

Morrisonide ameliorates lipopolysaccharide-induced inflammatory damage in iris pigment epithelial cells through inhibition of TLR4/JAK2/STAT3 pathway

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Abstract

• **AIM:** To investigate the effect of morroniside (Mor) on lipopolysaccharide (LPS)-treated iris pigment epithelial cells (IPE).

• **METHODS:** IPE cells were induced by LPS and treated with Mor. Cell proliferation was detected by cell counting kit (CCK) -8, apoptosis was detected by flow cytometry, the levels of tumor necrosis factor- α (TNF- α), interleukin (IL)-6, and IL-8 were measured by enzyme-linked immunosorbent assay (ELISA) kits, and the protein expression of TLR4, JAK2, p-JAK2, STAT3, and p-STAT3 was analyzed by Western blotting. In addition, overexpression of TLR4 and Mor treatment of LPS-stimulated IPE cells were also tested for the above indices.

• **RESULTS:** Mor effectively promoted the proliferation and inhibited the apoptosis of LPS-treated IPE cells. In addition, Mor significantly reduced the levels of TNF- α , IL-6, and IL-8 and significantly inhibited the expression of TLR4, p-JAK2, and p-STAT3 in LPS-treated IPE cells. The effect of Mor on LPS-treated IPE cells was markedly attenuated after overexpression of TLR4.

• **CONCLUSION:** These findings suggest that Mor may ameliorate LPS-induced inflammatory damage and apoptosis in IPE through inhibition of TLR4/JAK2/STAT3 pathway.

• **KEYWORDS:** morroniside; iris pigment epithelial cells; inflammatory; TLR4/JAK2/STAT3 pathway

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INTRODUCTION

Uveitis, a prevalent intraocular inflammatory disease affecting the iris, ciliary muscle, and choroid, poses a significant risk to vision, potentially resulting in visual impairment in approximately 35% of individuals^[1]. There are various forms of uveitis, but acute anterior uveitis (AAU) is the most common, it affects the iris and ciliary body, with 90% of cases involving the iris^[2]. The absence of a comprehensive comprehension regarding the pathogenesis of anterior uveitis has resulted in the absence of an optimal and efficacious treatment^[3]. Despite the effectiveness of immunosuppressants and hormones, they are unstable and prone to recurrence, eventually resulting in loss of visual function^[4]. As a result, new treatment methods are an important topic in uveitis research.

Cornus officinalis Sieb. et Zucc is a plant of the *Cornaceae* family, and further isolation of *Cornus officinalis* cyclic enol ether terpene glycosides yielded morroniside (Mor), which has been extensively used in traditional Chinese medicine^[5]. Research has demonstrated that Mor plays a role in modulating the down-regulation of apoptotic proteins in osteoarthritis, thereby exerting anti-apoptotic effects^[6], Mor reduces the volume of cerebral infarction and inhibits cortical lipid peroxidation in a rat model of focal cerebral ischemia-reperfusion^[7]. Furthermore, Mor can exert anti-inflammatory pharmacological properties by decreasing the phosphorylation level of nuclear factor kappa-B (NF- κ B) in rats with acute myocardial infarction as well as cerebral infarction^[8], and prevent acute colitis and type 2 diabetes mellitus in mice by inhibiting the NF- κ B pathway^[9-10], and also have a positive effect on the treatment of diabetic nephropathy in its early stages^[11]. The findings of our initial investigation indicate that Mor may possess a protective effect against lipopolysaccharide (LPS)-induced uveitis by modulating the polarization of

M1/M2 macrophages. Nonetheless, additional research is warranted to elucidate the precise mechanism through which Mor ameliorates uveitis.

Experimental evidence indicates that LPS, a pathogen-associated molecular pattern (PAMP), plays a significant role in the pathogenesis of uveitis by swiftly activating the innate immune system *via* Toll-like receptor 4 (TLR4) of the pattern recognition receptor family^[12-13]. Subsequent studies have found that selective interference with TLR4 function leads to a reduced response to LPS stimulation by macrophages in the peripheral blood of AAU patients, causing LPS tolerance^[14]. In addition, TLR4, when activated, stimulates inflammatory cytokine production^[15]. JAK2 can signal through the conserved JAK-STAT cell signaling pathway, and experiments have demonstrated that *H. pylori* contain LPS that recruits and activates JAK2, which catalyzes p-STAT3^[16]. Therefore, TLR4 may activate JAK2/STAT3 by inducing the production of inflammatory factors.

The human iris pigment epithelium (IPE) is a crucial cellular layer within the iris structure, anatomically linked to the ciliary epithelium *etc.*, and is a monolayer of epithelial cells, which can be separated from the stroma^[17]. Notably, it exhibits functional characteristics such as phagocytosis and the synthesis of cytokines and growth factors^[18]. Moreover, the expression of a functional LPS receptor complex is observed in IPE, leading to the release of proinflammatory mediators upon exposure to LPS. The cytokines released by IPE bear resemblance to those identified in samples obtained from patients with AAU, implying that IPE serves as a potential source of cytokines during disease activity^[19]. Furthermore, scholarly research has documented that TLRs present on the IPE and their reaction to PAMPs offer valuable insights into the underlying mechanisms of ocular inflammation, particularly anterior uveitis^[20]. Meanwhile, IPE is easy to take in the clinic and has a better prospect of application^[21].

Collectively, the excessive stimulation of TLR4 can result in inflammatory harm within the organism. Therefore, it is of utmost significance to devise diverse pharmaceutical interventions that impede or suppress the over-stimulation of the TLR4 signaling pathway, and subsequently employ them in the prevention and management of uveitis. Therefore, in this experiment, LPS will be used to treat human IPE cells, and Mor-treated cells will be used to compare cell proliferation, apoptosis, and inflammatory factors, as well as changes in TLR4/JAK2/STAT3 pathway in treated cells. Moreover, the effect of Mor on IPE after overexpression of TLR4 was observed to provide ideas and a theoretical basis for uveitis treatment.

MATERIALS AND METHODS

Cell Culture The IPE cell (Procell, cat No.CP-M118, Wuhan, China) was cultured in DMEM/F12 complete

medium supplemented with 10% fetal bovine serum (cat No. PM150312B, Wuhan Servicebio Co., Ltd., China) at 37°C with 5% CO₂ in an incubator. Upon reaching a confluence of 70%-80%, the cells were enzymatically dissociated using trypsin and subsequently passaged.

Cell Grouping and Transfection IPE cells in the logarithmic growth phase were harvested and the cell density was standardized to 4×10^4 cells/mL prior to inoculation into 96-well plates at a volume of 100 μ L per well. The experimental groups were designated as Mor (0, 5, 10, 20, 40 μ mol/L), with varying concentrations of Mor added for a duration of 48h. The cell counting kit (CCK)-8 method was used to screen the optimal Mor concentration for treating IPE cells. Subsequently, the experimental groups were set as 1) Control, LPS [from *Escherichia coli* (serotype O55:B4, Sigma-Aldrich, St. Louis, MO), 10 mg/mL], LPS+Mor; 2) Control, LPS, LPS+Mor, LPS+Mor+overexpression-negative control (ov-NC), and LPS+Mor+overexpression-Toll like receptor (ov-TLR4). The corresponding concentrations of Mor or transfected ov-NC or ov-TLR4 were added according to the grouping. The ov-NC and ov-TLR4 (Sangon Biotech, Shanghai) were transfected into the cells once they reached a cell density of 70% to 80%, following the instructions provided by Lipofectamine 2000 (cat No. 11668500, Gibco). In brief, a total of 125 μ L of culture medium containing either 2.5 μ g of ov-TLR4 or ov-NC was gently combined to form solution A. Additionally, 5 μ L of Lipofectamine 2000 transfection reagent was prepared as solution B. Solution A and solution B were then mixed together, and after allowing the mixture to stand at room temperature for 5min, an appropriate amount of culture medium was added. The resulting mixture was subsequently added to the corresponding plate at a volume of 2 mL per well. The transfection efficiency was assessed by Western blotting 48h post-transfection.

Cell Counting Kit-8 Assay to Detect Cell Proliferation The principle underlying CCK-8 detection involves the reduction of the WST-8 compound in the detection reagent by an intracellular dehydrogenase, resulting in the formation of a water-soluble orange-colored methyl saliva compound with a maximum absorption peak at 450 nm. In this particular study, CCK-8 was employed for the purpose of cell proliferation detection. Each group of cell samples was treated by the addition of 10 μ L of CCK-8 solution per well, followed by incubation at 37°C for a duration of 2h. The absorbance (A) value at 450 nm was subsequently determined using an enzyme marker.

Flow Cytometry to Detect Cell Apoptosis Cells in each group were treated with ethylene diamine tetraacetic acid (EDTA)-free trypsin, centrifuged at 3000 r/min for 5min, and the cell precipitates were suspended in 500 μ L of binding

buffer, and 5 μL each of Annexin V (cat No. KGA1030, KeyGEN Bio TECH, Jiangsu) and propidium iodide (PI; cat No. KGA1030, KeyGEN Bio TECH, Jiangsu Province) were added before and after. The reaction was carried out at room temperature for a period of 15min, while ensuring protection from light. Finally, the samples were analyzed using flow cytometry (cytoflex, Beckman).

ELISA to Detect the Levels of TNF- α , Interleukin-6, and Interleukin-8 in Cell Supernatants The supernatants of each group of cells after culture were collected, and the levels of TNF- α , interleukin (IL)-6 and IL-8 in the culture supernatant were detected according to the instructions of ELISA kit (ZCIBIO Technology Co., Ltd., Shanghai, China).

Western Blotting Cell lysates were prepared and protein concentration was measured by bicinchoninic acid (BCA) protein assay reagent (Beyotime, Beijing). Equal amounts of proteins were electrophoresed on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane. The transferred proteins were confined in 5% nonfat milk powder in phosphate buffered saline (PBS) containing 0.1% Tween 20 (PBST) for 2h at room temperature. The membranes were then incubated with against TLR4, JAK2, p-JAK2, STAT3 and p-STAT3 primary antibodies (dilution ratio 1:1000, Abclonal, Wuhan, China) at 4°C overnight. Then it was incubated with a 1:5000 dilution of each HRP-conjugated secondary antibody for 2h and washed again with PBST. The expressed proteins were displayed by ECL chemiluminescence detection kit (Affinity, Shanghai), and the gray value results were analyzed by Quality one.

Statistical Analysis The data were analyzed using SPSS 20.0 statistical software (SPSS Inc., Chicago, IL, USA). The results were presented as mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) was employed to compare multiple groups. A significance level of $P<0.05$ was used to determine statistical significance.

RESULTS

Effect of Mor on Cytokine Levels and Apoptosis in LPS-treated IPE As a first step, we screened the optimal Mor concentration on IPE cells using CCK-8, and we found that the proliferation of IPE was significantly reduced at Mor concentrations of 10, 20, and 40 $\mu\text{mol/L}$ ($P<0.01$), while 5 $\mu\text{mol/L}$ Mor had no significant effect on the cell proliferation of IPE ($P>0.05$), therefore, Mor at 5 $\mu\text{mol/L}$ was chosen for the subsequent experiments (Figure 1A). Second, we observed the effect of Mor on cytokine levels and apoptosis in LPS-treated IPE, and the results demonstrated that cell proliferation was significantly decreased ($P<0.01$; Figure 1B), while apoptosis (Figure 1C, 1D) and the levels of TNF- α , IL-6, and IL-8 (Figure 1E) were significantly increased in LPS-treated IPE compared

with the control group ($P<0.01$). After treatment with Mor, the above apoptosis and cytokine levels were significantly reversed, indicating that Mor may inhibit LPS-induced apoptosis and inflammatory responses.

Effect of Mor on the TLR4/JAK2/STAT3 Pathway in LPS-treated IPE Western blotting was employed to verify Mor's effect on the TLR4/JAK2/STAT3 pathway in LPS-treated IPE. A significant difference was found between the LPS group and the control group in terms of TLR4, p-JAK2, and p-STAT3 expression ($P<0.01$). As compared to the LPS group, the LPS+Mor group showed markedly decreased levels of TLR4, p-JAK2, and p-STAT3 ($P<0.05$), showing that Mor attenuated that the activation of TLR4/JAK2/STAT3 pathway induced by LPS (Figure 2).

Effect of Overexpression of TLR4 to Reactivate JAK/STAT Signaling Pathway on Mor Action on LPS-treated IPE Given that Mor demonstrated a reduction in the activation of the TLR4/JAK2/STAT3 pathway induced by LPS, we proceeded to overexpress TLR4 in order to restore the activity of the JAK/STAT signaling pathway. Subsequently, we investigated the impact of Mor on cells treated with LPS following the reactivation of the TLR4/JAK2/STAT3 pathway. The data showed that cell proliferation was significantly decreased ($P<0.01$; Figure 3A), while apoptosis (Figure 3B, 3C) and the levels of TNF- α , IL-6, and IL-8 (Figure 3D) were significantly increased in LPS+Mor+ov-TLR4 compared with the LPS+Mor group ($P<0.01$), suggesting that the excessive expression of TLR4 hindered the facilitation of proliferation and the suppression of apoptosis of Mor in LPS-treated IPE.

Effect of Overexpression of TLR4 to Reactivate JAK/STAT Signaling Pathway on the TLR4/JAK2/STAT3 Pathway in LPS-treated IPE by Mor Furthermore, using Western blotting, we also examined the expression levels of TLR4, JAK2, p-JAK2, STAT3, and p-STAT3 proteins. The findings revealed that the expressions of TLR4, p-JAK2 and p-STAT3 in the LPS+Mor+ov-TLR4 group were increased compared with that in the LPS+Mor group ($P<0.05$; Figure 4), indicating that overexpression of TLR4 inhibited the inhibitory effect of Mor on JAK/STAT signaling pathway.

DISCUSSION

In the current investigation, it was observed that Mor exhibited a promotion of LPS-treated IPE cell proliferation, inhibition of LPS-treated IPE cell apoptosis and TNF- α , IL-6, and IL-8 levels, and activation of the TLR4/JAK2/STAT3 pathway is attenuated by LPS. Moreover, Mor was less effective in IPE treated with LPS when TLR4 was overexpressed.

IPE is the pigment-containing layer of the iris' posterior layer^[22]. Under certain conditions, IPE cells can be transformed into neural retinal cells, lens epithelial cells, and so on^[18]. Under inflammatory conditions IPE cells can participate in the

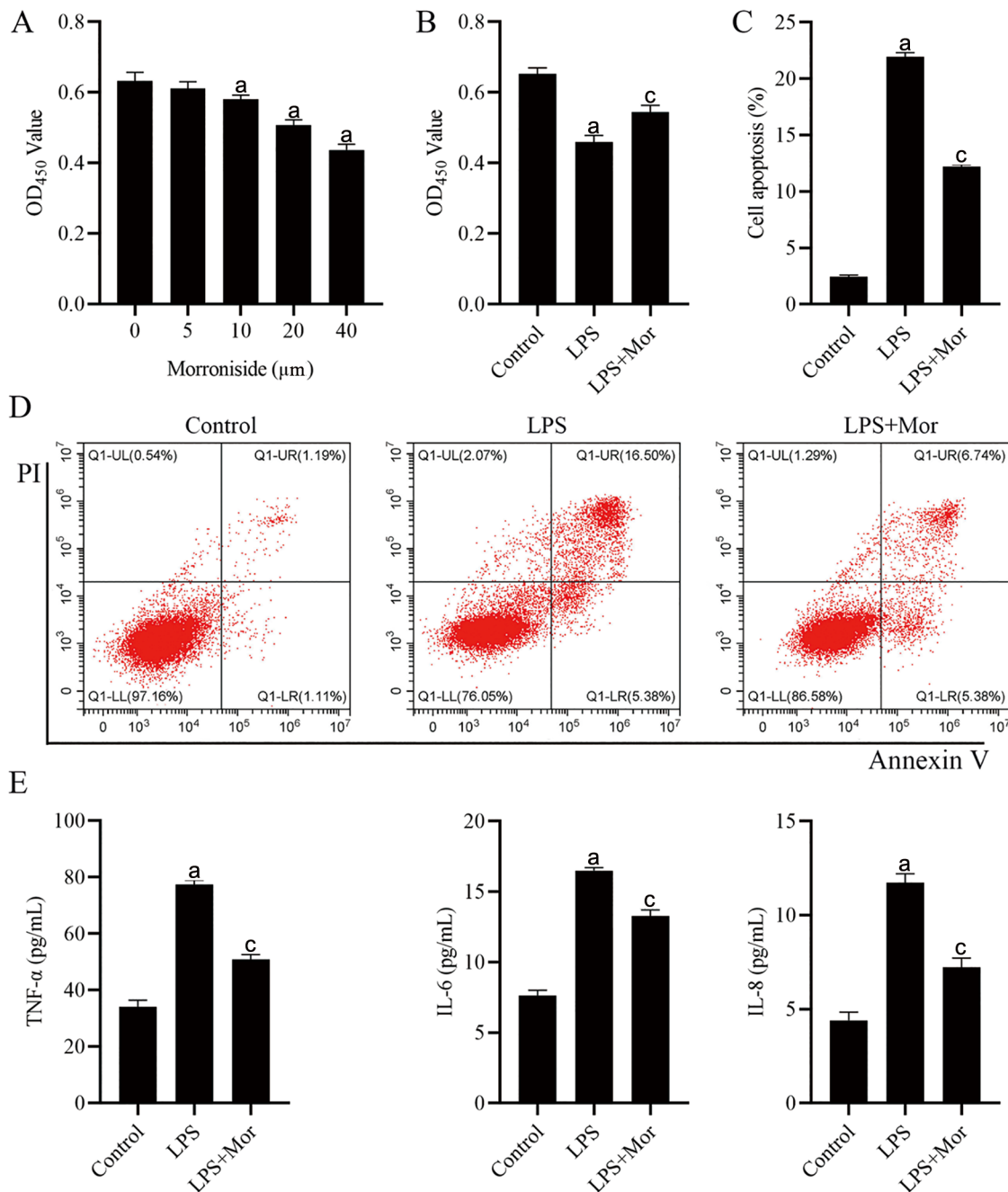


Figure 1 Effect of Mor on cytokine levels and apoptosis in LPS-treated IPE A: Cell proliferation in the groups treated with different concentrations of Mor ($n=4$); B: Cell proliferation in each treatment group ($n=4$); C: Apoptosis in each group ($n=3$); D: Flow diagram of apoptosis in each treatment group ($n=3$); E: The levels of TNF- α , IL-6, and IL-8 in each treatment group ($n=3$). The mean \pm SD of three independent samples is presented. ^a $P<0.01$ compared with the 0 or control group, ^c $P<0.01$ compared with the LPS group.

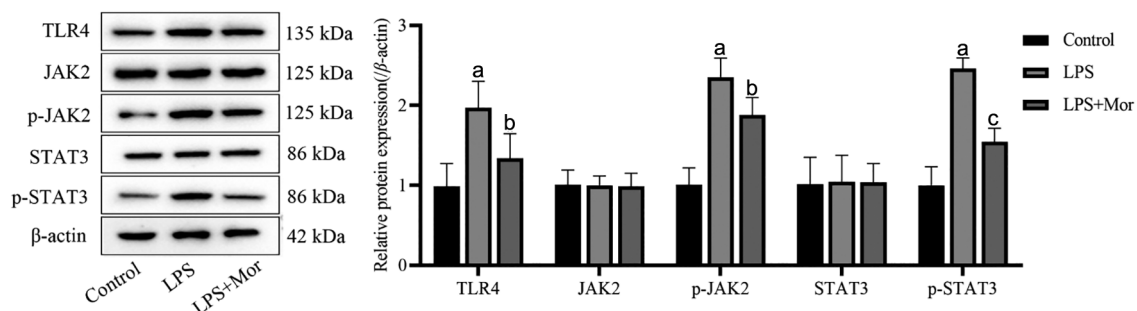


Figure 2 Effect of Mor on the TLR4/JAK2/STAT3 pathway in LPS-treated IPE The expression of TLR4, JAK2, p-JAK2, STAT3, and p-STAT3 in cells ($n=3$). Data were represented as mean \pm SD of three independent samples. The data in Western blot assays were showed after being normalized to β -actin. ^a $P<0.01$ compared with the control group, ^b $P<0.05$, ^c $P<0.01$ compared with the LPS group.

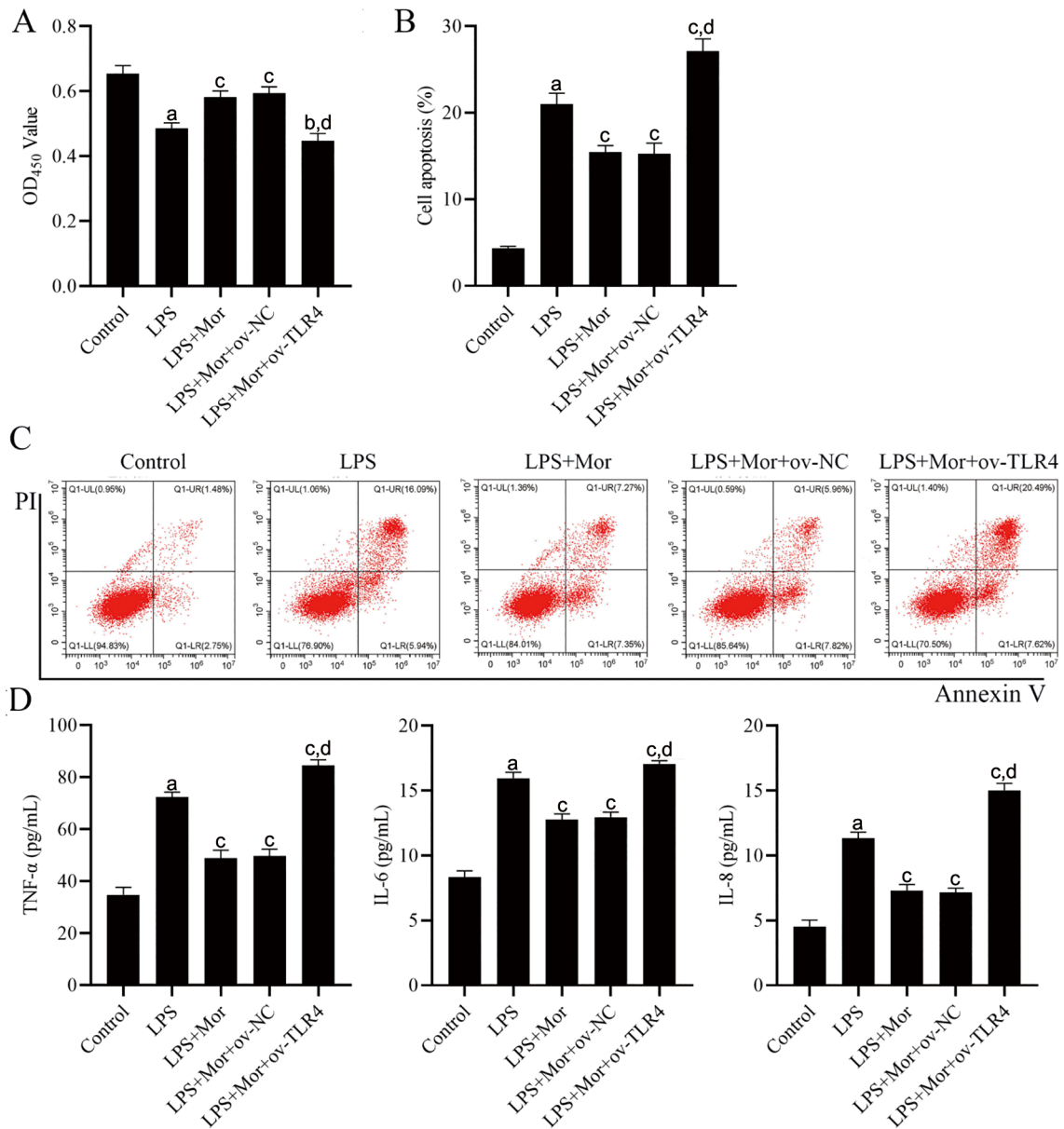


Figure 3 Effect of overexpression of TLR4 to reactivate JAK/STAT signaling pathway on Mor action on LPS-treated IPE A: Cell proliferation in each treatment group (n=4); B: Apoptosis in each treatment group (n=3); C: Flow diagram of apoptosis in each treatment group (n=3); D: The levels of TNF-α, IL-6, and IL-8 in each treatment group (n=3). The mean±SD of three independent samples is presented. ^aP<0.01 compared with the control group, ^bP<0.05, ^cP<0.01 compared with the LPS group, ^dP<0.01 compared with the LPS+Mor group.

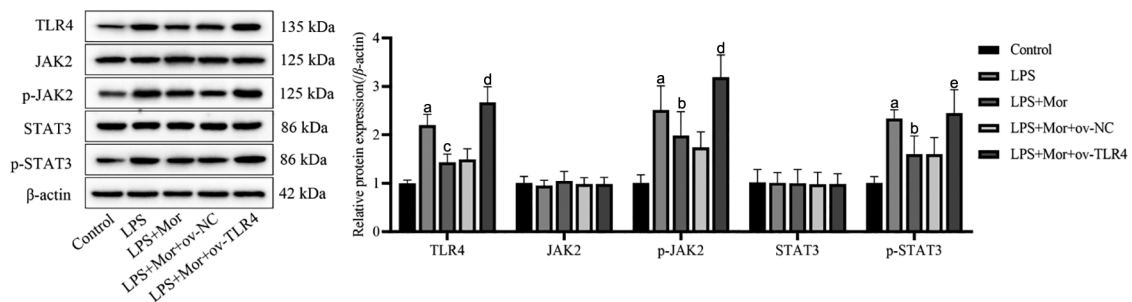


Figure 4 Effect of overexpression of TLR4 to reactivate JAK/STAT signaling pathway on the TLR4/JAK2/STAT3 pathway in LPS-treated IPE by Mor The mean±SD of three independent samples is presented. The data in Western blot assays were showed after being normalized to β-actin (n=3). ^aP<0.01 compared with the control group, ^bP<0.05, ^cP<0.01 compared with the LPS group, ^dP<0.05, ^eP<0.01 compared with the LPS+Mor group.

inflammatory response^[23]. In the current study, it was observed that IPE cells treated with LPS exhibited a notable inhibition of proliferation and a marked elevation in cell apoptosis. However, these effects were significantly counteracted upon treatment with Mor, suggesting its potential in inhibiting apoptosis induced by LPS. Moreover, inflammation assumes a crucial role in the pathogenesis of uveitis, and the therapeutic objective for this condition is to mitigate inflammation and safeguard ocular tissues from harm^[24]. And autoimmune inflammation caused by LPS is one of the more common pathogenic factors^[25]. It was found that cytokines TNF- α , IL-6, IL-8, and chemokines are involved in immune regulation, mediating cellular inflammatory responses and substance metabolism, and can regulate the pathogenesis of uveitis^[26]. The effects of Mor on LPS-treated IPE cells were attenuated in this study as it inhibited TNF, IL-6, and IL-8 levels. These findings align with prior investigations conducted on uveitis^[27-28]. The anti-inflammatory properties of Mor were hypothesized to be a result of its ability to inhibit the synthesis of TNF- α , IL-6, and IL-8.

The transmembrane protein TLR4, which belongs to the pattern recognition receptor family, has been implicated in acute endotoxic uveitis pathogenesis. According to research findings, LPS activates the TLR4 signaling pathway, resulting in the upregulation of pro-inflammatory cytokines, including TNF- α , IL-1 β , and IL-6, in the serum, consequently triggering an inflammatory response in the ocular tissues^[29]. Furthermore, the activation of TLR4 induces the activation of intricate signaling pathways, encompassing mitogen-activated protein kinases (MAPKs) such as p38, JNK, and ERK, phosphatidylinositol 3-kinase (PI3K), and GTPases^[30]. The Janus kinase 2 (JAK2) is a crucial regulator of various cellular processes such as proliferation, differentiation, survival, and senescence. Additionally, it exerts control over the activity of other signaling molecules, including signal transduction and activator of transcription (STAT)^[31]. Upon activation of JAK2, its own phosphorylation (p-JAK2) can create a docking site with STAT3, which activates the induction of STAT3 phosphorylation (p-STAT3)^[32]. Uveitis-prone rabbit retinas express TLR4 according to previous studies^[12]. In our study, it was observed that the treatment of IPE cells with LPS resulted in notable enhancements in the expression levels of TLR4, p-JAK2, and p-STAT3. Conversely, the presence of Mor exhibited inhibitory effects on the expression of TLR4, p-JAK2, and p-STAT3. More importantly, the effects of Mor on LPS-treated IPE cells were significantly inhibited after overexpression of TLR4, including cell proliferation, apoptosis, and levels of TNF- α , IL-6, IL-8, confirming that Mor may promote LPS-treated IPE cell proliferation, downregulate apoptosis and pro-inflammatory factor secretion

by inhibiting the phosphorylation level of TLR4/JAK2/STAT3 signaling pathway.

Overall, it was observed that Mor exhibited a stimulatory effect on cell proliferation, while also inhibiting apoptosis and the production of inflammatory cytokines in LPS-treated IPE cells. Additionally, Mor was found to suppress the activation of the TLR4/JAK2/STAT3 signaling pathway in these cells. However, it should be noted that the impact of Mor on LPS-treated IPE cells was diminished upon TLR4 overexpression. It is important to acknowledge that this study only represents an initial investigation into the regulatory role of Mor in LPS-treated IPE cells, and further comprehensive experimental validation is required to elucidate the precise mechanism by which Mor may be employed in the treatment of uveitis.

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Conflicts of Interest: Li WJ, None; Liu L, None; Lu H, None.

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