

Triptolide alleviates the development of inflammation in ankylosing spondylitis via the NONHSAT227927.1/JAK2/STAT3 pathway

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Abstract. Ankylosing spondylitis (AS) is a chronic inflammatory disease that can destroy the affected joints. Triptolide (TPL), a key active ingredient of the traditional Chinese medicine *Tripterygium wilfordii* exhibits promising efficacy in rheumatic immune disease with its anti-inflammatory effects. The present study aimed to elucidate the mechanism of TPL in treatment of AS by regulating the long non-coding RNA (lncRNA) NONHSAT227927.1. The role and underlying mechanisms of TPL in the development of inflammation in AS were assessed. *In vivo*, the expression of NONHSAT227927.1 in AS was detected by reverse transcription-quantitative (RT-q) PCR. Correlation analysis and binary logistic regression were performed between immune and inflammatory indicators, perception scale scores of patients and NONHSAT227927.1. *In vitro*, Cell Counting Kit-8 was used to evaluate the activity of AS-fibroblast-like synoviocytes (FLSs) following TPL exposure. AS-FLS inflammation was assessed by qPCR and ELISA. The interaction between TPL and JAK2 and STAT3 was verified by molecular docking and the JAK2/STAT3 pathway components were detected by western blotting. NONHSAT227927.1 was knocked down by small interfering RNA to determine its role. NONHSAT227927.1 was highly expressed *in vivo* and positively correlated with disease duration, disease duration, Body mass index (BMI), C-reactive protein (CRP), Visual analog scale (VAS), Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and Bath Ankylosing Spondylitis Metrology Index, among which ESR and VAS and BASDAI score were risk factors for NONHSAT227927.1. TPL downregulated pro-inflammatory factors in AS-FLSs and inhibited the

JAK2/STAT3 pathway via NONHSAT227927.1. TPL inhibited inflammatory factors in AS-FLSs and alleviated inflammatory responses via the NONHSAT227927.1/JAK2/STAT3 axis.

Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory disease that clinically manifests as chronic back pain and stiffness. AS inflammation tends to accumulate in the sacroiliac joints at the initial stage and then primarily affects the spine (1). AS has ~0.25% prevalence in China and its incidence in males is higher than that in females (~3:1) (2,3). Long-term spinal involvement may affect the biomechanical properties of the spine, accompanied by chronic inflammatory changes. As a key structure connecting the bone and joint capsule, the synovium often exhibits inflammatory erosion and hyperplasia in inflammatory disease (4). Under the stimulation of inflammatory cytokines [such as tumor necrosis factor (TNF- α)] (5), AS fibroblast-like synoviocytes (AS-FLSs) produce multiple inflammatory signaling molecules, including IL-6 and IL-4 (6,7), triggers cytokine cascade effects and recruits inflammatory cells, thereby aggravating inflammatory responses; these further induce ongoing joint inflammation and bone destruction (8). Therefore, it is key to control inflammation as early as possible, improve joint function and reduce deformities (9). To pathogenesis of AS is not fully understood. Due to lack of effective treatment methods, AS pain affects the health and daily life of patients. Hence, it is crucial to develop novel and effective drugs for relieving the symptoms of AS.

Key epigenetic regulators, including long non-coding RNAs (lncRNAs), have multiple biological functions (10,11). Certain aberrant lncRNA expression profiles contribute to AS pathogenesis (12,13). lncRNAs may directly regulate expression of protein-coding genes. For example, Han *et al* (14) found that upregulated lncRNA FOXA2 is associated with AS recurrence and poor outcome (14). Li *et al* (15) reported that lncRNA Maternally expressed gene 3 is downregulated in AS and associated with disease activity and hospital stay and disease duration. Our previous study revealed that NONHSAT227927.1 is a key lncRNA involved in AS inflammation via high-throughput sequencing and bioinformatics analysis (16).

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The Janus kinase/signal transducer and activator of transcription (JAK/STAT) axis is involved in regulation of cancer, inflammation and immunity. Numerous cytokines affect JAK/STAT signaling. When cells are subjected to pro-proliferative stimuli, JAK2 activates STAT3 to regulate cell survival and proliferation via its downstream targets (17,18). The role of JAK/STAT kinase signaling has also been studied in rheumatoid arthritis. For example, preliminary observations have suggested that the JAK/STAT kinase signaling cascade regulates the activation and proliferation of IL17⁺ effector memory T cells, showing a potential role in the pathogenesis of AS (19,20). lncRNA NONHSAT227927.1 is overexpressed in AS and regulates inflammatory factors by activating the JAK2/STAT3 signaling pathway and promotes development of AS (21).

Triptolide (TPL), a main compound extracted from the traditional Chinese medicine *Tripterygium wilfordii*, has strong biological activity for treating numerous types of tumor and autoimmune disease, especially rheumatoid arthritis, systemic lupus erythematosus and AS (22-24). Ji *et al* (25) found that TPL could inhibit osteoclastogenesis of the spine to alleviate arthritis in DBA/1 mice. Wang *et al* (26) reported the anti-ossification effects of TPL. To the best of our knowledge, however, little is known about whether TPL affects synovial cells to alleviate AS inflammation.

Our previous research has confirmed the role of the NONHSAT227927.1/JAK2/STAT3 combination in regulating inflammation in AS (21). The diagnostic potential of NONHSAT227927.1 on AS has also been confirmed through receiver operating characteristic curve analysis.

The present study aimed to investigate whether TPL regulates inflammation by targeting the NONHSAT227927.1/JAK2/STAT3 axis and whether it has an anti-inflammatory effect on AS.

Materials and methods

Characteristics of subjects. From March to May 2021, a total of 50 AS patients were recruited from the First Affiliated Hospital of Anhui University of Traditional Chinese Medicine in Hefei, Anhui Province, China. Among these patients, there were 32 males and 18 females, with ages ranging from 19 to 65 years. Additionally, 30 healthy controls were also recruited, who were carefully matched with the AS patients in terms of both gender and age. The inclusion criteria were as follows: i) Patients who met the diagnostic criteria of the 1984 American Society of Rheumatology (27), ii) aged 18-80 years and iii) patients with complete clinical data. The exclusion criteria were as follows: i) Severe mental illness or severe liver and kidney dysfunction, ii) pregnancy and iii) history of immunosuppressive drugs. All participants provided written informed consent. The present study was approved by the Medical Ethics Committee of Anhui University of Traditional Chinese Medicine (approval no. 2015-AH20).

Indicators collection. The general data of 50 cases in the AS group and 30 cases in the NC group were retrieved from the case system of the First Affiliated Hospital of Anhui University of Traditional Chinese Medicine: age, gender, disease duration,

height, weight, perception scale scores of patients indicators: Self Rating Anxiety Scale (SAS), Self-Rating Depression Scale (SDS), The Visual Analogue Scale (VAS), Bath Ankylosing Spondylitis Disease Activity Index (DASDAI), Bath Ankylosing Spondylitis Functional Index (BASFI) and Bath Ankylosing Spondylitis Metrology Index (BASMI), clinical indicators: Immunoglobulin A (IgA), Immunoglobulin M (IgM), Immunoglobulin G (IgG), Complement C3 (C3), Complement C4 (C4), erythrocyte sedimentation Rate (ESR), C-reactive Protein (CRP).

FLS culture. Human primary FLSs isolated from sacroiliac joints (cat. no. RAB-iCell-s004) and AS-FLSs (cat. no. JDBG200752) were purchased from Subikang iCell Bioscience, Inc. These cells were cultured in RPMI-1640 medium [Saibaikang (Shanghai) Biotechnology Co., Ltd.] containing 1% penicillin-streptomycin and 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in an incubator (37°C, 5% CO₂) with 100% humidity (11). The medium was refreshed every 2-3 days. At 80-90% confluence, cells were washed twice with phosphate-buffered saline, detached with 0.25% trypsin and observed under a light microscope (magnification, x200). When the cells adhered to the wall and became loose, trypsin was discarded. Complete medium was added and the cell layer was blown.

Culture of AS-Peripheral blood mononuclear cells (PBMCs). Venous blood (4 ml) from patients with AS was collected using an anticoagulant tube and mixed well with 4 ml PBS. Then, 4 ml Ficoll solution (cat. no. 17-1440-02; GE Healthcare) was added into a 15-ml centrifuge tube. The diluted blood was added slowly to the upper layer of the Ficoll solution, avoiding mixing the two solutions. Following tube centrifugation (1,150 x g, 37°C, 20 min) in a horizontal centrifuge, AS-PBMCs were located in the second white layer from the top. Next, cells were moved to a new centrifuge tube with PBS (10-15 ml) and then centrifuged (640 x g, 37°C, 10 min). After removing supernatant, PBS (5-10 ml) before repeating centrifugation. The cells were resuspended by adding 1 ml PBS, transferred to a 1.5-ml EP tube and set aside.

AS-PBMC induction and AS-FLS transfection. AS-PBMCs and AS-FLSs were seeded and cultured in a Transwell chamber at a ratio of 3:1. PBMCs were added to the apical chamber and FLSs were placed in the basolateral chamber. Cells were incubated for 24 h in 37°C. After growing to 70-90% confluence, cells in each Transwell well were removed for subsequent experiments. AS-FLSs were transfected with small interfering RNA (siRNA)-negative control (NC; cat. no. A06001) and siRNA-NONHSAT227927.1 (cat. no. A01001; both Shanghai GenePharma Co, Ltd.) using Lipofectamine[®] 2000 (cat. no. 11668-019; Thermo Fisher Scientific, Inc.; 37°C, 24 h). The transfection concentration of siRNA was 50 pmol/ml. A total of >5 µg nucleic acid was used and cells were collected following incubation (37°C, 48 h). Cell transfection efficiency was detected using reverse transcription quantitative polymerase chain reaction (RT-qPCR). The oligonucleotide sequences were as follows: siRNA-NC forward, 5'-UUCUCCGAA CGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUU

CGGAGAATT-3' and siRNA-NONHSAT227927.1 forward, 5'-CGACUGACUCGAUCUUUGAAG-3' and reverse, 5'-UCAAGAUCGAGUCAGUCGGG-3'.

RT-qPCR. The total RNA was extracted from AS-FLSs with TRIzol® (cat. no. 15596026; Thermo Fisher Scientific, Inc.). cDNA was synthesized using the PrimeScript™ RT Reagent kit (cat. no. RR047A, Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. Novostart SYBR qPCR SuperMix Plus (cat. no. E096-01B; Novoprotein Scientific, Inc.) was used for qPCR following the manufacturer's instructions. Thermocycling conditions were as follows: Initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 20 sec and annealing at 60°C for 1 min. Relative quantitative analysis was performed using the 2- $\Delta\Delta Cq$ method (24) with β -actin as an internal reference. The sequences of primers were as follows: NONHSAT227927.1 forward, 5'-TGGGAACCTCCTGAGCATACC-3' and reverse, 5'-ATGCTCCAGCAAGTCAGGAT-3' and β -actin forward, 5'-CCCTGGAGAAGAGCTACGAG-3' and reverse, 5'-GGAAGGAAGCTGGAAGAGT-3'.

ELISA. The levels of IL-4 (cat. no. JYM0142Hu), IL-10 (cat. no. JYM0155Hu) and TNF- α (cat. no. JYM0110Hu) in the serum of patients or the supernatant of AS-FLSs were evaluated using ELISA kits according to the manufacturer's instructions (Wuhan Genomics Technology Co., Ltd.). Each sample was examined three times independently.

Cell Counting Kit 8 (CCK-8) assay. The cell viability was measured using a CCK-8 assay kit (BIOSS) following the manufacturer's protocols. A total of 3×10^4 AS-FLSs was seeded into 96-well plates and cultured to 70-90% confluence. The cells were cultured for 0, 12, 24 and 48 h at 37°C. Then, 10 μ l CCK-8 solution was added to each well for 1-4 h at 37°C. The cell viability was assessed by measuring the optical density at 450 nm.

Molecular docking of TPL with JAK2/STAT3 proteins. Protein Data Bank (PDB) format files of JAK2 and STAT3 proteins were retrieved in the PDB protein structure database (rcsb.org/); the mol2 format files of the TPL structure were downloaded from the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) database (tcmsp-e.com). Before molecular docking, the software pymol 2.3.0 (DeLano Scientific LLC) was used to dehydrate the target protein receptor molecule and remove the ligand small molecule. The target protein was hydrogenated by Auto Dock 4.2.6 software (Molecular Graphics Lab at The Scripps Research Institute, La Jolla, USA). Finally, the receptor protein was molecularly docked with the ligand small molecule by Auto Dock Vina 1.1.2 software (Molecular Graphics Lab at The Scripps Research Institute), and visualized by PYMOL 2.3.0 (DeLano Scientific LLC). The binding energy of receptor protein and ligand small molecule energy <5 kcal/mol indicated strong binding force.

Western blot analysis. A total of 600 μ l RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) was used to extract total protein in the cells. SDS-PAGE preparation kit

(cat. no. S8010; Beijing Solarbio Science & Technology Co., Ltd.) was used to prepare the gel (5% stacking gel, 10% separating gel concentration) and 30 μ g protein/lane was added for electrophoresis on PVDF membranes. The membranes were blocked with 5% skimmed milk (0.1% Tween 20) for 2 h at room temperature and incubated (overnight, 4°C) with primary antibodies as follows: Anti-phosphorylated p-STAT3 (1:500; cat. no. ab76315; Abcam), p-JAK2 (1:1,000; cat. no. ab32101; Abcam), JAK2 (1:500; cat. no. ab39636; Abcam) and STAT3 (1:1,000; cat. no. ab68153; Abcam). Following washing, horseradish peroxidase-labeled secondary goat anti-mouse (cat. no. ZB-2305) and anti-rabbit (cat. no. ZB-2301; both ZSGB-bio) were added at a dilution of 1:1x10⁴ and membranes were re-probed at room temperature for 2 h. Following washing, the proteins were visualized using ECL kit (cat. no. 34094; Thermo Fisher Scientific, Inc.). Protein concentration was determined using the BCA protein concentration assay kit (catalog number P0012S; Beyotime). The expression of the target proteins was calculated relative to GAPDH (1:2,000, cat. no. TA-08; Zsbio). Image J 180 (National Institutes of Health) was used for band density analysis.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, Inc.; Dotmatics). Data are presented as mean \pm SD or median and interquartile range (IQR) and samples were compared using paired t test or Wilcoxon paired test based on normality. One-way ANOVA analysis of variance was used to analyze multiple groups. χ^2 test was used for analysis of categorical variables. Tukey's post hoc test or Dunn's post hoc test was used for multiple comparisons. Spearman correlation test was performed to evaluate the correlation between NONHSAT227927.1 and clinical data. Logistic regression analysis was used to analyze potential risk factors associated with NONHSAT227927.1. P<0.05 was considered to indicate a statistically significant difference.

Results

No difference in demographic characteristics between AS patients and healthy controls. A total of 30 healthy controls [median age, 34 years (IQR, 24-46)] and 50 patients with AS [median age, 35 years (IQR, 30-45)] were included. There was no significant difference in basic information (age, sex and BMI) between the two groups (Table I). ESR, CRP, IGA, IgG, IgM and SDS of the AS group were significantly higher than those of healthy controls, suggesting that patients with AS exhibited a stronger inflammatory response and higher risk of depression.

Correlation of NONHSAT227927.1 with demographic characteristics and clinical indicators in patients with AS. Our previous study demonstrated that NONHSAT227927.1 had significant diagnostic value in AS (21). To determine whether NONHSAT227927.1 serves as a biomarker in the process of AS, Spearman correlation analysis was performed to evaluate the correlation of NONHSAT227927.1 with clinical indicators and basic conditions of patients with AS. NONHSAT227927.1 was positively correlated with disease

Table I. Clinical immune-inflammatory markers and perception score of patients with AS and HCs.

Parameter	AS (n=50)	HC (n=30)	t/F/ χ^2 -value	P-value
Median age (IQR), years	35 (30-45)	34 (24-46)	0.84	0.40
Sex (%)				1.00
Male	32 (64)	19 (63.33)		
Female	18 (36)	11 (36.67)		
Median BMI (IQR)	21.19 (18.39-23.80)	20.34 (18.29-22.25)	0.58	0.36
Median disease duration (IQR), years	12.00 (9.75-15.25)	NA		NA
Median ESR (IQR), mm/h	39.5 (21.50-58.00)	5.00 (1.00-7.00)	0.03	<0.01 ^b
CRP, mg/l	34.20±28.30	6.35±4.57	51.50	<0.01 ^b
IgA, mmol/l	4.58±5.67	1.01±0.81	17.88	<0.01 ^b
IgG, mmol/l	11.06±2.98	8.41±1.07	12.08	<0.01 ^b
IgM, mmol/l	1.49±0.81	1.00±0.45	6.27	0.01
C3, g/l	1.27 (1.13,1.40)	1.21 (0.98,1.42)	1.90	0.25
C4, g/l	0.35±0.17	0.38±0.20	0.64	0.43
Median BASDAI score (IQR)	4.4 (4.00-5.00)	NA		NA
BASFI score	4.70±0.53	NA		NA
BASMI score	6.82±1.37	NA		NA
SAS score	49.85±11.24	5.19±1.57	0.32	0.57
Median SDS score (IQR)	51.19 (41.71-73.32)	7 (17.19-12.11)	0.09	<0.01 ^a
Median VAS score (IQR)	6.80 (5.82-7.28)	NA		

^aP<0.05, ^bP<0.001. Data are presented as the mean ± standard deviation. BMI, body mass index; SDS, Self-rating Depression Scale; SAS, Self-rating Anxiety Scale; VAS, Visual Analog Scale; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; BASMI, Bath Ankylosing Spondylitis Metrology Index; HC, Healthy control; NA, not applicable; ESR, Erythrocyte sedimentation rate; CRP, C-reactive protein.

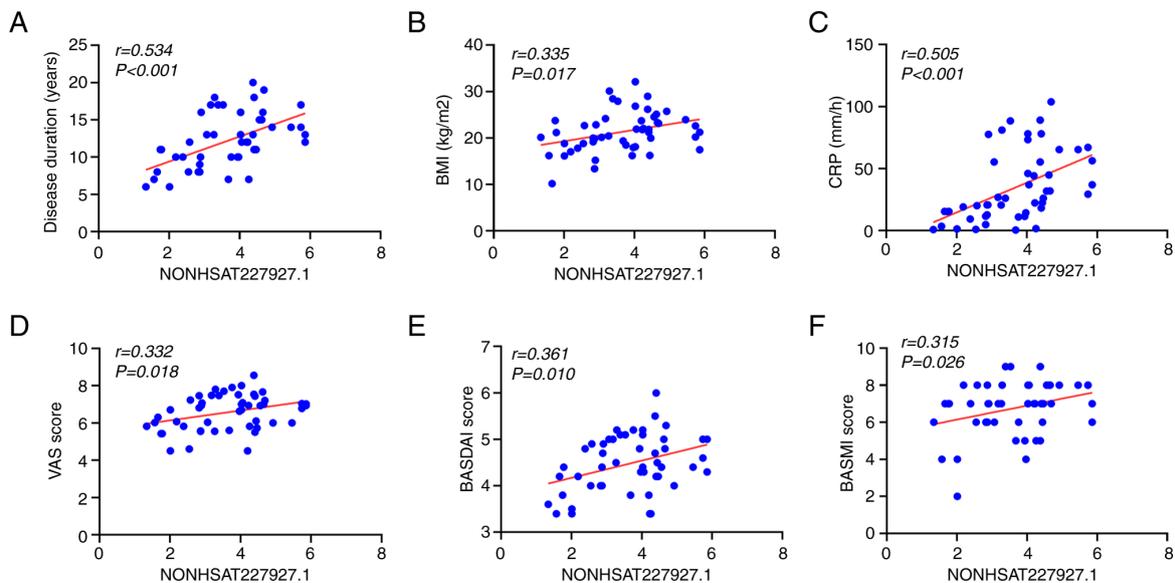


Figure 1. Correlation of NONHSAT227927.1 with clinical indicators in patients with AS patients. Correlation of NONHSAT227927.1 expression in peripheral blood mononuclear cell of patients with AS with (A) disease duration, (B) BMI, (C) CRP and (D) VAS, (E) BASDAI and (F) BASMI score. AS, ankylosing spondylitis; CRP, C-reactive protein; VAS, Visual analog scale; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASMI, Bath Ankylosing Spondylitis Metrology Index.

duration (Fig. 1A), BMI (Fig. 1B), CRP (Fig. 1C), VAS (Fig. 1D), BASDAI (Fig. 1D) and BASMI (Fig. 1E), which suggested that NONHSAT227927.1 was associated with the progression of AS.

Logistic-regression analysis of NONHSAT227927.1 with clinical characteristics of patients with AS. To identify risk factors associated with NONHSAT227927.1 in patients with AS, logistic regression analysis was performed. Significant

differences in NONHSAT227927.1 expression were associated with ESR (P=0.020), VAS (P=0.046) and BASDAI (P=0.009; Fig. 2). These findings indicated that ESR, VAS, and BASDAI were risk factors associated with NONHSAT227927.1.

TPL inhibits viability of AS-PBMC-stimulated AS-FLSs. The chemical structure of TPL is shown in Fig. 3A. To determine the optimal treatment concentration and duration, AS-FLSs were stimulated with AS-PBMCs to induce inflammatory response before measuring cell viability following TPL intervention (0, 25, 50 and 100 µg/ml). The inhibitory ability of AS-FLSs was strongest when TPL concentration was 100 µg/ml and the intervention time was 48 h (Fig. 3B). Therefore, this TPL concentration and treatment duration were used for subsequent experiments.

Effect of TPL on inflammation in AS-PBMC-stimulated AS-FLSs. To verify that TPL exerted therapeutic effects on AS-FLSs via NONHSAT227927.1, NONHSAT227927.1 was knocked down (Fig. 4A) and anti-inflammatory effect of TPL was assessed. RT-qPCR results revealed that TPL intervention led to a significant decrease in NONHSAT227927.1 expression in FLSs. Compared with the si-NONHSAT227927.1 group, TPL decreased levels of NONHSAT227927.1 (Fig. 4B). ELISA results indicated that the addition of TPL notably increased the contents of IL-4 but decreased the content of TNF-α compared with the PF +si-NC group. In response to si-NONHSAT227927.1-mediated knockdown of NONHSAT227927.1, the levels of IL-4 and IL-10 were significantly increased and levels of TNF-α were decreased; these changes were significantly greater following addition of TPL (Fig. 4C and D). Taken together, these data indicated that TPL may play an anti-inflammatory role in AS by regulating NONHSAT227927.1.

Molecular docking of TPL and JAK2/STAT3. Our previous study confirmed that activating the JAK2/STAT3 pathway promotes inflammation in AS (21). Therefore, molecular docking of TPL with JAK2 and STAT3 protein was performed to investigate the potential mechanism by which TPL may inhibit the inflammatory response in AS via the JAK2/STAT3 pathway. The molecular docking (Fig. 5) showed the binding energy of JAK2 was -6.5 kcal/mol and the binding energy of STAT3 was -7.9 kcal/mol. The binding effect of the compound and target JAK2 and STAT3 protein was stronger, indicated by lower binding energy. PyMOL 2.1 software was used to visualize the compound formed following docking with the protein and the binding mode between the compound and protein was obtained. According to the binding mode, active amino acid residues bound by TPL and JAK2 target proteins were ARG-335 and ASP-334 and the active amino acid residues bound by TPL and STAT3 target proteins were ASP-976 and ARG-938. This compound formed a strong reactive group with the aforementioned amino acid residues. These interactions improved the stability of the compound in JAK2 and STAT3 protein pockets, so the compound was a potentially active small molecule.

TPL regulates the JAK2/STAT3 pathway via NONHSAT227927.1. It was determined whether TPL participated in NONHSAT227927.1-regulated cellular inflammation via

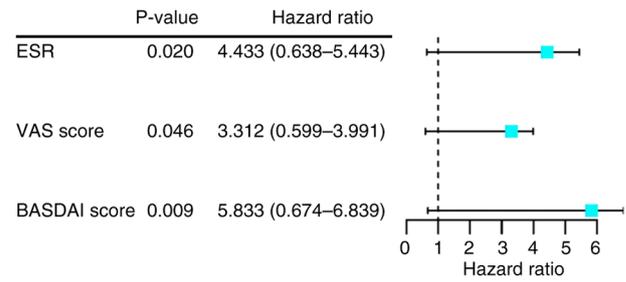


Figure 2. Logistic regression analysis of NONHSAT227927.1 with clinical characteristics of patients with Ankylosing spondylitis. ESR, Erythrocyte sedimentation rate; VAS, Visual analog scale; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index.

JAK2/STAT3 signaling in AS-PBMC-stimulated FLSs. Levels of p-JAK and p-STAT3 protein were significantly increased in FLSs, while these increased levels were inhibited by TPL treatment, implying that TPL inhibited pro-inflammatory pathways (Fig. 6). Moreover, knockdown of NONHSAT227927.1 reduced the phosphorylation levels of JAK2 and STAT protein in FLSs. These results suggested that TPL regulated the JAK2/STAT3 pathway by regulating NONHSAT227927.1.

Discussion

Considering the inflammatory nature of AS, most available treatments for AS focus on decreasing the inflammatory burden (28). Due to the impact of disease activity on structural damage and function, controlling inflammation is key in the treatment of AS (29,30). Despite the improvements in AS treatment, the pathogenesis of AS has not yet been elucidated. In addition, the treatment effect is often poor due to the lack of effective therapeutic targets, the diagnosis and treatment of AS remain a challenge. lncRNAs serve key roles in various types of autoimmune disease, including AS (31). lncRNAs can predict AS recurrence and poor outcomes, representing potential predictive biomarkers for AS (14,32,33). Currently, the therapeutic options for AS are limited compared with those for other rheumatoid diseases (such as rheumatoid or psoriatic arthritis) and traditional synthetic disease-modifying antirheumatic drugs or long-term corticosteroids are considered ineffective in treatment of axial spondyloarthritis (33,34). Evidence has indicated TPL as an effective oral agent for the treatment of active AS (35,36).

Our preliminary study demonstrated that lncRNA NONHSAT227927.1 is highly expressed in patients with AS and might be a potential AS-specific diagnostic marker (AUC was 0.8463) (21). The laboratory indicators (ESR, CRP, IgA, IgG, and IgM) and SDS score of the AS group were significantly higher than those of healthy controls. Pain is the main characteristic of inflammation. When there is an inflammatory reaction, physical pain can lead to anxiety and depression (37). In addition, NONHSAT227927.1 was positively correlated with disease duration, BMI, CRP, VAS, BASDAI and BASMI; these indicated that NONHSAT227927.1 expression was associated with the disease severity of AS. Logistic regression found that ESR, VAS and BASDAI were risk factors for high NONHSAT227927.1 expression.

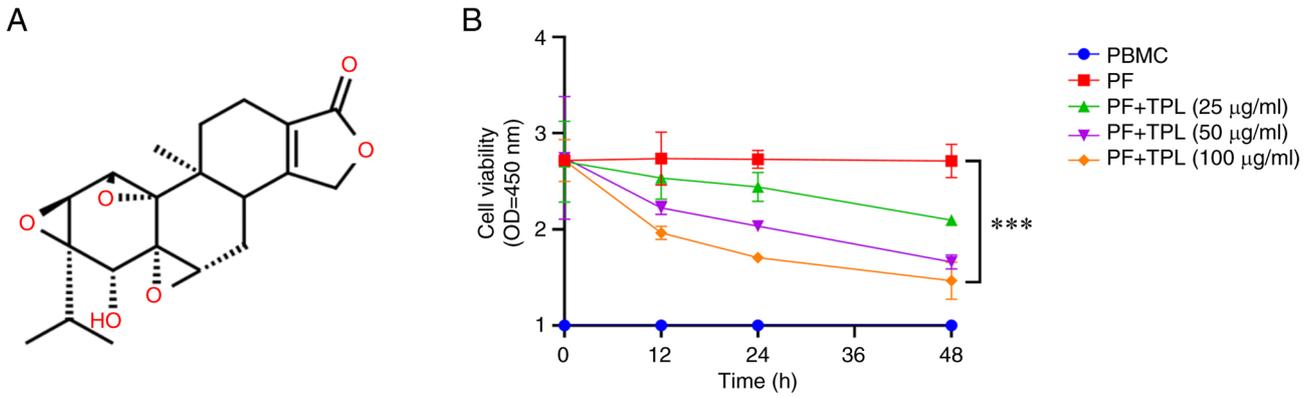


Figure 3. Effect of TPL on viability of AS-PBMC-treated AS-FLSs. (A) Chemical structure of TPL. (B) Inhibitory effect of TPL on the viability of AS-FLSs. Data are presented as the mean \pm SD of 3 independent repeats. *** P <0.001. TPL, Triptolide; AS-FLS, AS-Fibroblast-like synoviocyte; PBMC, Peripheral blood mononuclear cell; PF, AS-PBMC +AS-FLS.

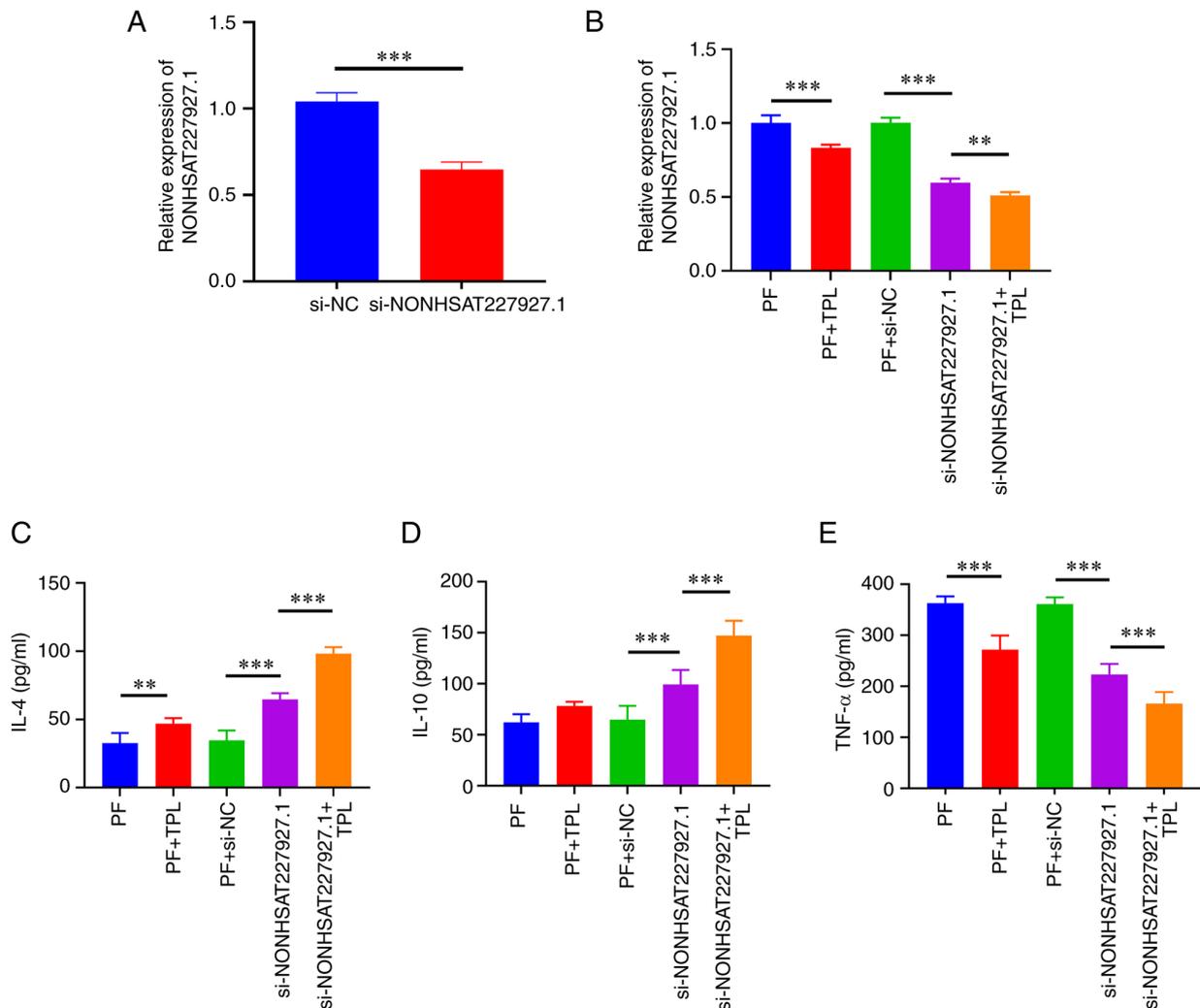


Figure 4. Effect of aberrant NONHSAT227927.1 expression on inflammatory factors in AS-FLSs. Expression of NONHSAT227927.1 in AS-FLSs after (A) siRNA transfection and (B) TPL treatment. Effect of NONHSAT227927.1 knockdown on inflammatory factors (C) IL-4, (D) IL-10 and (E) TNF- α in AS-FLSs. ** P <0.01, *** P <0.001. AS-FLS, Ankylosing spondylitis-Fibroblast-like synoviocyte; si-NC, small interfering negative control; TPL, Triptolide; PF, AS-PBMC+ AS-FLS

Studies have shown the key roles of lncRNAs in the differentiation and function of immune cells (38-40). When the body is subjected to internal or external stress responses,

non-specific immune cells specifically express hexamethylene bis-acetamide-inducible protein (HEXIM1) and Nuclear Enriched Abundant Transcript1 (NEAT1) to regulate immune

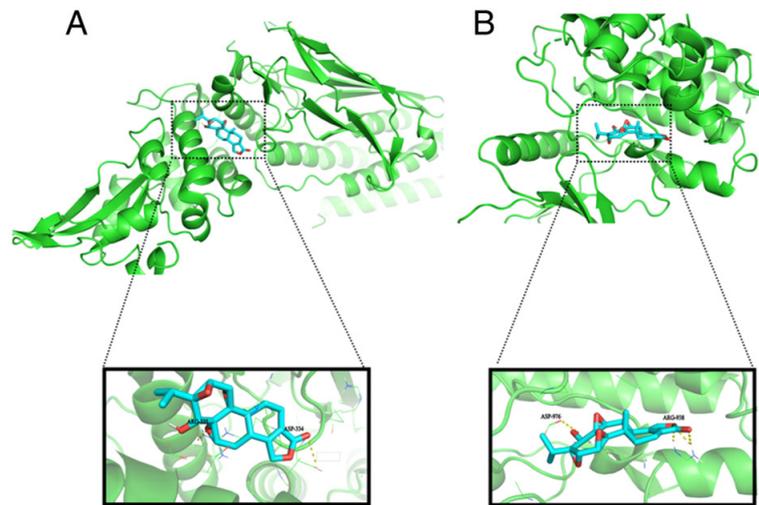


Figure 5. Molecular docking of TPL with JAK2 and STAT3 protein. TPL and (A) JAK2 (binding energy, -6.5 kcal/mol) and (B) STAT3 docking model (binding energy, -7.9 kcal/mol). TPL, Triptolide.

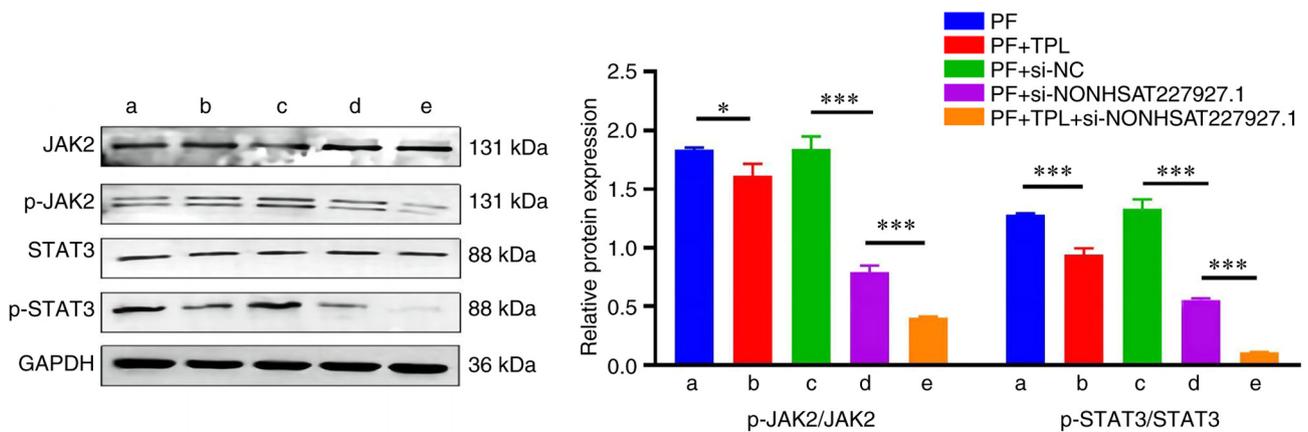


Figure 6. TPL regulates the JAK2/STAT3 pathway via NONHSAT227927.1. Effects of NONHSAT227927 on the expression of JAK2, STAT3, p-JAK2 and p-STAT3 were evaluated by western blot analysis. Data are expressed as mean \pm standard deviation of 3 independent repeats. * $P < 0.05$, *** $P < 0.001$. TPL, Triptolide; p-, Phosphorylation-; AS-PBMC, Ankylosing spondylitis-Peripheral blood mononuclear cell; FLS, fibroblast-like synoviocyte; PF, AS-PBMC+AS-FLS; si-NC, small interfering negative control.

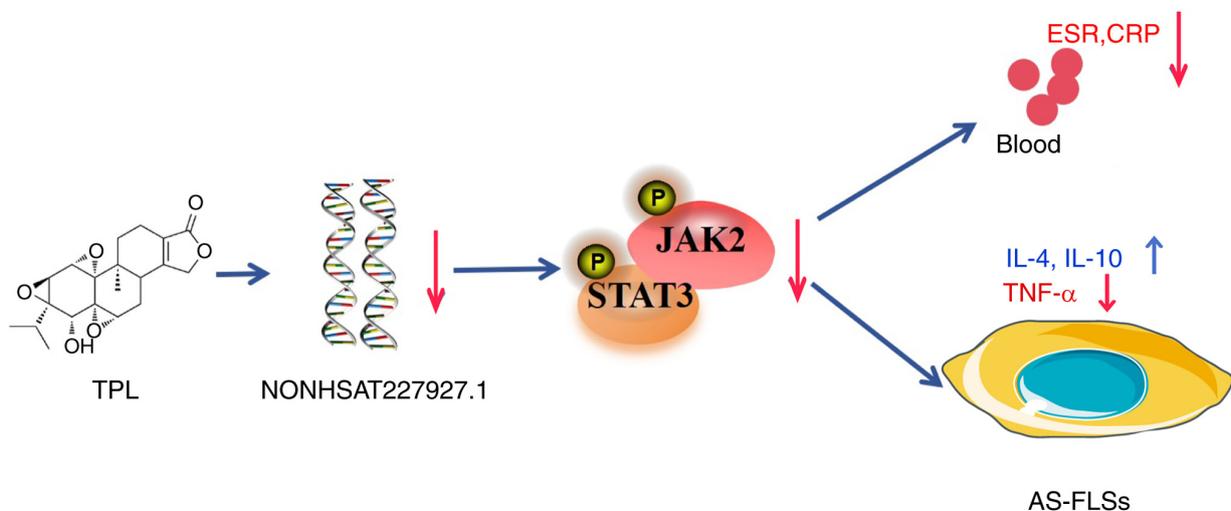


Figure 7. Schematic of the protective effect of TPL on AS-FLSs. TPL prevent the progression of AS by downregulating the expression of NONHSAT227927.1 and inhibiting the activation of the JAK3/STAT3 pathway, thereby improving the inflammatory response of AS-FLSs. TPL, triptolide; AS-FLS, Ankylosing spondylitis-Fibroblast-like synoviocyte; ESR, Erythrocyte sedimentation rate; CRP, C-reactive protein.

cell differentiation and function (41). Li *et al* (42) found that lncRNA AK001085 is poorly expressed in the serum of patients with AS and is negatively correlated with immune-inflammatory markers CRP and ESR. Ding *et al* (43) found that 36 lncRNAs are involved in the Spondyloarthritis/AS competitive regulation of the immune-validation reactor pathway. Our preliminary high-throughput sequencing of AS-PBMCs and lncRNAs enriched in immune-inflammatory responses (fold-change ≥ 2 ; $P \leq 0.05$) identified NONHSAT227927.1 for RT-qPCR verification.

T. wilfordii Hook F (TwHF), a traditional Chinese herb, has been used to treat rheumatoid arthritis and other autoimmune and inflammatory diseases for a long time (44,45). TPL ($C_{20}H_{24}O_6$) is a diterpenoid triepoxide purified from TwHF that possesses potent immunosuppressive and anti-inflammatory properties (46). According to pharmacological studies, TPL exerts anti-inflammatory, detoxification, heat-clearing and dampness-dispelling effects, as well as inhibitory effects on humoral and cellular immunity (47,48). A number of randomized controlled trials have shown that TPL is beneficial in treatment of AS (44,49). However, the therapeutic potential of TPL is limited due to its strong toxicity (50,51). Here, TPL at a concentration of 100 $\mu\text{g/ml}$ significantly inhibited cell viability at 48 h. The isolated AS-FLSs were exposed to TPL (100 $\mu\text{g/ml}$) to examine the inhibitory effect of TPL. The present results showed that TPL suppressed the cytokine metabolism disorder in AS-PBMC-stimulated AS-FLSs. In addition, the JAK2/STAT3 pathway was significantly inhibited after NONHSAT227927.1 knockdown.

The JAK2/STAT3 signaling pathway is primarily responsible for regulating inflammatory responses in AS (52). Together with several STAT proteins, JAK mediates signaling of extracellular cytokines and affects various cellular functions. STAT3 is a component of the acute phase response factor complex activated by IL-6 (53). The present study suggested that TPL inhibited AS progression by mediating JAK2/STAT3 pathway inactivation and TPL interacted with residues in the JAK2/STAT3 inhibitory interaction pocket.

The efficacy of TPL in treating AS has been confirmed by previous clinical studies (25,26,36). To the best of our knowledge, however, research on the specific mechanism of TPL in treating AS is relatively limited (54). The present study investigated inflammatory pathway to explore the potential of TPL in treatment of AS-FLSs, whereas previous studies have used chondrocytes (25,26). The present study used AS-PBMC and AS-FLS from patients to co-culture a cell model to verify the therapeutic effect of TPL. PBMC were taken from the patient's whole blood, and FLS was taken from the patient's joint. This cell model has a stronger inflammatory response and can better simulate the human body environment. The *in vitro* co-culture cell model can also provide a usable cell model for subsequent experiments. TPL prevent the progression of AS by downregulating the expression of NONHSAT227927.1 and inhibiting the activation of the JAK3/STAT3 pathway, thereby improving the inflammatory response of AS-FLSs (Fig. 7).

Finally, the present study revealed the binding modes and sites of TPL and pathway proteins based on molecular docking. These results provide a scientific basis for *T. wilfordii* as a potential therapeutic drug for AS.

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Availability of data and materials

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

Authors' contributions

XD and JL designed the study. YS and XC analyzed data. XD wrote the manuscript. JL and YS confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Anhui University of Traditional Chinese Medicine (approval no. 2015-AH20). Written informed consent to participate was obtained from all patients. All procedures were conducted in accordance with the Medical Ethics Committee of the First Affiliated Hospital of Anhui University of Traditional Chinese Medicine protocols.

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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