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ORIGINAL RESEARCH

# TNF/NF-κB Signaling Pathway is Involved in Mechanisms of Tongluo Decoction on Rat Models with Sequelae of Pelvic Inflammatory Disease

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**Purpose:** Traditional Chinese medicine can produce strong therapeutic activities for sequelae of pelvic inflammatory disease (SPID). This study aimed to investigate the efficacy and protective mechanisms of Tongluo Decoction (TLD) in the treatment of SPID.

**Patients and Methods:** The damage-protective and inflammation-inhibitory effects of TLD on SPID were investigated in rat models. The possible targets of TLD against SPID were predicted using network pharmacology. The hub targets were tested in SPID rat models. Rescue experiments were conducted in human endometrial epithelial cells (HEECs). Detection of necroptosis was used the flow cytometry. ELISA was used for the quantification of inflammatory cytokines. Protein levels were detected using a Western blot assay.

**Results:** TLD had a protective effect against uterus tissue damage caused during SPID. TLD inhibited the levels of adhesion cytokines (VEGF and TGF-β1), the tight junction proteins (ZO-1 and occludin), and inflammatory cytokines (TNF-α and IL-6). TNF, IKBKB, and NFKB1 were predicted as hub targets for TLD against SPID. TLD inhibited necroptosis of HEECs via TNF, IKBKB, and NFKB1. TLD inhibited the ratio of CD86+ M1 macrophages after differentiation induction of THP-1.

**Conclusion:** The therapeutic effects of TLD for SPID may be the result of a dual action on inflammation and necroptosis, via TNF, IKBKB, and NFKB1.

**Keywords:** herb, formula, uterus, inflammation, necroptosis

## Introduction

Pelvic Inflammatory Disease (PID) is an inflammation of the upper reproductive tract caused by infection in the female population.[1] PID is usually associated with sexually transmitted infections and travels upward from the lower genital tract to affect the uterus, fallopian tubes, and/or ovaries.[2] Infections of the female reproductive system can lead to long-term poor consequences such as chronic pelvic pain, infertility, or ectopic pregnancy.[3] These poor consequences were defined as sequelae of pelvic inflammatory disease (SPID).[4] In 2019, the age-standardized rate of PID prevalence was 53.19 per 100,000 population, with a decreased estimated annual percentage changes trend (- 0.50) from 1990.[5] However, the associated SPID continues to be a public health burden with a strong correlation with ectopic pregnancy.[5] In some women, SPID may initially be asymptomatic and untreated; the onset of SPID is only recognized through testing after infertility.[2] Western medical treatment for SPID is based on the different clinical manifestations. For example, infertility is treated with assisted reproductive technology;[6] tubal hydrosalpinx is treated surgically;[7] recurrent pelvic inflammatory disease is treated with antibiotics and, if necessary, surgery.[8] However, antibiotics have limited efficacy in the management of poorly circulating pelvic connective tissue, and long-term use often leads to drug resistance and dysbiosis. When the effectiveness of western medical treatment is limited, other effective interventions and strategies need to be established.

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Traditional Chinese medicine (TCM) has a long history of treating PID, with many ancient books describing the symptoms and treatment strategies.[9] Previous studies have found that TCM can shorten the course of PID and reduce its sequelae.[10,11] Based on a large number of clinical and experimental studies, TCM evidence-based and herbal treatments for SPID are being expanded.[12–14] For example, a Phase 2 randomized clinical trial shows that ZY5301 tablet (a preparation extracted from Ajuga decumbens Thunb.) is efficacious for the relief of one of the SPID known as chronic pelvic pain.[15] Based on the rat SPID model, a study reveals that Fuke Qianjin capsule can achieve a quadruple effect of antibacterial, anti-inflammatory, repair, and immune enhancement, providing early anti-inflammatory and late repair for SPID.[14] Tongluo Decoction (TLD) is a primary traditional Chinese medicine formula. Depending on the disease, additions and subtractions can be made according to the formula and transformed into different formulas. TLD contains Bupleuri Radix (*Bupleurum chinense* DC.), Aurantii Fructus Immaturus (*Citrus aurantium* L.), Paeoniae Radix Rubra (*Paeonia ladiflora* Pall.), Liquidambaris Fructus (*Liquidambar formosana* Hance), Salviae Miltiorrhizae (*Salvia miltiorrhiza* Bge.), Notoginseng Radix et Rhizoma (*Panax noto ginseng* (*Burk)*. F. H. Chen), Herba Salviae Chinensis (*Salvia chinensis* Benth.), Astragali Radix (*Astragalus membranaceus* (Fisch.) Bge), Eupolyphaga Steleophaga (*Eupolyphaga sinensis* Walker), Scolopendra (*Scolopendra subspinipes mutilans* L. Koch), and Gleditsiae Spina (*Gleditsia sinensis* Lam). Among these medicines, Bupleuri Radix, Aurantii Fructus Immaturus, Paeoniae Radix Rubra, Salviae Miltiorrhizae, Notoginseng Radix et Rhizoma, and Astragali Radix have been identified to be key medicines used in the TCM practice of Professor Xu Runsan for treatment of chronic pelvic pain caused by SPID.[9] However, the underlying mechanisms by which TLD counteracts SPID are unknown.

Therefore, first, we assessed the inhibitory effect of TLD on SPID rat models. Second, given the complex ingredients in TLD, we performed a network pharmacology analysis for TLD, to predict its potential targets against SPID. Then, using endometrial epithelial and THP-1 cells, we validated the potential targets for TLD.

# Materials and Methods

## Preparation of TLD

The liquid preparation of TLD was entrusted the company to China National Traditional Chinese Medicine Corporation (Beijing, China), which is an approved company that provides TCM decoction services for our hospital (China–Japan Friendship Hospital, Beijing, China). The liquid of TLD was then centrifugated. The supernatant was collected, concentrated to a paste, and then lyophilized to a powder.

## Animals

All animal care and procedures were approved by the Experimental Animal Ethics Committee of China–Japan Friendship Hospital. All animals received care following the guidelines outlined in the "Laboratory Animal-Guideline for ethical review for animal welfare" (GB/T 35892–2018). Specific pathogen-free-grade SD female rats, with 200 ± 10 g of body weight (aged 5 weeks), were purchased from the Beijing Laboratory Animal Research Center [SYXK(Jing)2022–0006]. The rats were reared in a separate cage, with free access to water and food. The housing condition was stable at 55–60% humidity and a 22–26°Ctemperature with a 12 h light/12 h dark cycle.

## Animal Grouping and Treatment

In accordance with the random digital table, thirty-six female rats were randomly divided into four groups, with six rats in each group. The dosage for rats is calculated based on the body surface area conversion algorithm for the dosage given to humans. The four groups are sham-operated group, SPID model group, SPID+L-dose TLD group (1.0 g TLD lyophilized powder), SPID+M-dose TLD group (1.5 g TLD lyophilized powder), SPID+H-dose TLD group (2.0 g TLD lyophilized powder) and SPID+Positive group (50 mg/kg levofloxacin). The sham operation group had not performed any surgical operation on the uterus. SPID modeling method was performed as described previously.[4] Before modeling, rats were fasted overnight and then anesthetized. After reaching the surgical anesthetic plane, the rat uterus was exposed after a laparotomy. A suspension containing 0.2 mL of bacterial suspension (counted by Macrobiotic method: $6 \times 10^7$/mL, E. coli mixed with Staphylococcus aureus at a ratio of 2:1) was gently injected into each uterine

horn. The needle was moved back and forth within the uterine cavity three times to cause mechanical damage to the endometrial tissue. After the operation, the abdominal cavity of the rats was closed, with skin and connective tissues sutured layer by layer. After awakening, the rats were gently transferred back to their cages and had free access to food and water, for 14 days. Fourteen days post-surgery, the TLD was dissolved in double-distilled water and administered at the indicated doses, for a 14-day treatment. The rats in the model group and the sham group were intragastrically administered equivalent double-distilled water.

## The Collagen Volume Fraction and Histopathological Scores

Paraffin blocks were made from rat uterine tissue. After dewaxing and rehydration, 5-μm sections were made. Sections were stained with Masson stain, and total collagen deposition was quantified and collagen volume fraction was calculated using ImageJ system software. Endometrial tissue histopathological scores were recorded based on previously reported criteria.[14] Scores were set on a scale of 0–3 depending on the extent of the lesion, including the number of glands, epithelial integrity, cellular degeneration and necrosis, and inflammatory cell infiltration.

## Enzyme-Linked Immunosorbent Assay (ELISA)

Serum was collected from three milliliters of blood of each rat using centrifugation at 4°C and 3000 r/min for 5 min. Tumor Necrosis Factor-α Assay Kit (for TNF-α), Transforming growth factor-β Assay Kit (TGF-β1), Vascular endothelial growth factor Assay Kit (for VEGF), and Interleukin-6 Assay Kit (for IL-6) were all purchased from Nanjing Jiancheng Bioengineering Institute (China). Determination of the content of each factor was carried out according to the respective kit instructions.

## Network Pharmacology Analysis for Potential Targets of TLD in SPID

The ingredients and target genes for herbs in TLD were collected from TCMSP (https://tcmsp-e.com/). The ingredients and target genes for Eupolyphaga Steleophaga and Scolopendra were collected from HERB (http://herb.ac.cn/). The disease-related genes for SPID were retrieved and downloaded from Genecards (http://www.genecards.org). Intersection of these two sets of targets were obtained using the Venn 2.1 to predict potential targets of TLD therapy for SPID. A TLD-medicine-ingredient-target network was constructed. The protein–protein interaction analysis of these therapeutic targets from STRING 11.0 database (https://string-db.org/) with "Homo sapiens" was re-analyzed by the "Network Analyzer" and "MCODE" plug-ins in Cytoscape, to display the nodes based on the degrees.

## Preparation of TLD-Mediated Serum

Rats after environmentally acclimatized rearing were taken and administered by gavage at high doses for 14 consecutive days using TLD. An additional group of Sham was set up using an equal dose of saline by gavage. Three hours after the last administration, the rats were anesthetized and blood was collected from the abdominal aorta. After the blood was stratified at room temperature, it was centrifuged (2000 r/min, 10 min) and serum was extracted. The serum was inactivated in a water bath at 56°C for 20 min and filtered through a 0.22 μm microporous membrane (Nest, China) for inactivation. The processed Sham serum and H-dose TLD serum were split and stored at −20°C.

## Cell Lines, Cell Culture, and Transfection

Human endometrial epithelial cells (HEECs) were obtained from Procell (Wuhan, China), and THP-1 cells were obtained from the American Type Culture Collection (Manassas, USA). These two cell lines were cultured in DMEM from Thermo Fisher, supplemented with 10% FBS and 1% penicillin and streptomycin. THP-1 cells were induced to M1 macrophages using 20 ng/mL LPS, 20 ng/mL IFN-γ, and 25 nM PMA. A triad overexpression vector of TNF, IKBKB, and NFKB1 was generated with pcDNA3.1 vector (YouBio, Changsha, China). The pcDNA vector was used as a negative control. Cells were transfected with the triad oligonucleotide or vector using Lipofectamine 3000 (Thermo Fisher, Wilmington, USA) for 24 hours.

## Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis

RNA-easy Isolation Reagent was used to extract total RNA (Vazyme, China). RT Master Mix for qPCR III (Medchemexpress, USA) was used for cDNA synthesis. Using FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics GmbH), PCR was performed on an Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher, USA). The relative quantification was analyzed based on the equation $2^{-\Delta\Delta Ct}$ method. The internal control GAPDH mRNA was used to normalize the Ct data.

## Flow Cytometry Detection of CD86 M1 Macrophages

Rat uterine tissue was ground to homogenate and prepared as a single cell suspension. THP-1 cells were differentiated. After incubation with or without TLD-containing serum, THP-1 cells were trypsinized. Then, THP-1 cells were resuspended in PBS with 20 μL of primary PE-anti-CD86 (BD Biosciences, USA) antibody, followed by an incubation at 37°C for half an hour. After incubation, cells were washed using PBS and recovered by centrifugation at 200 × g, 4°C for 5 min. CD86 M1 Macrophages were analyzed by using a CytoFLEX flow cytometer (Beckman Coulter, USA).

## Western Blot Analysis

For the detection of proteins by western analysis, rat uterine tissues were ground. Cells were lysed using RiPA buffer (Fisher Scientific, The Netherlands), supplemented with protease inhibitors (Roche, The Netherlands), on ice. After a determination by the Bradford method (Bio-Rad), aliquots of protein (20 μL) were loaded on a 10% polyacrylamide-SDS gel. Proteins were transferred from gels to nitrocellulose membranes using the trans-blot turbo system. Membranes were blocked, followed by an incubation with primary antibody at 4°C overnight. For detection, a horseradish peroxidase-conjugated anti-mouse IgG antibody (1:2000) was used to incubate with membranes for one hour at room temperature. The grayscale values of the target protein bands on membranes were detected and normalized to the results of anti-β-actin antibody (1:5000, Sigma-Aldrich, Germany).

## Cell Viability and Necroptosis Detection

Cell viability was assessed using Cell Counting Kit-8 (Dojindo, Japan). Cells pre-cultured for 24h were treated for 24 h by adding different concentrations of drugs. Then, 10 μL of CCK-8 solution was added to each of the cell culture wells, and the culture was continued for 2 hours. Absorbance at 450 nm was measured using an enzyme marker and normalized to the control.

Cell necroptosis was detected using Apoptosis and Necrosis Detection Kit with YO-PRO-1 and PI (Beyotime, China). Treated cells were assayed using the appropriate volume of YP1/PI assay work solution according to the instructions at Ex/Em = 535/617 nm, to characterize the number of $PI^+$ cells.

## Statistics

GraphPad Prism 8 software was used for statistical analysis. Two-way Analysis of Variance, post hoc test via Tukey's tests, were used for difference assessment among the experimental groups. Significance level was set at $P < 0.05$.

# Results

## Protective Effect of TLD on Uterine Damage During SPID

After observing the uterine tissues of the rats in each group after 21 days of TLD administration (with images of uterine histology showed in Supplementary Figure 1), it was found that the histopathological scores of the rats given TLD, both at low and medium-high concentrations, were significantly reduced (Figure 1A). The collagen volume fraction in the uterus of rats treated with TLD was significantly smaller than that of the model group (Figure 1B). Inhibition of VEGF expression can reduce tissue adhesion and attenuate tissue proliferation induced by the inflammatory response.[16] TGF-β1 regulates cell proliferation and matrix repair.[17] Our study found higher serum concentrations of VEGF and TGF-β1 in SPID model rats, which were reduced after TLD administration (Figure 1C and D). Moreover, the tight junction proteins ZO-1 and occludin were significantly reduced in the SPID model group, whereas TLD treatment significantly increased

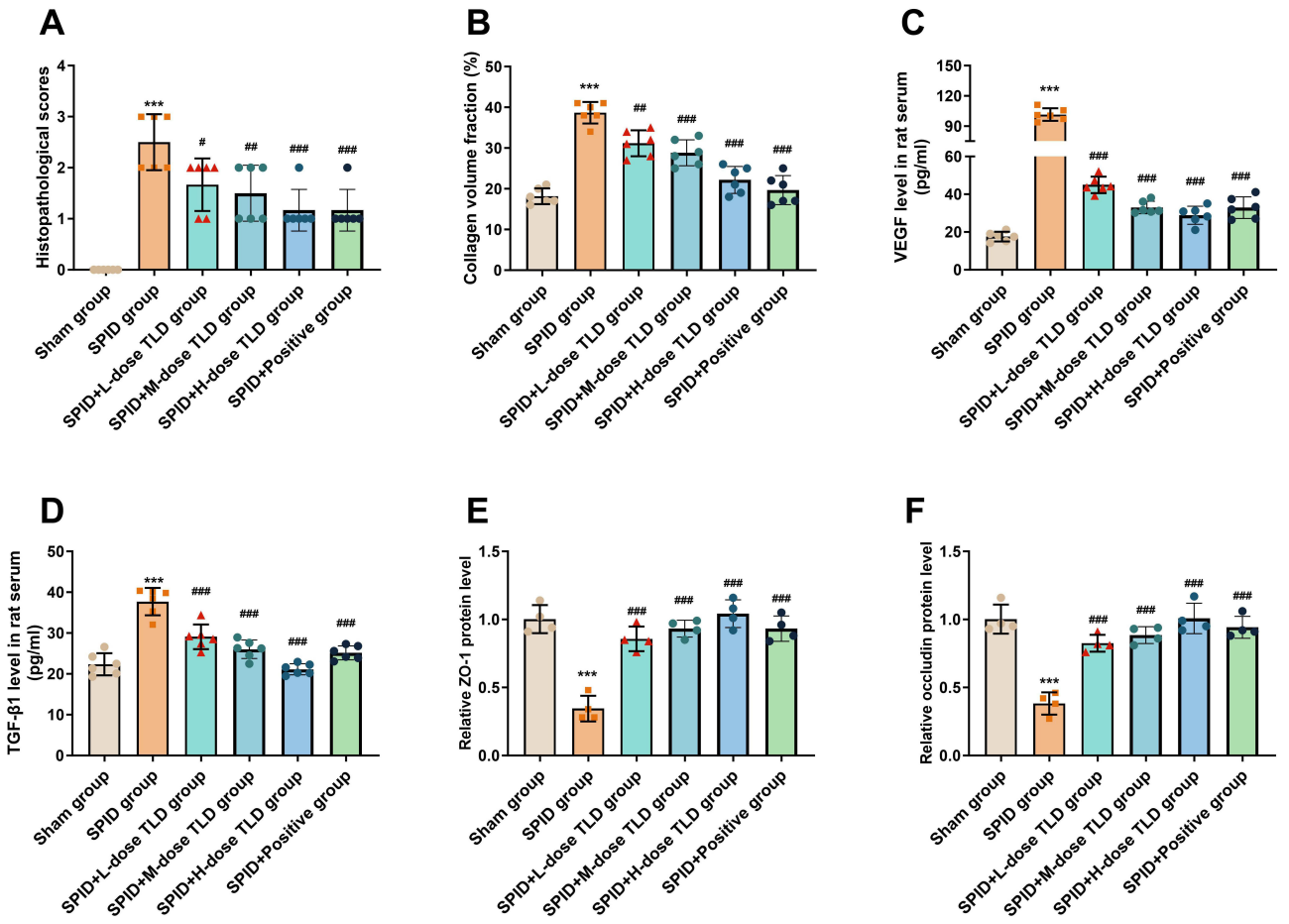


**Figure 1** Protective effect of Tongluo Decoction (TLD) on uterine damage in SPID rats. (**A**) Histopathological scores of rat uterine tissues in each group. (**B**) The quantification of collagen volume fraction after respective treatments. (**C**) VEGF levels in rat serum via ELISA. (**D**) TGF-β1 levels in rat serum via ELISA. (**E**) The levels of tight junction proteins ZO-1 in rat uterine tissues by Western blot assays. (**F**) The levels of tight junction proteins occludin in rat uterine tissues by Western blot assays. ***$P < 0.001$, vs Sham group. #$P < 0.005$, ##$P < 0.01$, ####$P < 0.001$, vs SPID group.

their expression (Figure 1E and F; Supplementary Figure 2). These results suggested an inhibitory effect of TLD on uterine damage during SPID.

## Inhibitory Effect of TLD on Inflammation During SPID

As highly pleiotropic cytokines, TNF-α and IL-6 are involved in a variety of biological functions, such as cell proliferation, inflammatory response, and cell death.[18] In this study, serum levels of TNF-α and IL-6 were substantially elevated in SPID rats, and their levels were reduced after TLD treatment (Figure 2A and B). Macrophages play a unique role in chronic inflammation and adhesion formation [6,8]. Cd86 is a highly glycosylated protein that is widely used as an M1 macrophage marker [8]. In this study, TLD reduced the number of $CD86^+$ macrophages in the uterus of SPID rats (Figure 2C). Moreover, TLD administration significantly reduced the lymphocyte ratios compared to the model group (Figure 2D). These results suggest that TLD has a significant inhibitory effect on the inflammatory response during SPID.

## TNF, IKBKB, and NFKB1 Were Key Targets of TLD Against SPID

Based on the parameter threshold of oral bioavailability ≥30% and drug-likeness ≥ 0.18, the TCMSP database screened a total of 147 active constituents of TLDs, and their targets were screened for 1950 target proteins after UniProt standardized names. There were 101 overlapping genes between TLD targets and SPID-related genes (Figure 3A). The constituent-target network of TLDs targeting SPIDs consisted of 225 nodes and 971 edges (Figure 3B). The STRING database was used for PPI network construction of the 101 overlapping genes. After visualizing the PPI network with Cytoscape software, we analyzed the degree


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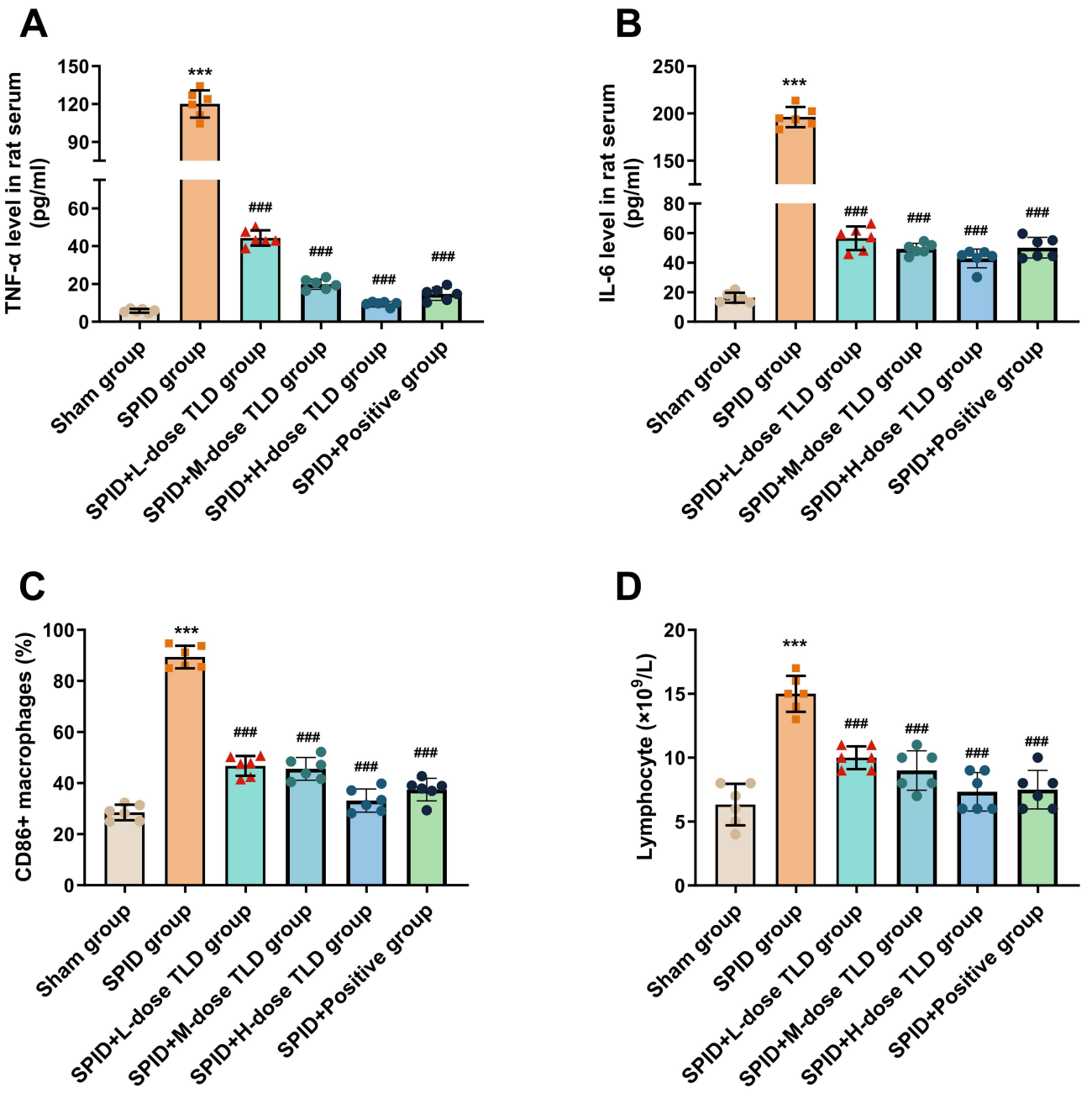


**Figure 2** Effects of Tongluo Decoction (TLD) on the inflammatory response in SPID rats. (**A**) TNF-α in rat serum via ELISA. (**B**) IL-6 in rat serum via ELISA. (**C**) The percentage of $CD86^+$ macrophages in uteri by flow cytometry. (**D**) The lymphocyte ratio in rat blood. ***$P < 0.001$, vs Sham group. ###$P < 0.001$, vs SPID group.

value of each node (Figure 4A). Where the size of the nodes varied from large to small according to the degree value, we found that the TNF degree value was ranked first (Figure 4A). Pathway enrichment analysis of 101 targets according to KEGG mapper showed that TNF was involved in the TNF/NF-κB pathway together with IKBKB and NFKB1 (Supplementary Figure 3). Therefore, we then examined the changes in the expression levels of these three factors in rat uterine tissues. The results showed that the mRNA expression levels of TNF, IKBKB, and NFKB1 were significantly elevated in the model group of rats; however, the intervention of TLD could significantly reduce the expression levels of these factors (Figure 4B–D).

## TLD Inhibited Necroptosis in HEECs via TNF, IKBKB, and NFKB1

Next, we explored and validated the possible mechanism of action of TLD in HEECs. mRNA expression levels of TNF, IKBKB, and NFKB1 induced by LPS were significantly decreased in HEECs after TLD treatment ($P < 0.001$, compared

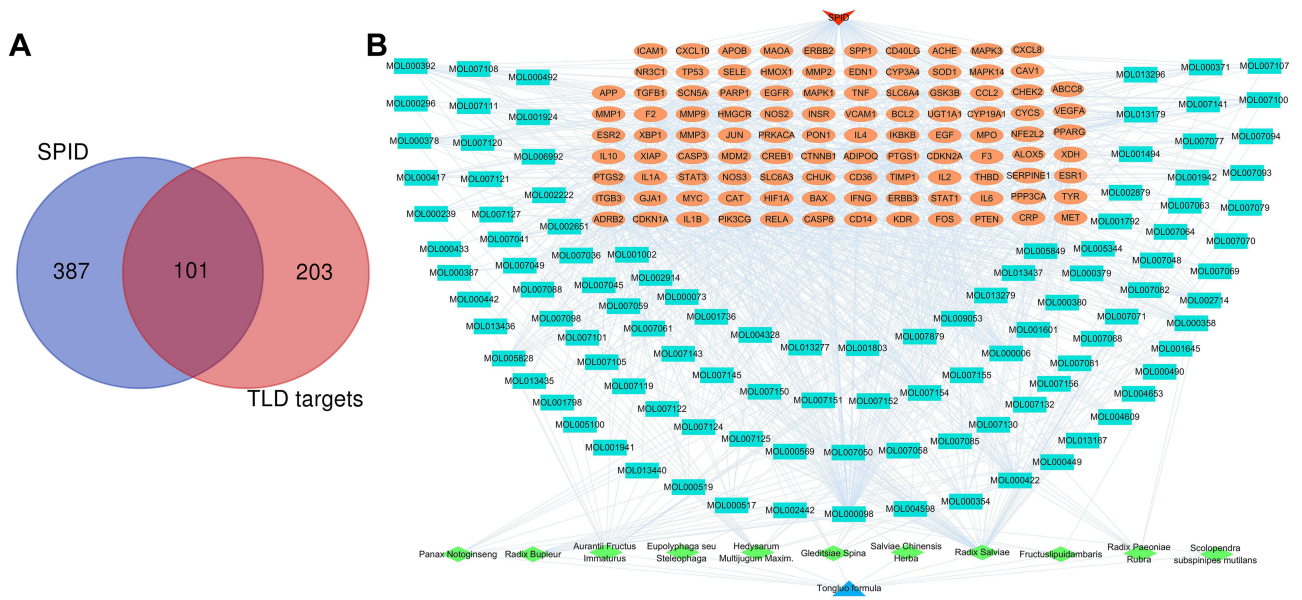


**Figure 3** Network pharmacology analysis for Tongluo Decoction (TLD). (**A**) VENN diagram for common targets between TLD targets and SPID-related genes. (**B**) The TLD-medicine- ingredient-targets-SPID network.

with the LPS+Sham serum group) (Figure 5A–C). However, this effect was offset by co-overexpression of TNF, IKBKB, and NFKB1 ($P < 0.001$, compared with the vector group) (Figure 5A–C). TLD serum reduced the elevated ratio of $PI^+$ HEECs, and this effect was reversed in TNF/IKBKB/NFKB1-overexpressed HEECs (Figure 5D). TNF can lead to apoptosis or necroptosis by activating canonical NF-κB signalling.[19] We then assessed the effect of TLD on LPS-induced necroptosis in HEECs by examining the expression of RIPK1, RIPK3, MLKL, and cystatinase 8 (cas8). The expression of RIPK1, RIPK3, and MLKL was significantly increased and the expression of cas8 was significantly decreased in HEECs in the LPS group ($P < 0.01$, compared with the FBS group); however, TLD significantly decreased the expression of LPS-induced expression of RIPK1, RIPK3 and MLKL and increased cas8 expression ($P < 0.01$, compared with the LPS+Sham serum group) (Figure 5E–H). Notably, the effects of TLD on RIPK1, RIPK3, MLKL, and cas8 expression were prevented by the co-overexpression of TNF, IKBKB, and NFKB1 ($P < 0.001$, compared with the vector group) (Figure 5E–5H; Supplementary Figure 4A). These findings indicate that SPID may inhibit necroptosis in HEECs via TNF, IKBKB, and NFKB1.

## TLD Inhibited Inflammation in Macrophages

The effect of TLD on macrophages was performed using the M1 polarization-induced THP-1 cell model. THP-1 cells without induced differentiation showed no statistically significant difference in cell survival after different treatments (Figure 6A), suggesting that a high concentration of TLD drug serum was not cytotoxic to THP-1. When THP-1 was induced to differentiate, a significant increase in cell survival was observed compared to the normal group ($P < 0.001$); however, treatment with TLD serum inhibited macrophage survival ($P < 0.001$, compared with the model + Sham serum group) (Figure 6B). Furthermore, after THP-1 was induced to differentiate, NFKB1 protein levels in cells were significantly elevated ($P < 0.001$, compared with the normal group), but TLD drug serum reversed this elevation ($P < 0.001$, compared with the model + Sham serum group) (Figure 6C; Supplementary Figure 4B). The release of IL-6 from THP-1 cells was increased after differentiation induction ($P < 0.001$, compared with the normal group), whereas the increase in IL-6 release was significantly suppressed after pretreatment with TLD drug serum ($P < 0.001$, compared with the model + Sham serum group) (Figure 6D). In addition, TLD-containing serum significantly inhibited M1 macrophage polarization in YHP-1, as evidenced by reduced the number of $CD86^+$ macrophages ($P < 0.001$, Figure 6E) and iNOS protein expression levels ($P < 0.001$, Figure 6F; Supplementary Figure 4B).


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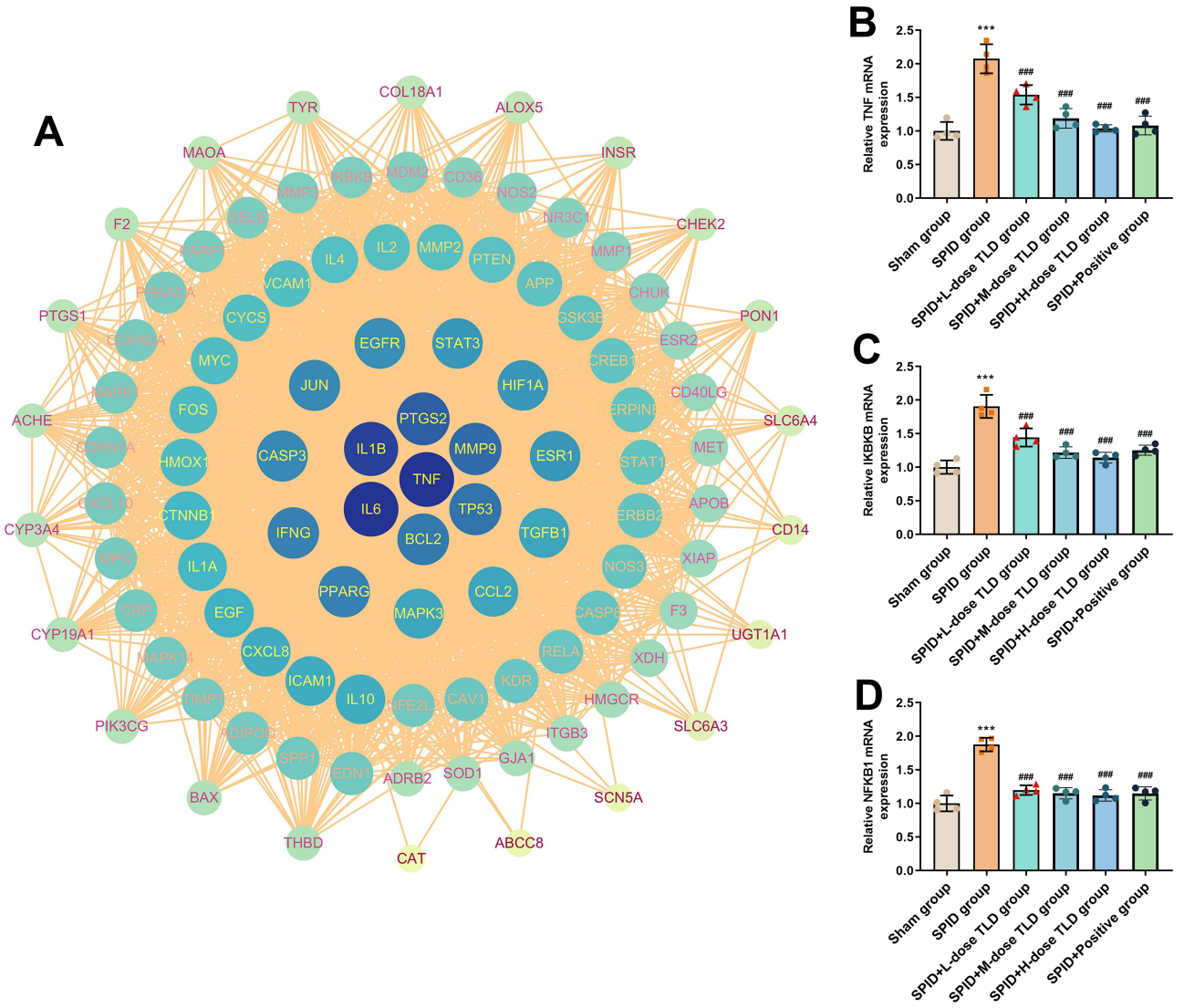


**Figure 4** Hub targets predicted for Tongluo Decoction (TLD). (**A**) Protein–protein interaction network. (**B–D**) The expression of TNF, IKBKB, and NFKB1 mRNAs by quantitative real-time PCR. ***$P < 0.001$, vs Sham group. ###$P < 0.001$, vs SPID group.

## Discussion

SPID is an infectious disease, and antibiotic treatment is currently the mainstay of Western medicine. However, the abuse of antibiotics is serious, and the potential dangers of bacterial dysbiosis, bacterial resistance, superbugs, and increased adverse reactions are becoming more and more prominent, so there is an urgent need to find ways and means of supplementing or substituting for antibiotics in the treatment of this disease. Traditional Chinese medicine (TCM), with its wide range of sources and diverse compositions, has unique advantages in the treatment of SPID.[10,20] In the treatment of SPID, Chinese medicines can be used as direct antibacterial agents, or they can stimulate the mobilization of the body's intrinsic antibacterial positive factors, as well as reduce bacterial virulence and the destructive capacity of bacteria on tissue cells.[21] Chinese medicines can also enhance the body's immunity by regulating the immune function to achieve the purpose of treating the disease.[22] TLD contains six medicines that are key herbs in the TCM practice of SPID.[9] For instance, Radix Paeoniae Rubra can improve chronic pelvic inflammatory disease by regulating the arachidonic acid pathway PTGS2.[23] In this study, we found that TLD can protect uterines from damage and inhibit inflammation during SPID. Using network pharmacology, we predicted that TLD may act its anti-SPID effects by inhibiting the expression

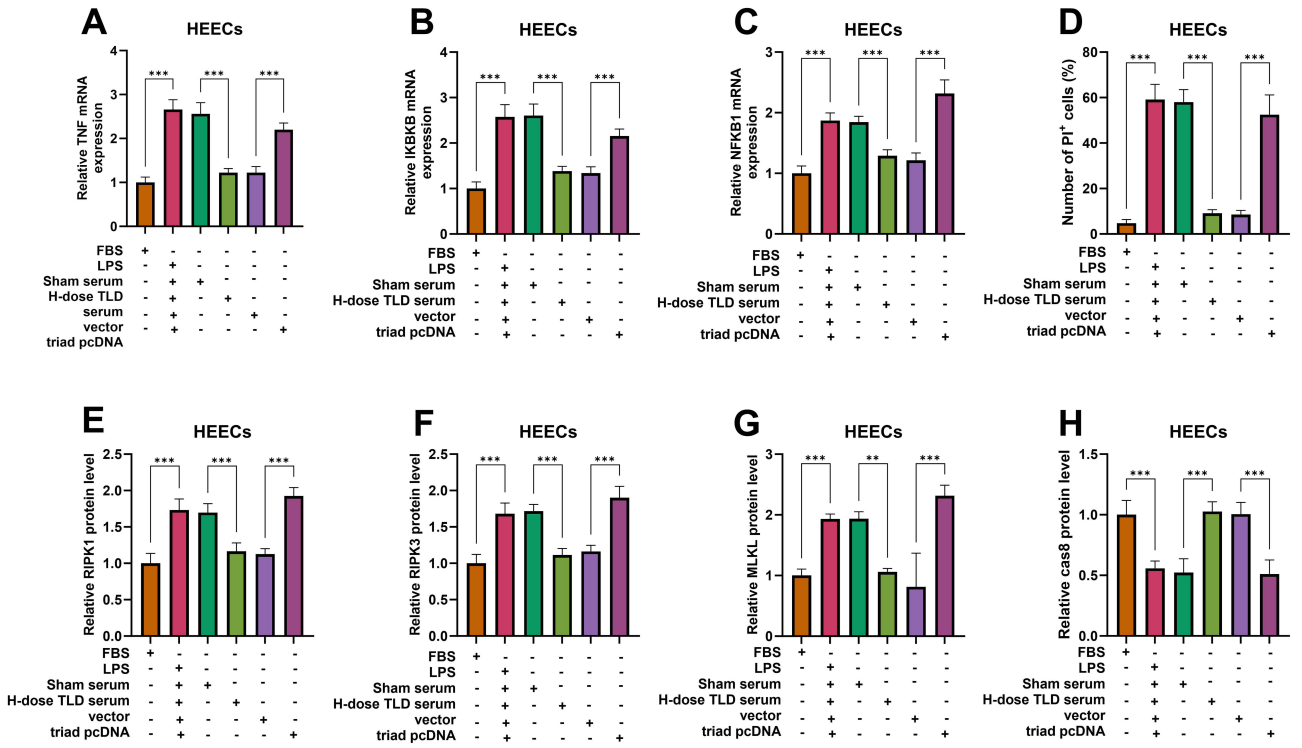


**Figure 5** TNF, IKBKB, and NFKB1 mediated the effect of Tongluo Decoction (TLD) on the necroptosis of human endometrial epithelial cells (HEECs). (**A** and **B**) (**C**) The expression of TNF, IKBKB, and NFKB1 mRNAs in HEECs by quantitative real-time PCR, after TLD serum treatment, with or without overexpression of TNF, IKBKB and NFKB1. (**D**) The necroptosis of HEECs characterized by number of $PI^+$ cell ratio using flow cytometry with Annexin V/PI staining. (**E**–**H**) The protein levels of RIPK1, RIPK3, MLKL, and cas8 in HEECs by Western blot, after TLD serum treatment, with or without overexpression of TNF, IKBKB and NFKB1. $^{**}P < 0.01$; $^{***}P < 0.001$.

levels of TNF, IKBKB, and NFKB1. Cell experiments in HEECs and macrophages validated that TNF, IKBKB, and NFKB1 mediated the anti-SPID effects of TLD maybe via necroptosis and M1 macrophage polarization.

Necroptosis is a pathway of necrotic cell death that is genetically encoded.[24] Unlike apoptosis, necroptosis, as a cellular suicide mechanism, permits the release of immunogenic cellular contents, including damage-associated molecular patterns and inflammatory cytokines, to trigger inflammation.[25] Enhanced necroptosis and inflammation caused by NF-κB activation have been found in an Escherichia coli-induced endometritis animal model.[21] Currently, it is not possible to use standard histological techniques to distinguish between cells that undergo necroptosis and those that undergo necrosis.[26] Distinct from apoptotic markers, receptor-interacting protein kinases (RIPKs) are important mediators of necroptosis.[27] Caspase-8, on the other hand, can antagonise the ability of necroptosis to sag through necroptosis mediators.[28] Studies have shown that necroptosis in inflammatory diseases can lead to tissue damage.[25,29] Inhibition of necroptosis has therapeutic potential in inflammatory diseases, such as SPID. In this study, TLD treatment significantly inhibited necroptosis in HEECs. This function may be achieved by targeting TNF, IKBKB, and NFKB1. Current understanding of necroptosis considers TNF to be a potent molecule that triggers necroptosis.[30] RIPK1/NF-kappaB-dependent TNF-α secretion and RIPK3-dependent ROS generation contribute to neoalbaconol-induced necroptosis. p50 (Nfkb1), one of the subunits in the NF-κB heterodimer, has been shown to promote cell survival by inhibiting apoptosis and necroptosis.[31] RIPK1/NF-κB-dependent TNFα secretion and RIPK3-dependent ROS generation contribute to increased necroptosis.[32] Furthermore, in addition to elevated levels of RIPK1 and RIPK3, deletion of cas8 alters the complex to IIb (also known as necrosome). This necrosome enables the cell to undergo necroptosis through direct phosphorylation of the mixed-spectrum kinase structural domain-like protein (MLKL).[33] We observed elevated RIPK1, RIPK3, and MLKL, as well as decreased cas8, in a cellular model of HEECs with SPID, which is evidence of increased necroptosis. This is consistent with previous findings.[21] Most notably, we found that TLD serum can be therapeutic for SPID by restoring the expression levels of these abnormal factors related to necroptosis.


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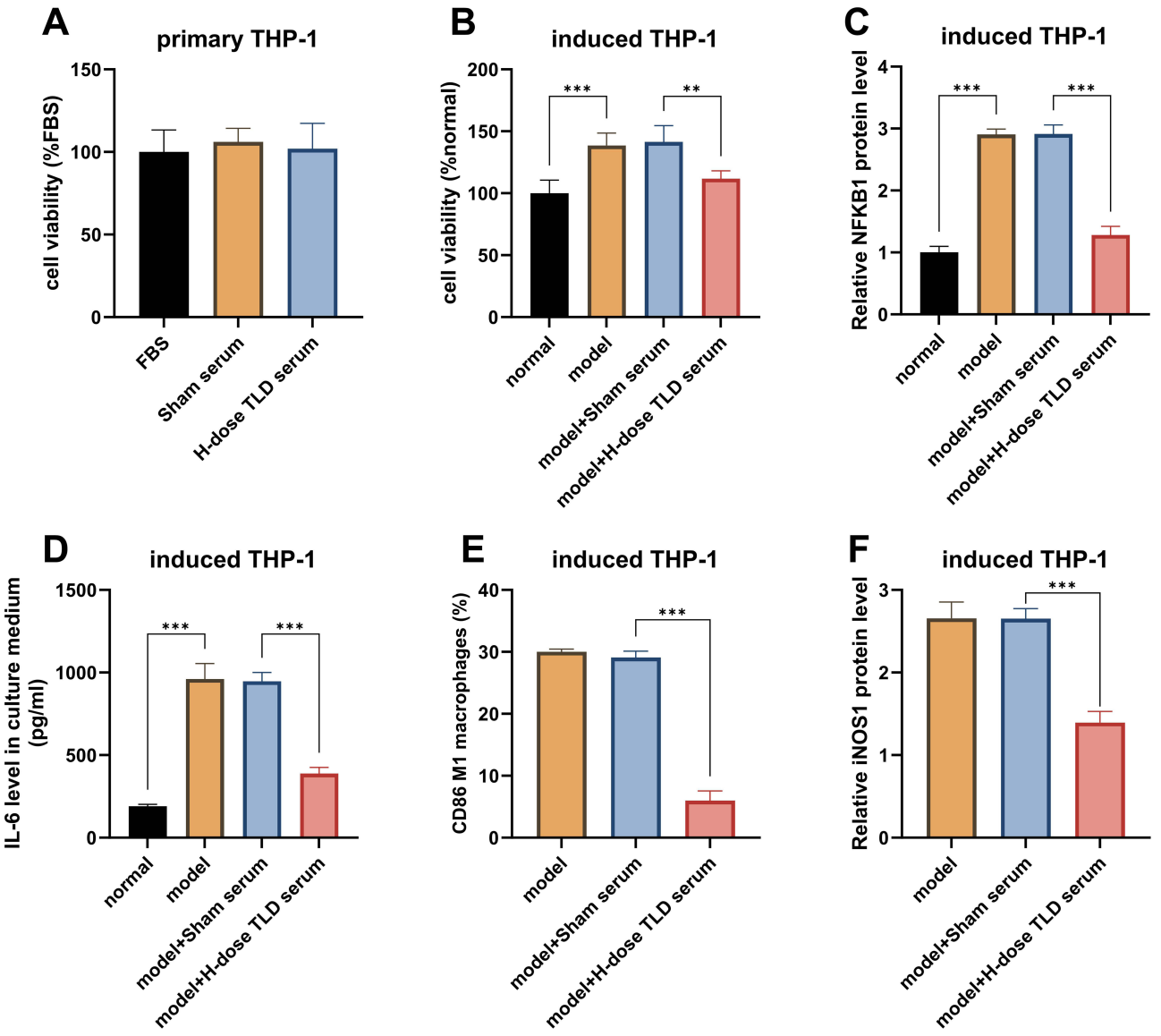


**Figure 6** Effect of Tongluo Decoction (TLD) THP-1 cells. (**A**) Effect of TLD-medicated serum on cell viability of normal THP-1 cells. (**B**) Effect of TLD-medicated serum on cell viability of M1-induced THP-1 cells. PYJ medicated serum inhibited cell proliferation of RAW 264.7 cells stimulated by LPS. (**C**) The protein levels of NFKB1 in THP-1 by Western blot, after different treatments. (**D**) The releases of IL-6 in THP-1 cells by ELISA. (**E**) The number of $CD86^{+}$ macrophages after differentiation induction of THP-1 by flow cytometry. (**F**) The protein levels of iNOS in THP-1 after differentiation induction. $^{**}P < 0.01$; $^{***}P < 0.001$.

It has been shown that TNF/NF-κB p50 (Nfkb1) can be involved in the regulation of macrophage polarization rearrangement.[34] Modulating the shift of macrophages from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype is an attractive treatment for pelvic inflammatory diseases.[35] This study used a combination of cellular and mouse models to demonstrate the significant anti-inflammatory and macrophage M1 polarization-modulating effects of TLD. In the rat and THP-1 cell SPID models, TLD was found to inhibit M1-like polarization, as demonstrated by the suppression of CD86+ cell numbers. More studies should be conducted to determine the potential effects of the components on the medicinal effects of the phytochemicals from the plants in TLD. Our future studies would focus on clinical trial with this formula to prove its efficacy in the human body. This process requires a multidisciplinary approach with ethical and clinical insights.

## Conclusion

In conclusion, this study validated the therapeutic effect of TLD in SPID rats. Its ability to regulate necroptosis and macrophage M1 polarization by targeting TNF, IKBKB, and NFKB1 was revealed through the use of network

pharmacological approaches and cellular experiments. We speculate that TLD may reduce endometrial damage and inflammatory responses caused by SPID by targeting the TNF/NF-κB signaling pathway. This would be further validated using more experiments, such as clinical trials in human body.

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## Disclosure

The authors report no conflicts of interest in this work.

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