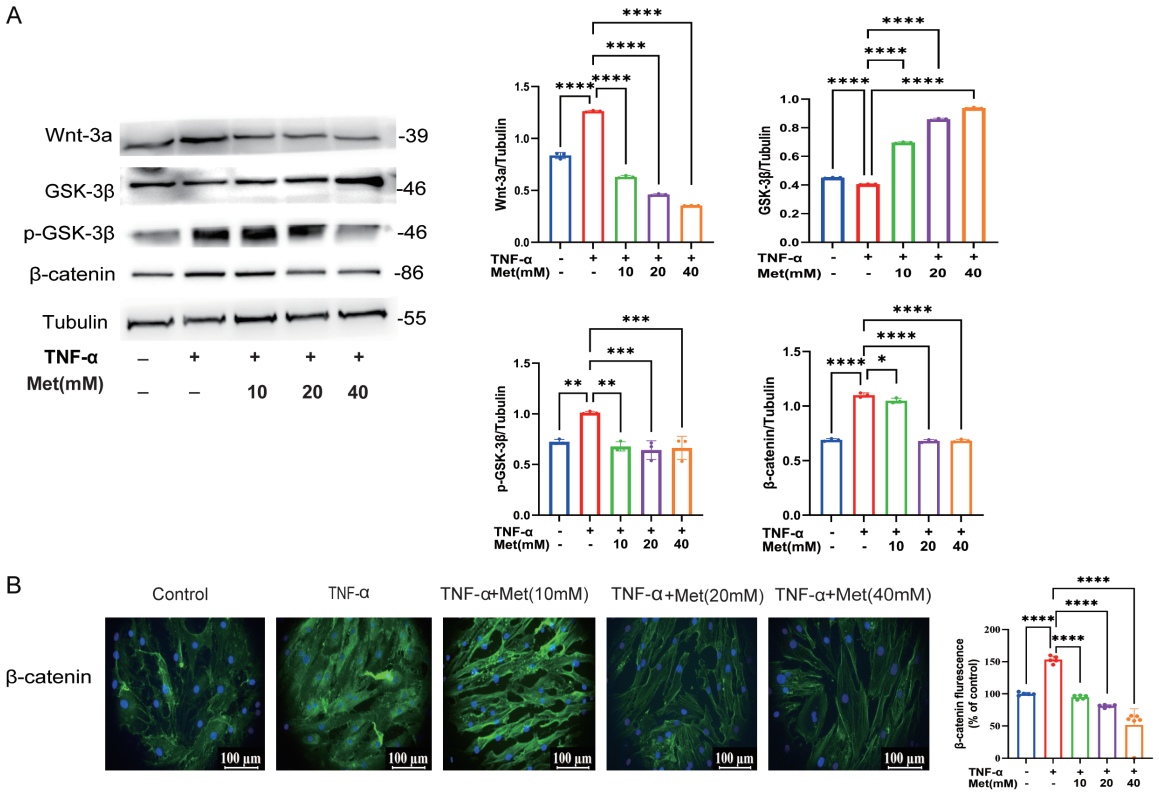


Figure 3. Metformin attenuated aberrant activation of canonical Wnt-3a/β-catenin signaling in TNF-α-exposed RA-FLS. A: Immunoblot profiles and quantitative analysis of expression changes in core pathway proteins across all experimental treatment groups. B: Inverted fluorescence microscopy images of β-catenin immunofluorescent labeling (green) with DAPI nuclear counterstaining (blue). Three independent cell batches were used for staining validation. All data are reported as mean ± SD. $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

signaling. Western blot analysis showed that TNF-α stimulation increased the expression of Wnt-3a and phosphorylated GSK-3β (Ser9) levels, accompanied by enhanced β-catenin expression and altered GSK-3β expression. Metformin treatment reversed these changes in a concentration-dependent manner, characterized by decreased Wnt-3a and phosphorylated GSK-3β levels, restored total GSK-3β expression, and suppressed β-catenin accumulation (**Figure 3A**).

Immunofluorescence analysis further showed that TNF-α stimulation promoted β-catenin accumulation in the nucleus, indicating activation of canonical Wnt signaling. In contrast, metformin treatment reduced nuclear β-catenin fluorescence intensity and increased cytoplasmic retention of β-catenin in a concentration-dependent manner, confirming that metformin inhibits nuclear translocation of β-catenin and inhibits downstream Wnt-3a signaling activity (**Figure 3B**).

LiCl reversed the anti-inflammatory effects of metformin in RA-FLSs

To investigate whether inhibition of Wnt-3a/β-catenin signaling mediates the anti-inflammatory effects of metformin, a pharmacologic rescue assay was performed using LiCl (GSK-3β inhibitor, Wnt pathway activator). TNF-α-stimulated RA-FLSs were co-treated with 40 mM metformin with LiCl.

Western blot results showed that LiCl partly reversed the inhibitory effects of metformin on Wnt-3a, p-GSK-3β and β-catenin, thereby restoring Wnt pathway activation (**Figure 4A**). Moreover, ELISA and western blot analyses demonstrated that LiCl co-treatment restored the secretion of inflammatory cytokines that had been suppressed by metformin alone (**Figure 4B**, **4C**). Collectively, these rescue experiments indicated that the anti-inflammatory effects of metformin in RA-FLSs are mediated, at least partially, through inhibition of the canonical Wnt-3a/β-catenin pathway.

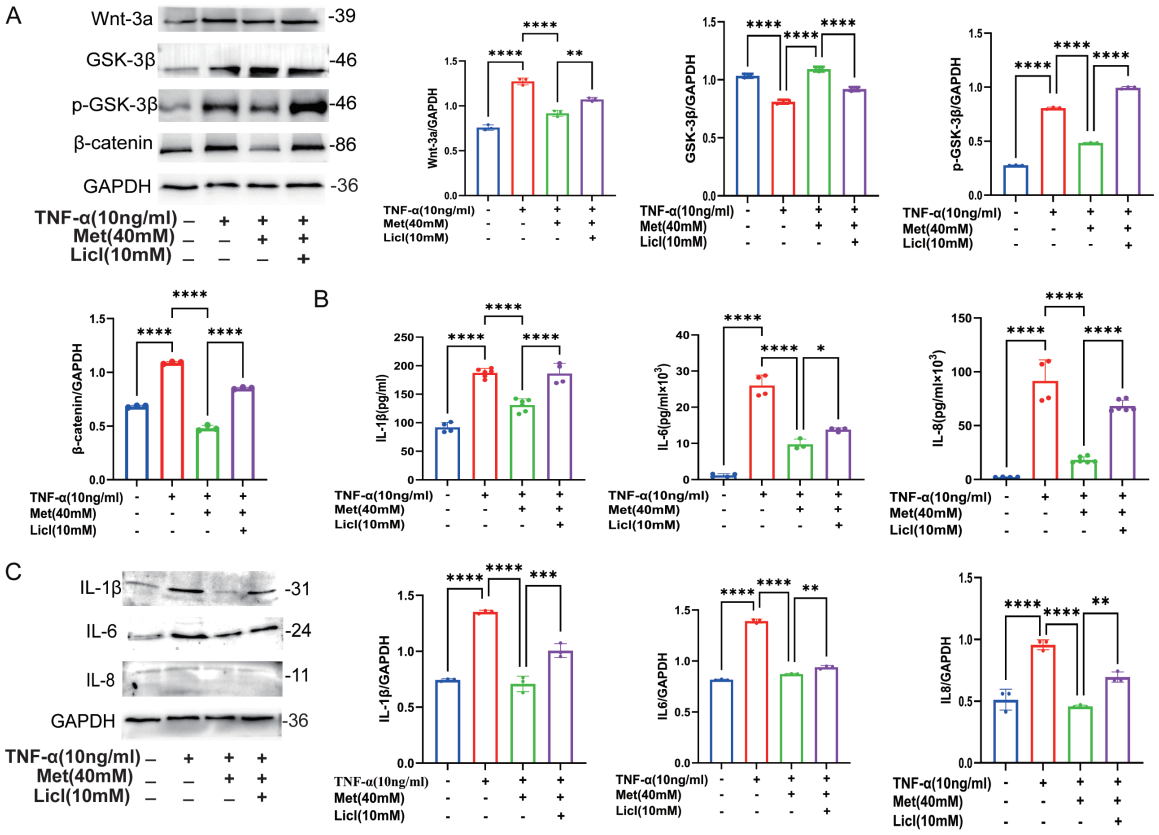


Figure 4. LiCl-mediated activation of Wnt/β-catenin signaling reversed the inhibitory effects of metformin in TNF-α-stimulated RA-FLSs. A: Representative western blot images and quantitative analysis of key Wnt/β-catenin pathway proteins in RA-FLSs co-incubated with 40 mM metformin and 10 mM LiCl. B: ELISA quantification of extracellular IL-1β, IL-6 and IL-8 levels in the cell culture supernatants following combined drug treatment. C: Western blot analysis and quantitative assessment of intracellular IL-1β, IL-6, and IL-8 protein levels following combined treatment. Data are expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Metformin and MTX were well tolerated in AIA mice

Before evaluating therapeutic efficacy *in vivo*, we assessed the safety of metformin and MTX in AIA mice. We hypothesized that daily intraperitoneal administration of metformin (100, 200 mg/kg) and twice-weekly administration of MTX (2 mg/kg) would not induce systemic weight loss or liver and kidney organ damage during a 42-day intervention. The experimental timeline for AIA modeling and drug administration are shown in **Figure 5A**. From day 7 to day 42 after arthritis induction, dynamic measurement of hind paw swelling thickness and arthritis clinical score showed severe joint swelling and high inflammatory score in untreated AIA model group. However, continuous monitoring of body weight demonstrated steady weight gain in all groups without significant weight loss (**Figure 5B**). Blood biochemical analysis demonstrated that the typical liver and kidney function indicators alanine transaminase (ALT), aspartate aminotransferase (AST), and creatinine (CR) of mice in all groups were within normal ranges (**Figure 5C**). Histologic examination of liver and kidney tissues revealed preserved cellular morphology without apparent edema, inflammatory infiltration, or structural damage in any treatment group (**Figure 5D**, **5E**). These findings suggest that metformin and MTX were well-tolerated at the administered doses during the experimental period.

Metformin improved clinical and histologic arthritis in AIA mice

Both MTX and metformin (100 and 200 mg/kg) treatment significantly alleviated paw swelling

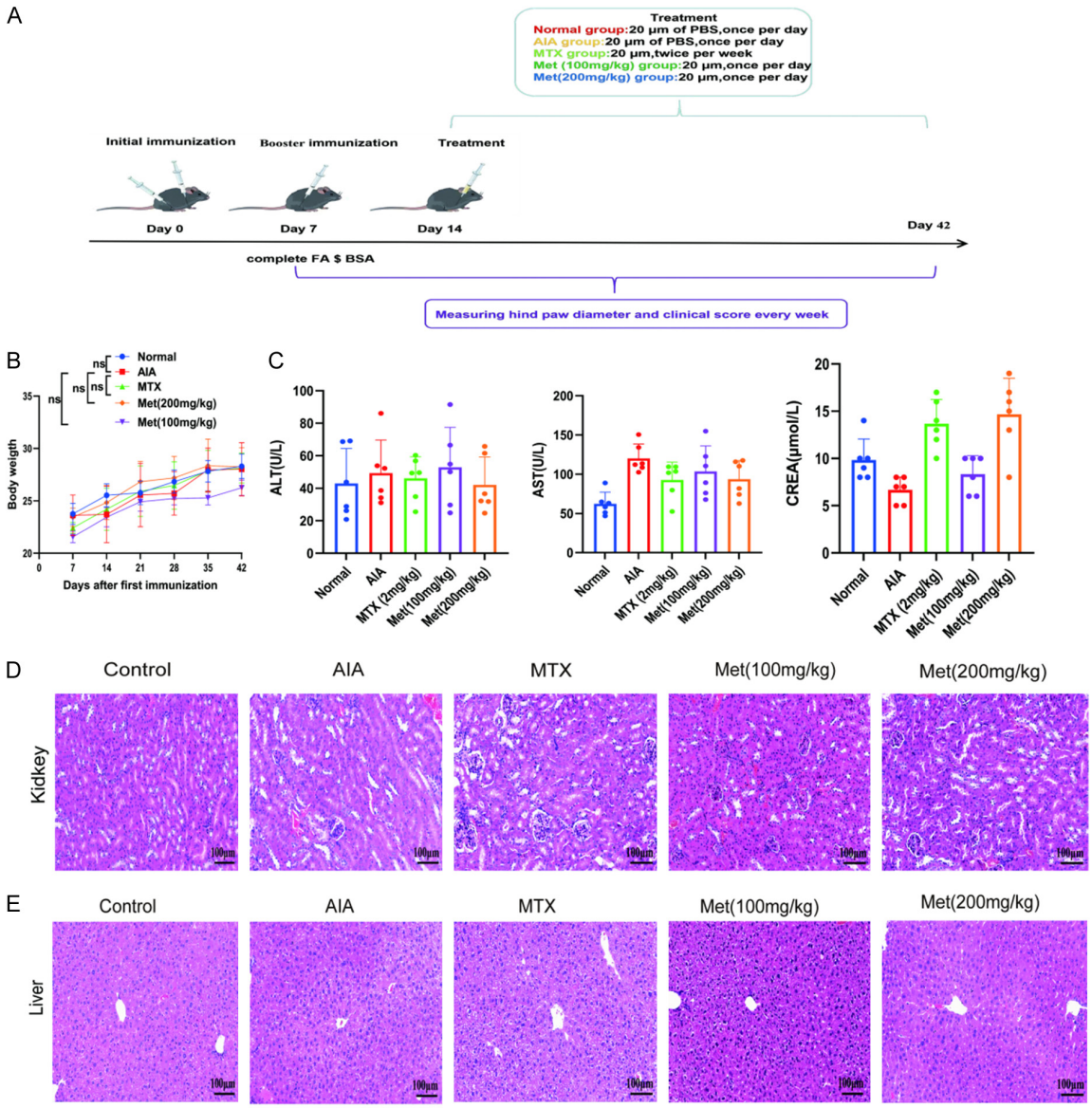


Figure 5. MTX and Metformin exhibited no apparent systemic toxicity in AIA mice. (A) Schematic workflow illustrating AIA induction, drug administration, and scheduled sample collection. (B) Changes in body weight during the experimental period. (C) Serum biochemistry values (ALT, AST, creatinine). Representative hematoxylin-eosin stained tissues of kidney (D) and liver (E) (n=3). All longitudinal quantitative data are expressed as mean ± SD. ns, non-significant intergroup comparison.

and reduced clinical arthritis scores in AIA mice, with high-dose metformin (200 mg/kg) achieving the greatest therapeutic improvement (**Figure 6A**, **6C**). As shown in **Figure 6B**, H&E staining of mouse knee joint sections revealed severe synovial hyperplasia, massive inflammatory cell infiltration, pannus formation, and joint space narrowing in AIA model mice. In contrast, MTX and low-dose metformin groups only presented mild synovial proliferation without severe cartilage damage; notably, the high-dose metformin group displayed joint tissue morphology that more closely resembled that of healthy control mice.

Metformin reduced systemic and synovial inflammatory mediator levels in AIA mice

We further evaluated the systemic and local anti-inflammatory effects of metformin in AIA mice. ELISA analysis demonstrated that serum levels of IL-1β, IL-6, and TNF-α were significantly

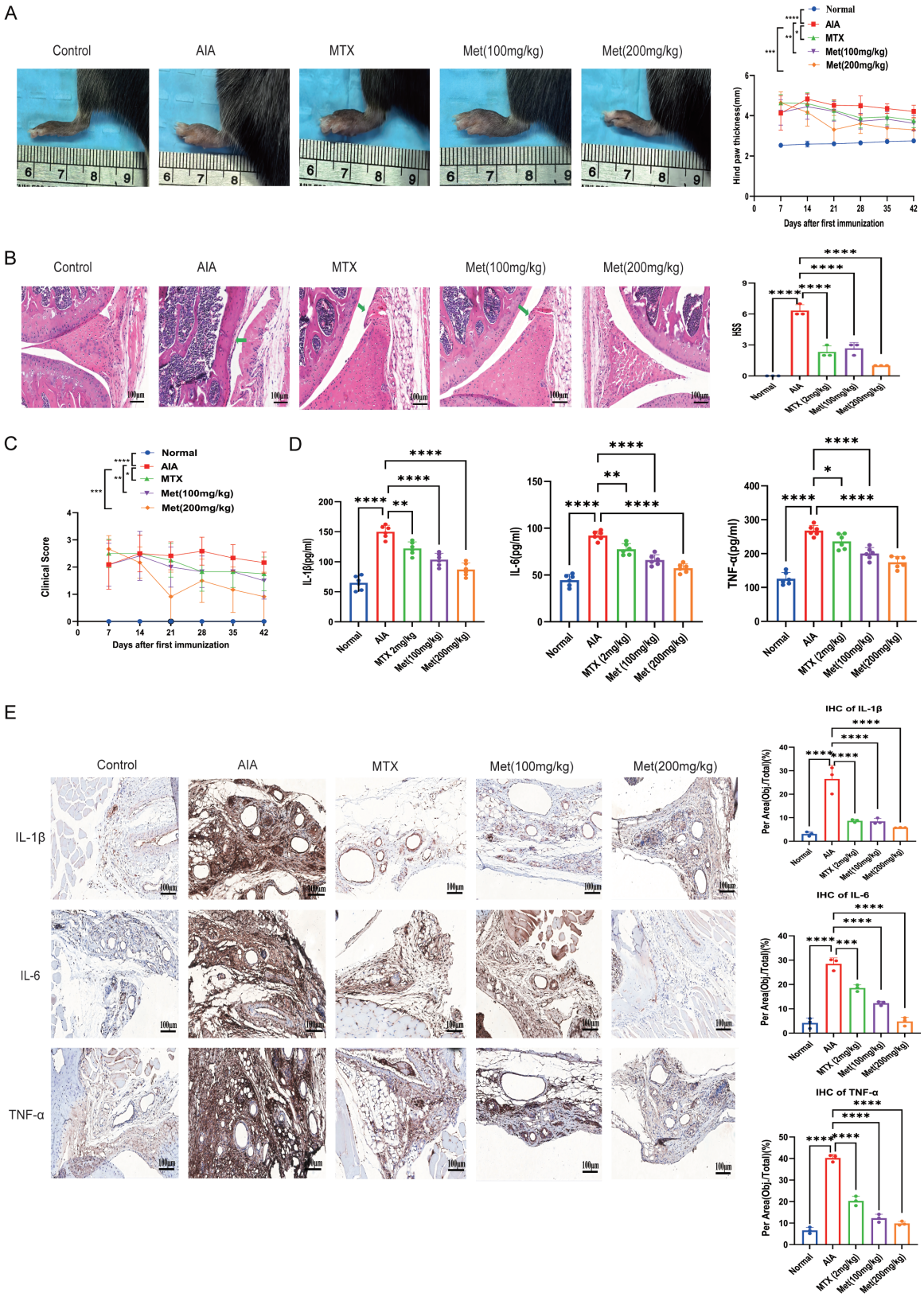


Figure 6. Metformin (Met) alleviated the severity of AIA mice. A: Representative photographs showing morphological swelling of bilateral hind paws captured on day 28 of treatment intervention. B: H&E-stained tissue sections displaying pathological alterations, including synovial lining hyperplasia, inflammatory immune cell infiltration, and

invasive pannus formation within joint cavities. C: Temporal curves of cumulative clinical arthritis scores assessed weekly to quantify overall disease severity in each experimental group. D: ELISA-based quantitative measurement of systemic circulating IL-1β, IL-6 and TNF-α levels in whole mouse serum samples. E: Immunohistochemical staining and quantitative analysis of the same antigens in paraffin-embedded knee synovial sections from each treatment cohort. All data are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

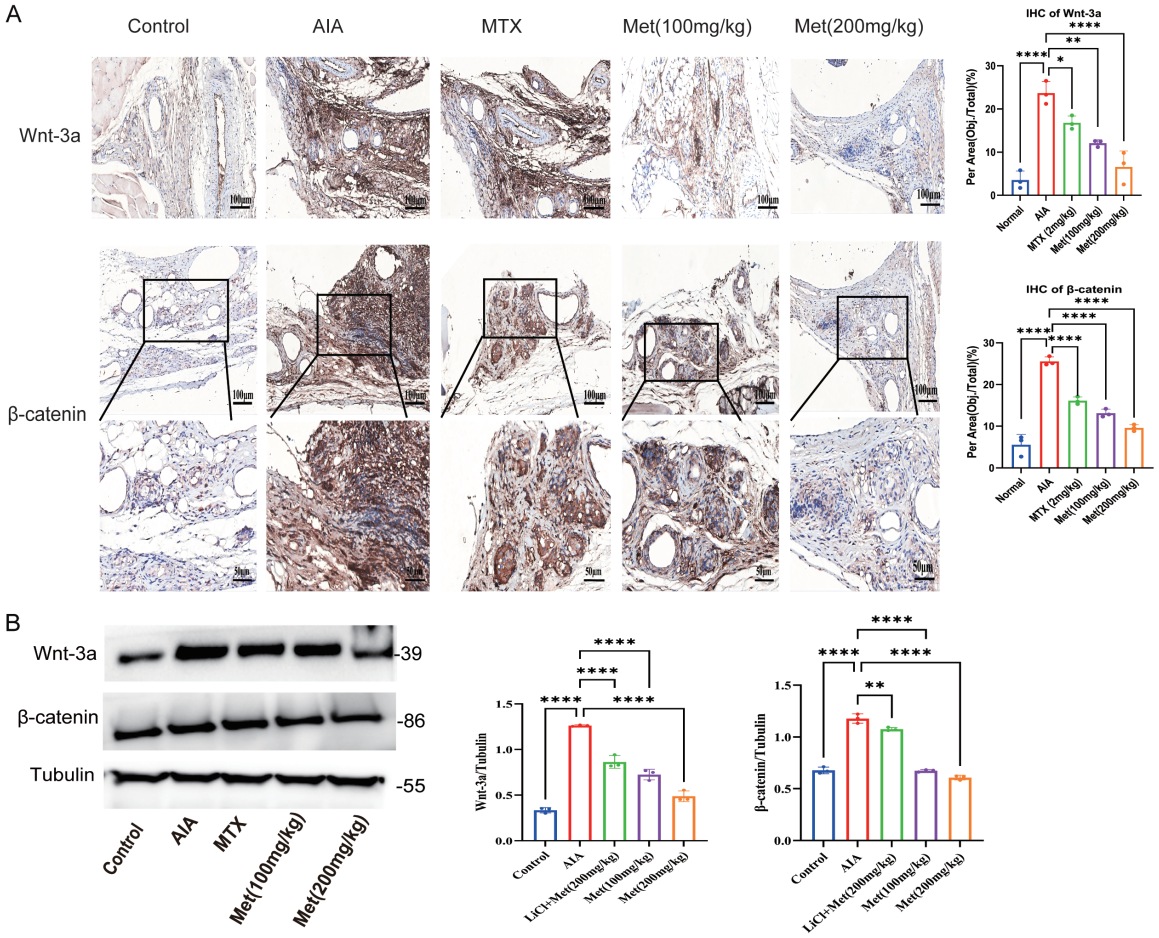


Figure 7. Intraperitoneal metformin delivery attenuated excessive Wnt-3a and β-catenin protein expression in the inflamed synovial membrane of AIA model mice. A: Immunohistochemistry staining and quantitative analysis showing Wnt-3a and β-catenin positive cell populations in joint synovial sections. B: Western blot images and quantitative analysis of core pathway proteins in the synovial membranes of the knee joints of each group of mice. All data are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

elevated in AIA mice compared with healthy controls (**Figure 6D**). MTX or metformin treatment markedly reduced circulating inflammatory cytokine levels, with metformin showing a dose-dependent inhibitory effect. IHC staining of mouse knee synovial tissues also demonstrated increased expression of inflammatory mediators in AIA synovium. Metformin treatment significantly decreased the proportion of positively stained cells and reduced staining intensity in the synovial membrane (**Figure 6E**).

Metformin inhibited Wnt-3a/β-catenin signaling in the synovium of AIA mice

Finally, we investigated whether metformin suppressed Wnt-3a/β-catenin signaling in arthritic synovial tissue of AIA mice. ICH staining revealed strong Wnt-3a and β-catenin staining in synovial slices from AIA mice, indicating enhanced activation of this pathway within inflammatory synovium. Intraperitoneal metformin (100 and 200 mg/kg) injection markedly reduced Wnt-3a and β-catenin expression in

synovial tissues, with 200 mg/kg metformin producing a stronger inhibitory effect than 100 mg/kg MTX. Quantitative analysis further confirmed that metformin reduced Wnt-3a/β-catenin pathway activation in AIA synovium in a concentration-dependent manner (**Figure 7A**). Western blot analysis of knee joint synovial tissues further confirmed that metformin dose-dependently suppressed the expression of key proteins involved in the Wnt-3a/β-catenin signaling pathway (**Figure 7B**).

Discussion

Persistent synovial inflammation and remodeling are key pathologic processes driving RA progression. Fibroblast-like synoviocytes (FLSs) are the dominant stromal cell population in synovial tissues, and their sustained activation contributes to multiple pathological features of RA [25]. TNF-α is a critical mediator of synovial fibroblast activation; therefore, TNF-α-stimulated primary RA-FLSs are widely used as an *in vitro* model to investigate RA pathogenesis and evaluate potential treatments [26, 27]. Previous studies have shown that metformin reduces IL-1β and TNF-α secretion by cultured RA-FLSs and decreased circulating proinflammatory cytokine levels in animal models [19, 20]. However, the molecular mechanisms underlying these effects, particularly the involvement of Wnt/β-catenin signaling, had been incompletely understood. In the present study, metformin attenuated inflammatory activation in TNF-α-stimulated primary RA-FLSs, and this protective effect was mediated through downregulation of the Wnt-3aWnt-3a/β-catenin signaling. In AIA mice, metformin also alleviated synovial inflammation and reduced arthritic severity in AIA mice, supporting its potential therapeutic value in RA [28].

Persistent inflammatory activation of the synovial lining is a major contributor to RA progression. Upon abnormal activation, RA-FLSs acquire an aggressive inflammatory phenotype and secret abundant pro-inflammatory mediators, including TNF-α, IL-1β, IL-6, and IL-8 [29-31]. Excessive production of these mediators forms a positive feedback loop that sustains synovial inflammation, ultimately contributing to irreversible cartilage destruction and bone erosion [32]. Therefore, suppression of excessive synovial inflammation represents a central treatment strategy for RA management [33]. Previous studies have suggested that metformin inhibits the secretion of IL-1β and TNF-α in cultured RA-FLSs and reduces circulating inflammatory cytokine levels in rat arthritis models [19, 20]. However, the mechanisms through which metformin regulates TNF-α-mediated inflammatory responses in human primary synovial fibroblasts remained unclear. To fill this research gap, the present study integrated in vitro and in vivo experiments to systematically investigate the anti-inflammatory effects and underlying mechanisms of metformin in RA.

TNF-α is a key upstream mediator that initiates inflammatory cascades in RA and promotes the transition of quiescent RA-FLSs toward an activated inflammatory phenotype. Consistent with previous findings, our *in vitro* experiments confirmed that TNF-α stimulation significantly increased the expression of IL-1β, IL-6, and IL-8 in RA-FLSs. Notably, treatment with three non-cytotoxic concentrations of metformin (10, 20, and 40 mM) reduced TNF-α-induced overproduction of these cytokines in a concentration-related manner, further supporting its anti-inflammatory activity in TNF-α-activated RA-FLSs. Consistent with these *in vitro* findings, intraperitoneal metformin administration in AIA mice significantly decreased circulating IL-1β, IL-6, and TNF-α levels and reduced inflammatory mediator expression in joint synovial tissues. Within the tested non-cytotoxic dose range, metformin exhibited substantial anti-inflammatory activity in AIA mice, with effects comparable to or greater than those observed with MTX treatment.

A considerable body of preclinical evidence has demonstrated hyperactivation of the canonical Wnt/β-catenin pathway in synovial tissues from RA patients, cultured primary RA-FLSs, and experimental arthritis models [9-12]. This signaling axis exerts broad regulatory effects, including modulation of inflammatory responses in synovial fibroblast [8], making it a potential therapeutic target for RA. Under physiologic conditions without Wnt ligand stimulation, cytoplasmic β-catenin is phosphorylated by GSK-3β and subsequently targeted for ubiquitin-proteasome-mediated degradation. Upon Wnt ligand binding to the Frizzled/LRP5/6 receptor complex, GSK-3β is

phosphorylated at Ser9 and loses its enzymatic activity, thereby disrupting the formation of the β-catenin destruction complex (composed of p-GSK-3β, AXIN, and APC). Consequently, β-catenin escapes phosphorylation and degradation, accumulates in the cytoplasm, and translocates into the nucleus, where it interacts with TCF/LEF transcription factors to activate downstream target gene transcription.

Our western blot analyses of cellular and animal samples illustrated that metformin treatment reduced Wnt-3a and p-GSK-3β expression, restored total GSK-3β levels, and decreased intracellular β-catenin accumulation. These findings suggest that metformin suppresses excessive activation of the Wnt3a/β-catenin pathway in TNF-α-stimulated RA-FLSs and arthritic synovial tissues. Immunofluorescence staining further validated reduced β-catenin nuclear translocation, a core functional event in Wnt signaling activation. Lithium chloride (LiCl), a pharmacologic inhibitor of GSK-3β, promotes phosphorylation of GSK-3β at Ser9, thereby stabilizing β-catenin and facilitating its nuclear translocation to reactivate Wnt signaling [34]. In the present study, LiCl rescue experiments were performed to verify metformin's molecular target. LiCl co-treatment partially reversed the inhibitory effects of metformin on Wnt-3a/β-catenin signaling activation and restored inflammatory responses in RA-FLSs. Collectively, these rescue experiments, *in vivo* and *in vitro* consistently indicated that metformin alleviates RA pathologic manifestations, at least partially, through suppression of the canonical Wnt3a/β-catenin signaling pathway.

Previous studies investigating metformin in RA-FLSs have mainly focused on cell proliferation and general anti-inflammatory activity [35], whereas the underlying molecular mechanisms remain incompletely understood. In the present study, we systematically investigated the role of Wnt-3a/β-catenin signaling in the anti-inflammatory effects of metformin using a TNF-α-induced RA-FLS model. Furthermore, we confirmed metformin-mediated suppression of IL-1β expression and quantitatively demonstrated dose-dependent inhibitory effects on IL-6 and IL-8 expression.

This study had several limitations. First, whether metformin regulates the Wnt-3a/β-catenin pathway through AMPK signaling remains to be further investigated. In preliminary experiments, we used AICAR (200 μM; 5-Aminoimidazole-4-carboxamide ribonucleoside, an AMPK activator) and Compound C (20 μM; dorsomorphin, an AMPK inhibitor) to modulate AMPK phosphorylation. Neither treatment significantly altered β-catenin expression (Supplementary Figure 2). These findings suggest that metformin-mediated modulation of Wnt-3a/β-catenin signaling may not depend primarily on the AMPK pathway, and further research is needed. Second, the Wnt pathway rescue experiments in this study used LiCl drug intervention. Given the potential off-target effect of LiCl, future studies using genetic methods such as GSK-3β knockdown by small interfering RNA or gene knockout models, are needed to establish a more specific causal relationship between Wnt-3a/β-catenin signaling and the therapeutic effects of metformin. Third, the concentration of metformin used in this in vitro study (10-40 mM) was substantially higher than the plasma concentration typically observed in clinical settings (10-40 μM); although high concentrations can partially reflect local tissue accumulation of metformin through transporter-mediated uptake, caution is required when extrapolating *in vitro* experimental results to clinical applications. Fourth, we were unable to perform an in vivo LiCl rescue experiment in AIA mice. The causal relationship between Wnt-3a/β-catenin pathway inhibition and the *in vivo* therapeutic effects of metformin require further validation. Fifth, the comparison between metformin and methotrexate in the animal experiments should be interpreted with caution: the two drugs were not administered at pharmacokinetically equivalent doses, and the twice-weekly MTX administration regimen used in this study does not fully reflect clinical MTX treatment protocols, which may not reflect the optimal therapeutic efficacy of methotrexate. Sixth, the relatively small sample size of human synovial tissues (6 RA patients and 6 control subjects) may restrict the generalizability of clinical observations. This limitation is inherent to the mechanistic nature of the present study and should be addressed in larger clinical cohorts.

Conclusion

Metformin reduced inflammatory cytokine production in TNF-α-stimulated RA-FLSs and alleviated histologic arthritis in AIA mice. These

effects were associated with suppression of Wnt-3a/β-catenin pathway activity, and LiCl rescue experiments further suggested the involvement of this pathway in the anti-inflammatory effects of metformin. These findings extend previous studies on the effects of metformin on RA-FLS proliferation and provide a rationale for further preclinical evaluation of metformin as an adjunctive treatment for RA.

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Disclosure of conflict of interest

None.

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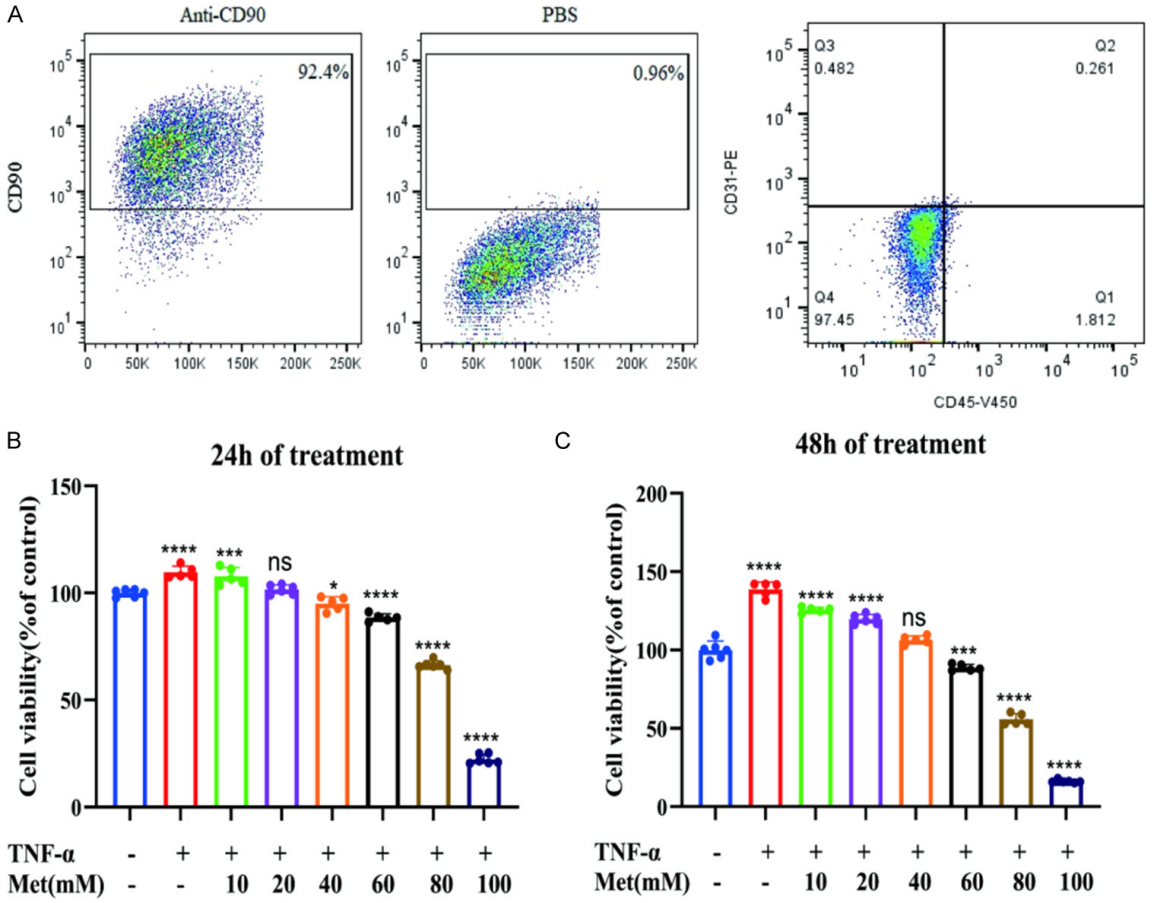
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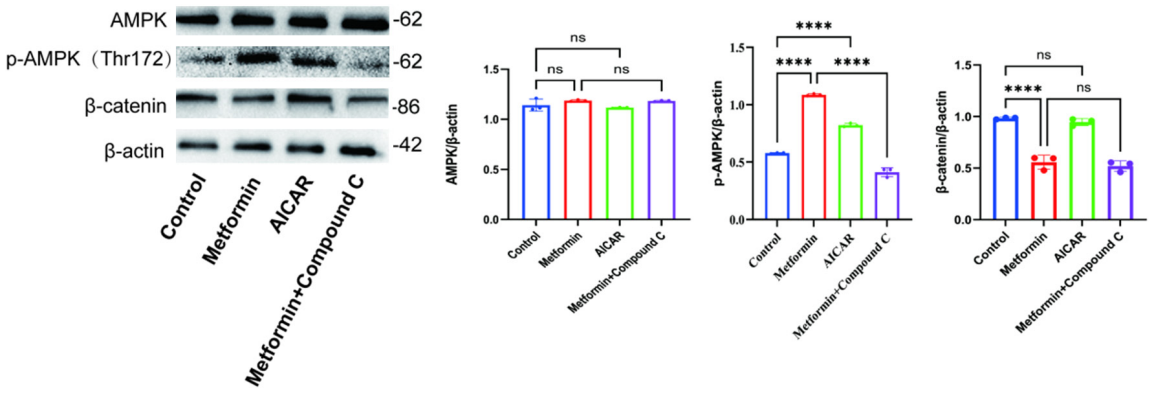
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Supplementary Table 1. Key clinicopathologic characteristics of the two groups

Patients	Rheumatoid arthritis (n=6)	Healthy controls (n=6)
Age in years	61, 72, 67, 68, 70, 65	50, 30, 40, 46, 53, 28
Female, n (%)	5 (83)	3 (50)
Duration of RA (year)	5, 7.5, 6, 4, 5.5, 6	
Anti-CCP positive n (%)	6 (100)	0 (0)
DAS28 scores mean (SD)	4.9 (0.2)	N/A
RF positive, n (%)	6 (100)	0 (0)
NSAID, n (%)	4 (67)	0 (0)
DMARD, n (%)	5 (83)	0 (0)
Immunosuppressive or biologic therapies	0 (0)	0 (0)



Supplementary Figure 1. Characterization of primary RA-FLS purity and cytotoxicity of metformin under TNF-α stimulation using CCK-8 assay. A: Positivity of CD90, CD31, and CD45 determined using Flow cytometry. B: Quantitative analysis of cellular viability after 24 h co-treatment with 10-100 mM metformin alongside 10 ng/mL TNF-α. C: Viability of RA-FLSs exposed to gradient metformin combined with TNF-α for 48 h. All quantitative results are reported as mean ± standard error of the mean (SEM). *P < 0.05, ***P < 0.001, ****P < 0.0001; ns, no significant difference compared to the untreated control group.



Supplementary Figure 2. Metformin regulated β-catenin through a non-AMPK pathway. Western blot images and quantitative analysis of AMPK, p-AMPK and β-catenin. Data are presented as mean ± SD. ****P < 0.0001. ns, non-significant intergroup comparison.