Clinical Complementary Medicine and Pharmacology 2 (2022) 100039

Contents lists available at ScienceDirect

Clinical Complementary Medicine and Pharmacology

journal homepage: www.elsevier.com/locate/ccmp

Jieduquyuziyin Prescription Suppresses the Inflammatory Activity of Macrophages via NOTCH1/NF-κB Pathway

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ARTICLE INFO

Keywords:
Jieduquyuziyin prescription
Systemic lupus erythematosus
Macrophages
NOTCH1
NF-κB

ABSTRACT

Background: Jieduquyuziyin prescription (JP) is a traditional Chinese medicine (TCM) formula, which has been applied to the treatment of systemic lupus erythematosus (SLE) for decades, and its efficacy and safety have been confirmed in clinical practice. However, little is known about its molecular mechanism.
Objective: To explore the effects of JP on macrophages' inflammatory activity and NOTCH1/NF-κB pathway.
Methods: The JP-treated serum was prepared to determine its optimal concentration. Given the fact that active components in rats' serum might affect the results, control serum without JP components was prepared simultaneously. Lipopolysaccharide (LPS) was used to activate RAW264.7, and the cells were interfered with DAPT (NOTCH1 blocker), control serum, and JP-treated serum, respectively. After the above intervention, the expression of NOTCH1 and RBPJ, the nuclear translocation of NF-κB, and the extracellular release of IL6, TNFα, and NO, was evaluated by real-time reverse transcription-polymerase chain reaction (RT-PCR), western blotting (WB), enzyme-linked immunosorbent assay (ELISA), and Griess method.
Results: Both DAPT and JP-treated serum could significantly suppress the expression of NOTCH1 and RBPJ induced by LPS, as well as the nuclear translocation of NF-κB, leading to the decreased release of IL6, TNFα, and NO, while control serum had little effect on macrophage activity and NOTCH1/NF-κB pathway.
Conclusion: These results demonstrated the effects of JP on macrophage activation and pro-inflammatory response and suggested that the molecular mechanism of JP might attribute to the inhibition of the NOTCH1/NF-κB pathway. Besides, previous studies suggested that paeoniflorin and ferulic acid are two major effective components in JP. In subsequent experiments, we would further explore the effects of these two components on MRL/lpr mice and macrophage activity.

1. Introduction

Jieduquyuziyin prescription (JP) is a traditional Chinese medicine (TCM) formula, which has been applied to the treatment of systemic lupus erythematosus (SLE) for decades and has been proven to be effective and safe in clinical practice (Ding et al., 2014). As a conventional therapy for SLE, glucocorticoid might aggravate health issues, such as cardiovascular or cerebrovascular diseases (Jung et al., 2019; Kostopoulou et al., 2020). It has been demonstrated that the combined treatment with JP can increase efficacy and reduce adverse effects caused by glucocorticoid, leading to reduced intake of glucocorticoid and better control of SLE (Wen et al., 2007c).

SLE is an autoimmune disease characterized by the presence of diverse autoantibodies and chronic inflammation (Tsai et al., 2019). Chronic inflammation responses, mediated by various immune cells and pro-inflammatory factors, would cause significant tissue damage and promote the progression of diseases (Duan et al., 2019). Macrophages play crucial roles in injury-induced responses (such as autoantibodies response) (Oishi and Manabe, 2018). Once activated, macrophages can produce numerous inflammatory cytokines, which initiate a cascade of inflammatory mediator release, thus eventually leading to tissue destruction (Hamidzadeh et al., 2017). It has been suggested in many studies that there is a correlation between the functional status of macrophages and the severity of SLE (Herrada et al., 2019).

Both NOTCH signals and nuclear factor kappa-B (NF-κB) signals are involved in the activation of macrophages (Zhou et al., 2014). NOTCH pathway is highly evolutionarily conserved and governs many cellular core processes, including participating in the development and differentiation of organs, tissues, and cells (especially immune system cells), and particularly playing a significant role in the functions of mature immune cells (Radtke et al., 2013). The NOTCH signaling is mainly mediated by

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https://doi.org/10.1016/j.ccmp.2022.100039
Received 1 August 2021; Received in revised form 15 April 2022; Accepted 19 April 2022

recombinant recognition sequence binding protein at the Jκ site (RBPJ), which is a sequence-specific transcription factor. NF-κB complexes are decisive transcription factors in the process of immune responses, and dysregulation of NF-κB activity is closely related to inflammatory and immune disorders (Oeckinghaus et al., 2011). It has been revealed in some studies that the NOTCH signals and NF-κB signals work synergistically. For instance, during the activation of macrophages, NOTCH signaling is necessary for the maintenance of NF-κB activity (Liang et al., 2019; An et al., 2020; Yan et al., 2019).

In this study, the molecular mechanism of JP for the treatment of SLE was explored by investigating its effects on macrophages' inflammatory responses. RAW264.7, a commonly selected inflammation model cell, was adopted as the experimental object. Besides, lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, which can initiate potent innate immune responses via Toll-like receptor4 (TLR4), was used to stimulate RAW264.7. In addition, to explore the effects of JP on the NOTCH1/NF-κB pathway and downstream inflammatory factors, the optimal concentration of JP was determined by cell counting kit-8 (CCK8); NOTCH1/NF-κB pathway was detected by real-time reverse transcription-polymerase chain reaction (RT-PCR) and western blotting (WB); the inflammatory factors was estimated by enzyme-linked immunosorbent assay (ELISA) and Griess reagent.

2. Materials and Methods

2.1. Herbal extract

JP was composed of ten kinds of herbs in total, including Dihuang (the dried root of *Rehmannia glutinosa* Libosch., batch No. 191101), Baihuasheshecao (dried whole herb of *Hedyotis diffusa* Willd., batch No. 191101), Jixuecao (dried whole herb of *Centella asiatica* (L.) Urb., batch No. 191101), Qinghao (dried aerial parts of *Artemisia annua* L., batch No. 191001), Chishao (dried root of *Paeonia lactiflora* Pall. or *Paeonia veitchii* Lynch., batch No. 191001), Mudanpi (dried root cortex of *Paeonia suffruticosa* Andr., batch No. 191101), Biejia (carapace of *Trionyx sinensis* Wiegmann, batch No. 191001), Shengma (dried rhizome of *Cimicifuga heracleifolia* Kom., *Cimicifuga dahurica* (Turcz.) Maxim. or *Cimicifuga foetida* L., batch No. 191101), Foshou (dried fruit of *Citrus medica* L. var. sarcodactylis Swingle, batch No. 191001) and Gancao (dried root and rhizome of *Glycyrrhiza uralensis* Fisch., *Glycyrrhiza inflata* Bat. or *Glycyrrhiza glabra* L., batch No. 191001), at a weight ratio of 5:5:5:4:4:4:4:3:3:2. The herbs mentioned above were all purchased from *Zhejiang Chinese Medical University TCM Decoction Pieces Co., Ltd* (Hangzhou, China) and identified and authenticated by Professor Yongsheng Fan at Zhejiang Chinese Medicine University (Hangzhou, China). These herb samples were preserved in the Public Platform of Medical Research Center, Academy of Chinese Medical Science, Zhejiang Chinese Medical University (Hangzhou, China), and the voucher numbers of them were presented as follows: GDH-2019-0010, BHSSC-2019-0010, JXC-2019-0010, QH-2019-0010, CS-2019-0010, DP-2019-0010, ZBJ-2019-0010, SM-2019-0010, FS-20190010, and SGC-2019-0010. JP with a total weight of 117 g was soaked in 10-fold distilled water for 1 h, and then boiled for 2 h. The crude drug decoction was collected and the residue was boiled again with the same volume of water for another 2 h. These two decoctions were combined, filtrated, and then concentrated to 1.56 $g{\cdot}mL^{-1}$ for later use.

In a previous study, it has been confirmed through high-performance liquid chromatography (HPLC) that JP contains gallic acid, paeoniflorin, ferulic acid, and isoferulic acid, and the concentration of these four components is 11.34, 88.02, 3.02, and 2.57 $mg{\cdot}mL^{-1}$, respectively (Ji et al., 2020).

2.2. Animals and cells

Twenty 6-week-old male Sprague-Dawley (SD) rats (180–220 g) were purchased from the Experimental Animal Center of Zhejiang Chinese Medical University (Hangzhou, China). All animals were maintained under specific pathogen-free (SPF) conditions in an environment with 50–55% humidity and daylight 12 h at 20 ± 2°C and had free access to food and water. This study was performed in accordance with the National Institutes of Health's guidelines for laboratory animal care and use, as approved by the Experimental Animal Health Ethics Committee of Zhejiang Chinese Medical University (permission No. SYXK-ZHE-2018-0012; Hangzhou, China).

RAW264.7 cells were purchased from American Type Culture Collection (ATCC). These cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, California, USA) containing 10% fetal bovine serum (FBS, Gemini, California, USA) and 1% penicillin/streptomycin solution (Biosharp, Hefei, China) at 37°C in an incubator with 5% CO_2 and passaged when their density reached 80–90%.

2.3. Preparation of JP-treated serum and control serum

With the consideration of the fact that active ingredients in rats' serum might affect the expression of proteins and cytokines in RAW264.7 cells, control serum without JP components was prepared simultaneously. After adaptive feeding for 1 week, twenty SD rats were randomized by random digits table and divided into the JP group and the control group. The maximum daily dose for rats was calculated based on the clinical equivalent dose of adults, namely, 2 mL/100 g each time and twice a day. JP and an equal dose of distilled water were administered with gavage to these rats in the JP group and control group for five consecutive days, respectively (Zhang et al., 2015). Blood samples were collected from the abdominal aorta under aseptic conditions one hour after the last gavage. After being separated, the serum was further inactivated in a water bath at 56°C for 30 min, filtered through a 0.22 μm membrane for sterilization, and aliquoted and stored at -80°C for later use.

The identification of chemical constituents and quality control of JP-treated serum had already been performed by liquid chromatography-mass spectrometry (LC-MS) in advance. Paeoniflorin (2.539 ± 0.656 $ng{\cdot}mL^{-1}$) and ferulic acid (0.350 ± 0.203 $ng{\cdot}mL^{-1}$) were detected in JP-treated serum, but they were not found in control serum under the same conditions (Ji et al., 2020).

2.4. Determination of the optimal concentration of JP-treated serum by CCK8

CCK8 kits (Biosharp, Hefei, China) were utilized to assess the effect of both JPtreated serum and control serum on cell viability in an attempt to determine the optimal concentration. According to the instruction, 100 μL cells (10^5/mL) were assigned to each hole of 96-well plates. After 4 h, the supernatant was removed and different volume fractions (0%, 2.5%, 5%, 10%, 15%, 20%, and 30%) of JP-treated serum and control serum were applied to each well. Meanwhile, blank wells (containing the same amount of culture medium and CCK8 but without cells) were set as control. After 24 h, 10 μL CCK8 solution was added to each well (avoid bubbles). The reagent was placed at 37°C for 1 h for reaction. Finally, the absorbance at the wavelength of 450 nm was measured by a microplate reader (Thermo Scientific, Massachusetts, USA).

2.5. Cell grouping and interventions

Cells (10^6/mL) in the phase of logarithmic growth were inoculated in culture plates, grouped and imposed with corresponding interventions as follows: the control group, the LPS (Yuanye, Shanghai, China) group (1 $\mu g{\cdot}mL^{-1}$ LPS), the DAPT (Beyotime, Shanghai, China) group (1 $\mu g{\cdot}mL^{-1}$ LPS + 10 μM DAPT), the JP-serum group (1 $\mu g{\cdot}mL^{-1}$ LPS + JP-treated serum whose concentration would be determined by CCK8 test) and the control-serum group (1 $\mu g{\cdot}mL^{-1}$ LPS + control serum whose concentration would be the same as that of JP-treated serum). After

6 h, 16 h, and 24 h, cells or supernatants were collected respectively for RT-PCR, WB, and ELISA.

2.6. Detection of Notch1 and Rbpj mRNA by RT-PCR

Total RNA was extracted with RNAiso Plus (TAKARA, Shiga, Japan) according to the manufacturer's instructions. Subsequently, cDNA was synthesized with PrimeScript™ RT Master Mix (TAKARA, Shiga, Japan) by T100™ Thermal Cycler (Bio-Rad, California, USA), and RT-PCR was performed with TB Green® Premix Ex Taq™ (TAKARA, Shiga, Japan) by LightCycler® 96 (Roche, Basel, Switzerland). GAPDH acted as endogenous control, and the equation $2^{-\Delta\Delta Ct}$ was calculated to evaluate gene expression. Nucleotide sequences of required primer pairs (Sangon Biotech, Shanghai, China) are presented as follows: the forward primer of *Notch1*: 5′-GAT GGC CTC AAT GGG TAC AAG-3′; the reverse primer of *Notch1*: 5′-TCG TTG TTG TTG ATG TCA CAG T-3′; the forward primer of *Rbpj*: 5′-CTC CAC CCA AAC GAC TCA CTA-3′; the reverse primer of *Rbpj*: 5′-TCC AAC CAC TGC CCA TAA GAT A-3′; the forward primer of *Gapdh*: 5′-AGG TCG GTG TGA ACG GAT TTG-3′; the reverse primer of *Gapdh*: 5′-TGT AGA CCA TGT AGT TGA GGT CA-3′.

2.7. Detection of NOTCH1, RBPJ, and NF-κB p65 by WB

Radio-immunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) and nuclear and cytoplasmic protein extraction kit (Beyotime, Shanghai, China) were employed to extract total protein, cytoplasmic protein, and nucleoprotein as per the instructions. β-actin was used as the internal control for total protein and cytoplasmic protein, and Lamin B1 as the internal control for nucleoprotein. After quantification and denaturation, cell lysates were subjected to SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore, Massachusetts, USA), and PVDF membranes were blocked for 1h at room temperature in 5% skim milk before being incubated with anti-β-actin antibody (1:5000 dilution, Bioker, Hangzhou, China), anti-Lamin B1 antibody (1:10000 dilution, Abcam, Cambridge, UK), anti-NOTCH1 antibody (1:1000 dilution, Abcam, Cambridge, UK), anti-RBPJ antibody (1:2000 dilution, Abcam, Cambridge, UK), anti-NF-κB p65 antibody (1:2000 dilution, Abcam, Cambridge, UK), and anti-NF-κB p65 (phospho S536) antibody (1:1000 dilution, Abcam, Cambridge, UK) overnight at 4°C. Blots were developed with preabsorbed secondary antibodies (goat anti-rabbit IgG IRDye 800, 1:10000 dilution, LI-COR Biosciences, Nebraska, USA) for 1 h at room temperature before being imaged with Odyssey infrared imaging system (LI-COR Biosciences, Nebraska, USA).

2.8. Detection of interleukin-6 (IL6), tumor necrosis factor α (TNFα) by ELISA and nitric oxide (NO) by Griess reagent

The levels of IL6, TNFα, and NO in cell supernatants were tested with ELISA kits (Multi Sciences, Hangzhou, China) and Griess kits (Beyotime, Shanghai, China) according to instructions. In addition, the concentration of the cytokines in the control-serum and JP-serum without any cells was measured as a control.

2.9. Statistical analysis

Data were presented as mean ± standard deviation (SD) from at least three independent experiments. Independent-sample *t*-test and one-way analysis of variance (ANOVA) were performed with *IBM SPSS Statistics 23*, and images were drawn with *GraphPad Prism 7.0*. $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of JP-treated serum on the proliferation of RAW264.7

CCK8 test was performed to evaluate the effects of JP-treated serum and control serum on cell proliferation, with the results shown in Fig. 1. There was a similar trend for the effects of these two serums on cell proliferation. Cell proliferation could be promoted by a low concentration but inhibited by a high concentration; compared with the blank control, the promotion effect was the most significant at the concentration of 2.5%; both serums could considerably inhibit cell proliferation when the concentration exceeded 15%; there was no significant difference in the effects on cell proliferation between both serums at the same concentration. Therefore, 2.5% was selected as the optimal concentration and applied to the following experiments.

3.2. Effects of JP-treated serum on NOTCH1 and RBPJ

Moreover, the effects of JP-treated serum on NOTCH1 and RBPJ expression were evaluated by RT-PCR and WB, respectively. Meanwhile, the DAPT (an inhibitor of γ-secretase) group was arranged to block the NOTCH1 pathway as the positive control, and the control-serum group to estimate the effects of serum without JP ingredients on the NOTCH1 pathway. The effects of JP-treated serum on NOTCH1 and RBPJ were presented in Fig. 2. LPS could significantly upregulate mRNA and protein expression of both NOTCH1 and RBPJ. Both DAPT and JP-treated serum could inhibit the increase in the expression of NOTCH1 and RBPJ induced by LPS, while the control serum had no significant effect on both. The above results indicated that JP-treated serum could inhibit the NOTCH1 pathway activation of macrophages triggered by LPS.

3.3. Effects of JP-treated serum on nuclear translocation of NF-κB p65

Once stimulated by LPS, NF-κB p65 in the cytoplasm of macrophages would transfer to the nucleus and regulate the transcription of various pro-inflammatory genes, thus mediating the pro-inflammatory response. The quantity of NF-κB p65 in the cytoplasm and nucleus, as well as the expression of p65 and phosphorylated p65 of total protein extracts, were measured separately to evaluate the effects of JP-treated serum on the nuclear translocation of NF-κB p65. As shown in Fig. 3, after being stimulated by LPS, there was no significant difference in the level of whole and cytoplasmic NFκB p65 among the five groups. But both phosphorylated NF-κB p65 and nuclear NF-κB p65 increased substantially. However, the increase was notably inhibited by DAPT and JP-treated serum, which demonstrated that DAPT and JP-treated serum could block the pro-inflammatory response mediated by NF-κB p65.

3.4. Effects of JP-treated serum on IL6, TNFα, and NO

After the activation of macrophages and nuclear translocation of NF-κB p65, proinflammatory factors such as IL6, TNFα, and NO would be secreted outsides cells and participate in subsequent inflammation. The level of IL6, TNFα, and NO in supernatants was detected by ELISA and Griess methods to assess the activation of macrophages. As revealed in Fig. 4, it could be seen that LPS dramatically promoted the release of IL6, TNFα, and NO, and both DAPT and JP-treated serum could remarkably keep these inflammatory factors under control, namely that DAPT and JP could suppress the inflammatory activity of macrophages.

4. Discussion

TCM, featuring individualized therapy, is popular in China and plays an important role in the treatment of clinical diseases (Sang et al., 2018). The advantages of combined therapy of TCM and modern medicine in the treatment of SLE have been confirmed in several studies. First of all, as a kind of auxiliary treatment for SLE, JP could decelerate the disease progression without causing significant side effects. Besides, it has been demonstrated that JP has a protective effect on the hypothalamic-pituitary-adrenal axis of SLE patients, contributing to the decrement of glucocorticoid and reducing its side effects (Yang et al., 2021; Wen et al., 2007a, 2007c). In addition, JP could increase both endogenous glucocorticoid and glucocorticoid receptor α in MRL/lpr mice (Cao et al.,

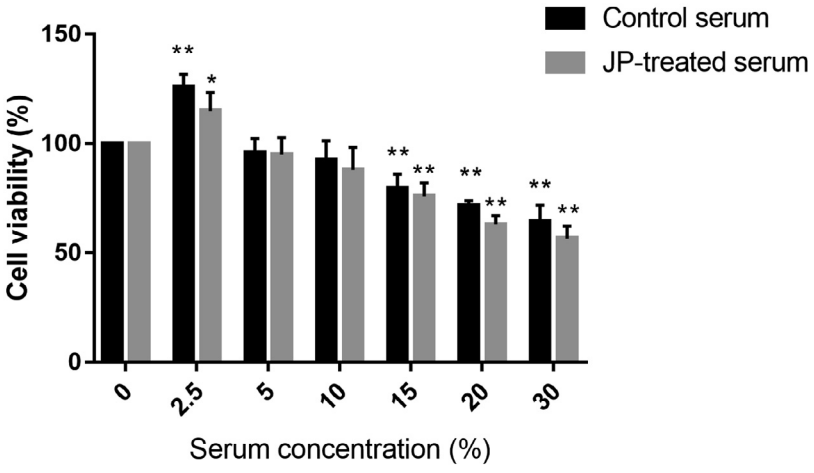

Fig. 1. Effects of control serum and JP-treated serum on the proliferation of RAW264.7 evaluated with CCK8. Data were presented as mean ± SD. * $P < 0.05$, ** $P < 0.01$, compared with the blank control.

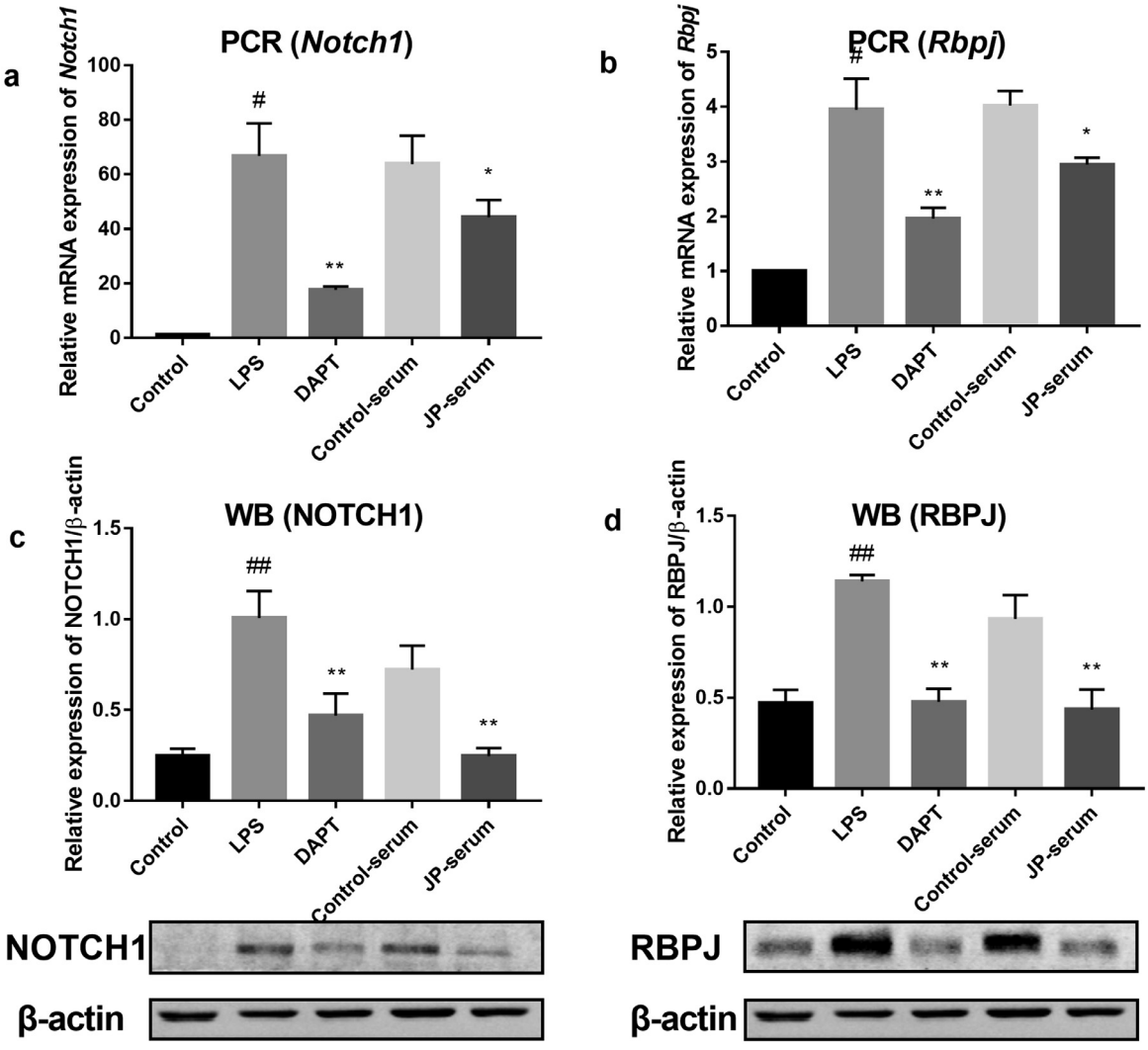

Fig. 2. Effects of JP-treated serum on NOTCH1 and RBPJ. The expression of NOTCH1 and RBPJ was evaluated by PCR and WB. Cell grouping and interventions from left to right respectively: the control group (without intervention), the LPS group (1 μg·mL^{-1} LPS), the DAPT group (1 μg·mL^{-1} LPS + 10 μM DAPT), the control-serum group (1 μg·mL^{-1} LPS + 2.5% control serum) and the JP-serum group (1 μg·mL^{-1} LPS + 2.5% JP-treated serum). Data were presented as mean ± SD. Compared with the control group, # $P < 0.05$, ## $P < 0.01$; compared with the LPS group, * $P < 0.05$, ** $P < 0.01$.

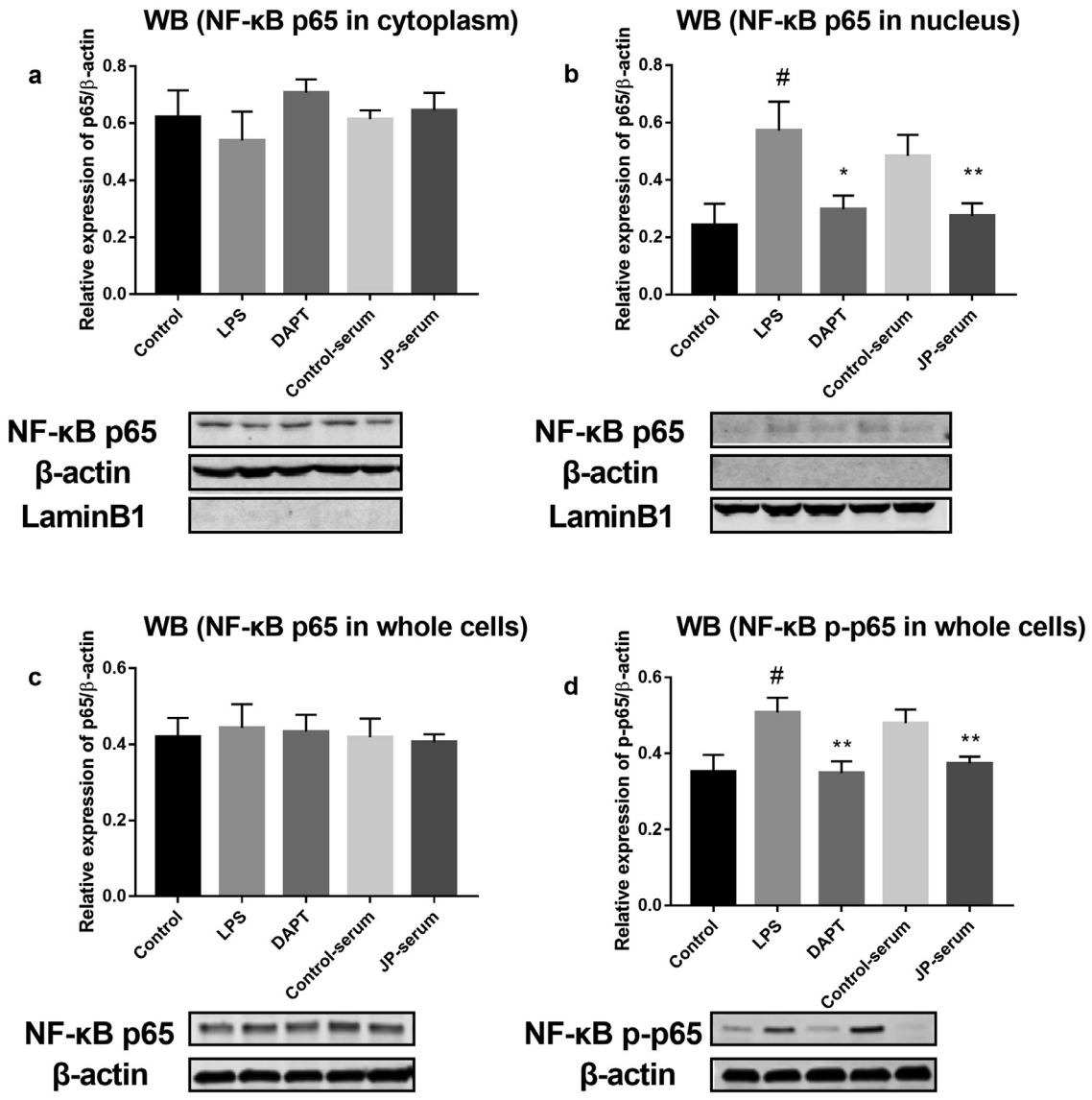

Fig. 3. Effects of JP-treated serum on nuclear translocation of NF-κB p65. The expression of cytoplasmic and nuclear NF-κB p65, as well as the expression of p65 and phosphorylated p65 of total protein extracts, were detected by WB. Cell grouping and interventions from left to right respectively: the control group (without intervention), the LPS group (1 μg·mL^{-1} LPS), the DAPT group (1 μg·mL^{-1} LPS + 10 μM DAPT), the control-serum group (1 μg·mL^{-1} LPS + 2.5% control serum) and the JP-serum group (1 μg·mL^{-1} LPS + 2.5% JP-treated serum). Data were presented as mean ± SD. Compared with the control group, $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$; compared with the LPS group, * $P < 0.05$, ** $P < 0.01$.

2010; Xu et al., 2011; Wen et al., 2007b), and inhibit macrophages' pro-inflammatory responses induced by LPS (Ji et al., 2020). This study tried to explore the effects of JP on the upstream mechanism of pro-inflammatory responses in macrophages.

NOTCH signals are activated through NOTCH ligands (Jagged1/2 and DLL 1/3/4) binding to receptors (NOTCH1/2/3/4) (Li et al., 2015). Once ligated by ligands, NOTCH intracellular domain (NICD) would be cleaved by A disintegrin and metalloproteinase domain (ADAM)-type proteinase and γ-secretase complex; subsequently, NICD is released to the cytoplasm and located in the nucleus; then, it combines with the transcription factor RBPJ to form a transcriptional activator complex, and induces the transcription of NOTCH target genes (Wang et al., 2010). There is accumulating evidence indicating that NOTCH signals are closely related to the dysregulation of macrophage activation and function. Once activated, macrophages would be polarized into two phenotypes, namely the classic activation type (M1 type) and the alternative activation type (M2 type), and the former could promote inflammation, and the latter could suppress inflammation (Orecchioni et al., 2019). LPS-induced activation of M1 macrophages depends on NOTCH signals. LPS could up-regulate the expression of NOTCH1 in macrophages through MYD88-dependent or independent pathways and activate the expression of the downstream genes, such as Hes1 and Deltex. Moreover, LPS could intervene with DAPT or eliminate RBPJ to block the NOTCH signals, which would result in the failure of LPS to activate macrophages (Wang et al., 2010; Palaga et al., 2008). According to the findings in this study, JP-treated serum, the same as DAPT, could suppress NOTCH1 signals and reduce the expression of pro-inflammatory factors (such as IL6, TNFα, and NO), which indicates JP might regulate the inflammatory activity of macrophages via the NOTCH1 pathway.

In mammals, the NF-κB family is composed of five members, namely, RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) (Oeckinghaus and Ghosh, 2009). NF-κB transcription factors, usually in the form of dimers (p65 and p50), could combine with its inhibitor IκB to form a trimer in the cytoplasm being inactive (Weng et al., 2018). Bacterial and viral infection, inflammatory cytokines, and antigen receptors engagement could trigger the activation of NF-κB, which sug-

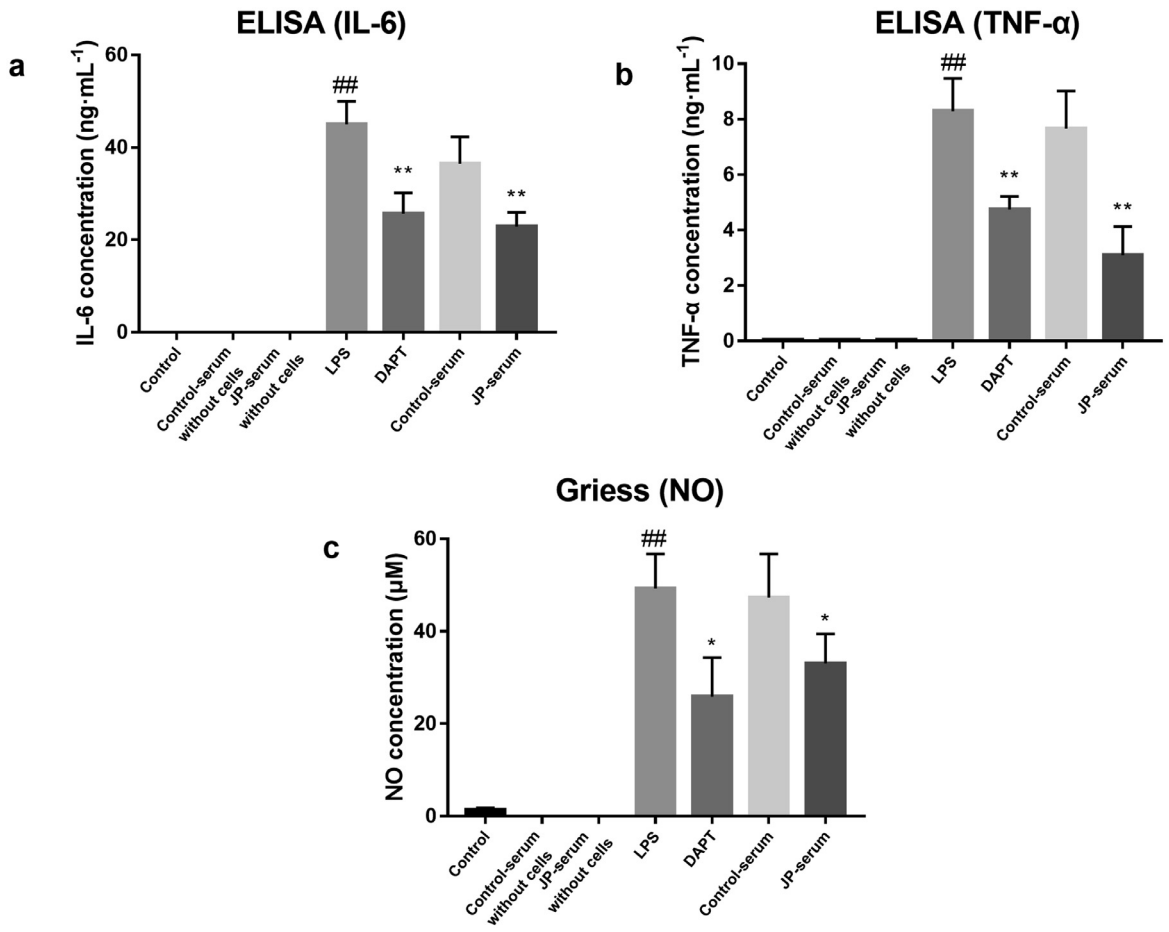

Fig. 4. Effects of JP-treated serum on IL6, TNFα, and NO. The level of IL6, TNFα, and NO were measured by ELISA and Griess methods. Data were presented as mean ± SD. Compared with the control group, $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$; compared with the LPS group, * $P < 0.05$, ** $P < 0.01$.

gests the NF-κB complex is crucial in the orchestration of immunity (Oeckinghaus et al., 2011). It has been demonstrated in some studies that the NF-κB complex is a downstream target of the NOTCH pathway (Fig. 5) (Liang et al., 2019; Osipo et al., 2008; Schwarzer et al., 2012; Hai et al., 2018). By selectively augmenting IRAK2-dependent TLR4 signaling, RBPJ could indirectly promote the nuclear translocation of NF-κB (Xu et al., 2012).

Besides, NICD1 could activate p65 phosphorylation and acetylation by combining ANK areas with p65 to adjust the activation state of the signaling pathway (Huang et al., 2018; Cheng et al., 2019). As shown in this study, both JP-treated serum and DAPT could promote the nuclear translocation of NF-κB in macrophages, demonstrating that JP could control the activation of macrophages by regulating the NOTCH1/NF-κB pathway.

Multiple pro-inflammatory factors are released to aggravate SLE after the activation of macrophages, including IL6, TNFα, and NO. IL6 and TNFα are two pleiotropic cytokines with significant functions in the regulation of the immune system; the increased expression of these two pro-inflammatory cytokines significantly contributes to the pathogenesis of SLE (Su et al., 2012; Yap and Lai, 2010; Rönnblom and Elkon, 2010). Besides, activated M1-type macrophages could produce more inducible nitric oxide synthases (iNOSs) to catalyze L-arginine to form NO. Both iNOS and NO share a positive correlation with SLE (Pan et al., 2020). NO can mediate apoptosis and regulate the balance among T cell subsets (Gonzalez-Gay et al., 2004). Besides, its product, peroxynitrite $ONOO^-$ (one of the reactive nitrogen species), can not only induce tissue injury, but also run through the cellular membranes and oxidate protein side-chains, thus generating neo-epitopes and contributing to T cells activation and autoimmune attack (Negre-Salvayre et al., 2008; Ahmad and Ahsan, 2014). This study indicated that JP might alleviate SLE by regulating the expression of proinflammatory mediators.

So far, we still know little about the mechanism of JP in the treatment of SLE. In this experiment, we focused on NOTCH signals and NF-κB signals, which are closely related to immune disorders and inflammation. Besides, there is crosstalk between these two pathways. Therefore, we selected RAW264.7 as an inflammation model to explore the influence of JP on the NOTCH1/NF-κB pathway. Our study indicated that both DAPT and JP-treated serum could significantly reduce the expression of NOTCH1, RBPJ mRNA, and protein induced by LPS, as well as inhibit the nuclear translocation of NF-κB and the extracellular release of IL6, TNFα, and NO. However, there was no statistical difference between the LPS group and the control-serum group, demonstrating that the expression changes in the JP-serum group should be owed to JP ingredients. In all, our study suggested that the molecular mechanism of JP might attribute to the inhibition of the NOTCH1/NF-κB pathway.

Paeoniflorin and ferulic acid are two major effective components in the JP formula. Paeoniflorin could exert extensive anti-inflammatory and immune regulatory effects (Zhang and Wei, 2020), and ferulic acid could perform various physiological functions, such as anti-inflammatory, antioxidant, antithrombotic, anti-cancer, and antidiabetic effects (Lin et al., 2010; Zduńska et al., 2018). Our previous studies have proved that paeoniflorin could inhibit the activation of the IRAK1/NF-κB signaling pathway in peritoneal macrophages from lupus-prone MRL/lpr mice. In subsequent experiments, we would further explore the effects of paeoniflorin, as well as ferulic acid, on the NOTCH

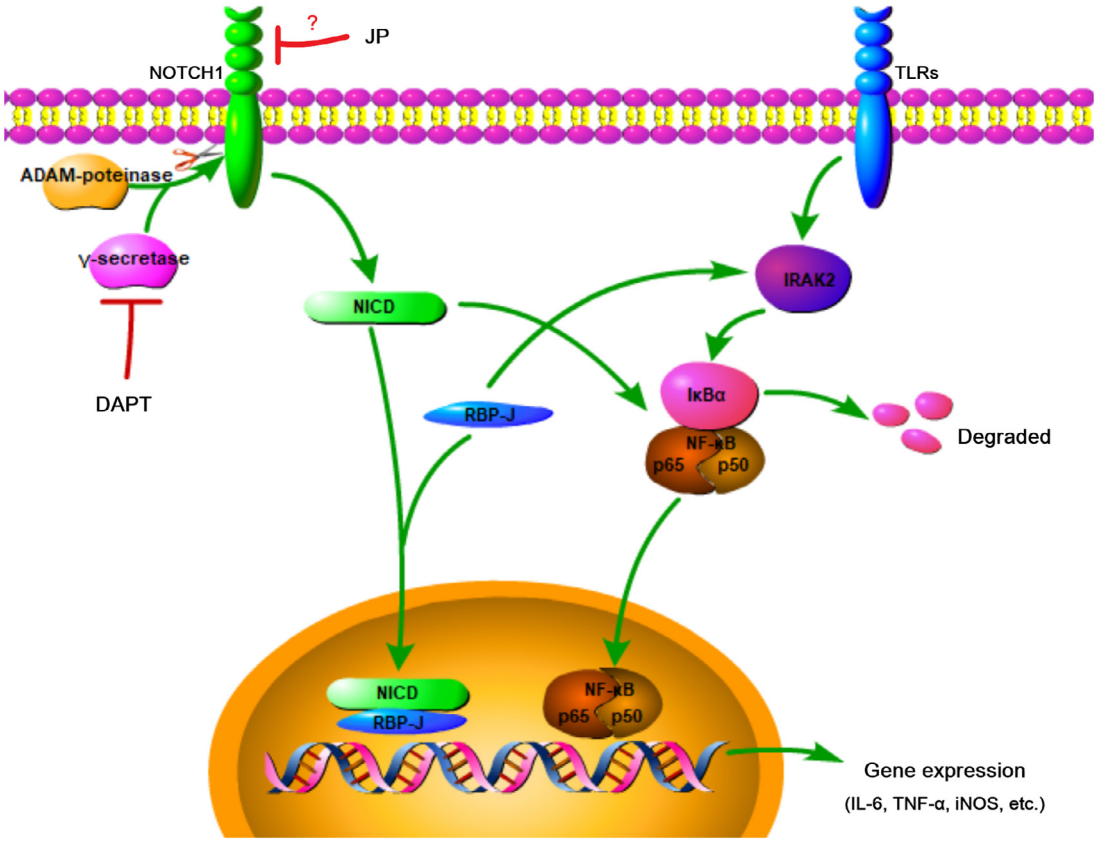

Fig. 5. Crosstalk between NOTCH1 signals and NF-κB signals. Once ligated by ligands, NOTCH intracellular domain (NICD) would be cleaved by ADAM-proteinase and γ-secretase complex; subsequently, NICD is released to the cytoplasm and located in the nucleus; then, it combines with RBPJ to form a transcriptional activator complex, and induces the transcription of NOTCH target genes. Meanwhile, by augmenting IRAK2 signals and activating p65 phosphorylation and acetylation, RBPJ and NICD could respectively promote the nuclear translocation of NF-κB.

pathway in vivo and in vitro with the consideration of the crosstalk between NOTCH signals and NF-κB signals.

5. Conclusion

Our study demonstrated that the effects of JP on macrophage activation and proinflammatory responses might attribute to the inhibition of the NOTCH1/NF-κB pathway. Besides, it has been confirmed in previous studies that paeoniflorin and ferulic acid are two major effective components in JP. Thus, it is necessary to explore further the effects of these two components on MRL/lpr mice and macrophage activity.

Ethical Approval

The research was following the National Institutes of Health's guidelines for laboratory animal care and use, as approved by the Experimental Animal Health Ethics Committee of Zhejiang Chinese Medical University (Permission Number: SYXK-ZHE-2018-0012; Hangzhou, China).

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request. The processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Funding

This work was financially supported by the Graduate Science Research Fund of Zhejiang Chinese Medical University (Grant No: 2020YKJ03), and the Young and Middle-aged Science Innovation Research Fund of Zhejiang Chinese Medical University (Grant No: KC201941).

Declaration of Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

CRediT authorship contribution statement

Sijia Fang: Investigation, Formal analysis, Writing – original draft. **Lina Ji:** Visualization, Data curation, Writing – original draft. **Shan Wu:** Investigation. **Xiaoxuan Yang:** Investigation. **Kepeng Yang:** Supervision. **Yongsheng Fan:** Supervision.

Acknowledgement

We thank Kepeng Yang and Yongsheng Fan for conceiving and supervising the experiments, and we thank all the colleagues in this study for providing useful discussions and technical assistance.

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Supplementary Materials

Nil.

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