• Basic Research •

Inhibitive effect of TAK-242 on Tenon's capsule fibroblasts proliferation in rat eyes

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Abstract

• AIM: To study the inhibition effect of TAK-242 on the proliferation of rat eye Tenon's capsule fibroblasts *via* the toll-like receptor 4 (TLR4) signaling pathway.

• METHODS: SD rat Tenon's capsule fibroblasts were extracted and cultured, then the cells were divided into normal control group, lipopolysaccharide (LPS) group (10 g/mL LPS) and TAK-242 group (1 μmol/L TAK-242, and 10 μg/mL LPS after 30min). The expressions of TLR4, transforming growth factor-β1 (TGF-β1) and interleukin-6 (IL-6) in each group were detected by Western blot and reverse transcriptase-polymerase chain reaction (RT-PCR). Cell proliferation was detected by cell counting kit-8 (CCK-8).

• RESULTS: Double immunofluorescent labeling in the extracted cells showed negative keratin staining and positive vimentin staining. Western blot showed that the LPS group had the highest expression of TLR4 and TGF-β1 (P<0.01). Enzyme linked immunosorbent assay (ELISA) also showed that the secretion of IL-6 was the highest in LPS group (P<0.01). But there was no significant difference in TLR4 and TGF-1, as well as IL-6 expressions between the TAK-242 group and the normal control group (P>0.05). RT-PCR showed that the IL-6 mRNA expression in LPS group was the highest in the three groups (P<0.01).

• CONCLUSION: TAK-242 inhibits the proliferation of LPS-induced Tenon's capsule fibroblasts and the release of inflammatory factors by regulating the TLR4 signaling pathway, providing a new idea for reducing the scarring of the filter passage after glaucoma filtration surgery.

• KEYWORDS: Tenon's capsule fibroblasts; fibrosis; TAK-242; rat
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INTRODUCTION

Glaucoma filtration surgery (GFS) is the golden standard for lowering intraocular pressure (IOP) in glaucoma[1]. The success rate is often limited by postoperative scarring of the filter passage[2]. Tenon's capsule fibroblasts are the main cellular components of filtration tract scar, which have been studied to reduce scar formation by inhibiting the proliferation of human Tenon's cystic fibroblasts[3-4]. TAK-242 is a cyclohexene derivative that blocks toll-like receptor 4 (TLR4) signal path specifically and inhibits the production of cytokines mediated by TLR4 selectively[5]. There are studies proved that TLR4 signal path plays an important role in various organ fibrosis diseases[6-8]. Moreover, TLR4 may be associated with the pathological development of glaucoma[9], but there was no report about its effect on the scarring of the filter passage after glaucoma surgery.

In the study, we examined the effect of TAK-242, a specific antagonist of TLR4, on the secretion of inflammatory cytokines and cell proliferation by Tenon's capsule fibroblasts, in order to verify the role of TAK-242 in inhibiting the postoperative scarring of the filter passage in glaucoma.

MATERIALS AND METHODS

Ethical Approval All animals were conducted in line with the Chinese Ministry of Science and Technology Guidelines on the Humane Treatment of Laboratory Animals and the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. This study was approved by the Animal Care Committee of China Three Gorges University.

Primary Culture of SD Rat Tenon's Capsule Fibroblasts Two SD rats aged 5-6wk were selected and weighed. Intraperitoneal injection of chloral hydrate 10% anesthesia (chloral hydrate 10% / rat body weight =0.35 mL/100 g). Drop

eye with local anesthetic proparacaine hydrochloride, then disinfect eye area with iodine volt. Under the microscope, the subconjunctival Tenon's capsule tissue was removed. After soaked in sterile phosphate buffered solution (PBS) solution containing double antibodies for about 30min, the tissue was transferred to the ultra-clean platform. Washing the tissue twice with PBS and shred them with an ophthalmic scissors. Finally, place the tissue fragments in a centrifuge tube. Primary cells were extracted by trypsin digestion: Pancreatic enzymes with a volume of 5 to 10 times of tissue fragments were added to centrifuge tube (0.25%), digest about 18min in 37℃ water pot until the tissue becomes flocculent and floats in the trypsin. For terminate digestion, added 8 mL Dulbecco's modified eagle media (DMEM) medium containing 15% fetal bovine serum (FBS). After repeated gentle blowing and beating, cell suspension was divided into equal parts and put into a cell culture bottle. Cells were cultivated in 37℃ containing 5% CO_2 incubator, exchange of the cell medium after every 3-4d, inoculated cells can be full of culture bottle about 10 to 15d.

SD Rat Tenon's Capsule Fibroblast Identification After the rat Tenon's cystic fibroblast slide was taken, the fixed slides were rinsed with PBS for 3 times. After the glass slide was dried, the cells were evenly distributed in the cover glass slide with the histochemical pen to circle the appropriate range, and the membrane breaking liquid was added to 50-100 μL, and incubated at room temperature for 20min. Cell slides were removed and rinsed with PBS for 3 times. The slides were placed in 5% milk and sealed at room temperature for 1h. Then slides into a diluted with PBS containing 1% bovine serum albumin (BSA) resistance of incubation, 4℃ for the night. Cell slides were taken out the next day and rinsed with PBS on a shaker for 3 times. After a little drying, the secondary antibody corresponding to the primary antibody was added to the circle in the brush group, and the cell slide was incubated at room temperature for 50min. The cell slide was removed and rinsed with PBS for 3 times on the shaking table. After the glass slide was dried, 4',6-diamidino-2-phenylindole (DAPI) dye solution was added to the circle and incubated at room temperature for 10min under dark conditions. Cell slides were removed and rinsed with PBS on a shaker for 3 times, 5min each. The slides were dried and sealed with anti-fluorescence quenching sealing tablets. The slides were observed under an inverted fluorescence microscope and the images were taken.

Western Blot Analysis of TLR4, Transforming Growth Factor-β1 Protein After the primary cells of Tenon's sac were cultured in groups, the total protein was extracted, and then the TLR4 protein expression was detected by Western blot. Western blot was used to detect the expression of transforming growth fautor (TGF)-β1 protein in three groups of cells by the same method.

Enzyme Linked Immunosorbent Assay After administration of Tenon's cystic fibroblasts in groups, the content of IL-6 in cell supernatants of each group was detected by enzyme linked immunosorbent assay (ELISA) kit.

Reverse Transcriptase-Polymerase Chain Reaction The relative transcriptional levels of IL-6 in the three groups of cells were further determined. Total RNA was extracted from Tenon's cystic fibroblasts after grouping. The expression of IL-6 was detected by reverse transcriptase-polymerase chain reaction (RT-PCR).

Cell Counting Kit-8 Detect the Proliferation Activity of the Three Groups Tenon's capsule fibroblasts were inoculated into 96-well plate. After cell adherence, the inoculated cells were divided into three groups: normal control group, lipopolysaccharide (LPS) group and TAK-242 group. The normal control group did not do any treatment. The LPS group was treated with 10 μg/mL LPS. The TAK-242 group was treated with 1 μmol/L TAK-242. Next, cell counting kit-8 (CCK-8) was administered and cultured in the absence of light. After a series of operations, the OD values of the three groups were detected by enzyme-labelled instrument.

RESULTS

Morphologic Observation Microscopically, small pieces of tissue digested by trypsin as chylous are seen scattered in the medium, the cells dissociate from the tissue in a clear, round shape. On the second day, a small number of cells start to adhere to the wall and continue to move out of the tissue mass. After adherent to the wall, the cells become elongated fusiform, divide and proliferate. About 10d after inoculation, when the cells covered 75%-80% of the bottle bottom, the cells were digested, centrifuged and subcultured. After passage, most of the cells were elongated fusiform and distributed in vortices, and some cells were irregular in shape. The cell morphology is shown in Figure 1. The results showed that the cells accord with the morphology of Tenon's capsule fibroblasts.

Cell Identification Cy3-labeled keratin should be red fluorescence, FITC-labeled vimentin should be green fluorescence, and DAPI labeled nucleus should be blue fluorescence (Figure 2A). Cy3 labeled keratin does not colored (Figure 2D). The nuclei of DAPI labeled cells showed blue fluorescence (Figure 2B, 2E). The immunofluorescence results showed that the primary cells extracted in the experiment expressed vimentin but not keratin, which was consistent with the biological characteristics of Tenon's capsule fibroblasts and was Tenon's capsule fibroblasts of SD rats.

Western Blot It was found that the TLR4 protein expression level of LPS group was significantly higher than that of normal control group and TAK-242 group. The relative gray value of each band was determined and statistically analyzed (Figure 3). The results showed that LPS could cause the increase of TLR4

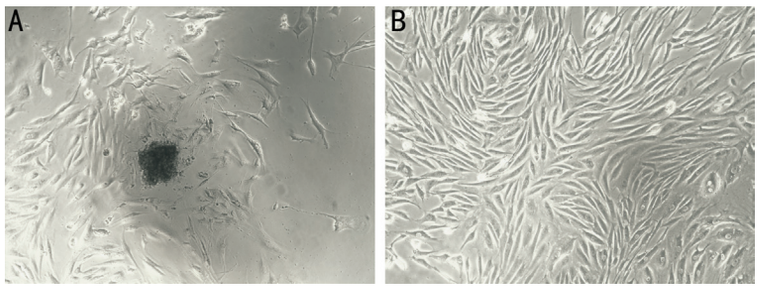

Figure 1 Primary culture of SD rat Tenon's capsule fibroblasts (100×) A: In the center, tissue fragments digested by trypsin. The cells migrate from the tissue fragments and divide and proliferate. The cells are spindle shaped or spindle-shaped, and a few are triangular. B: The cells are arranged in bundles or spirals after confluence.

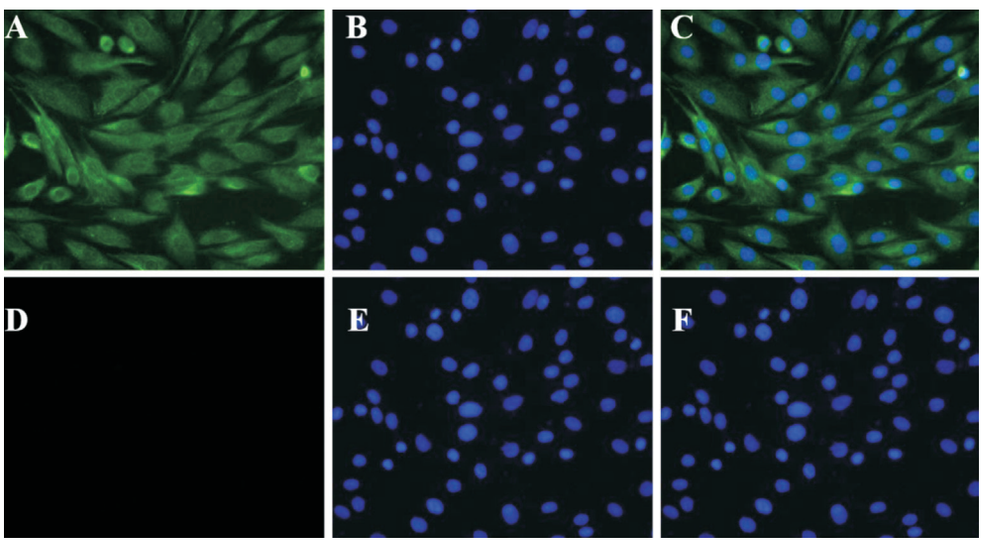

Figure 2 Identification of keratin vimentin: double immunofluorescent labeling (400×) A-C: Vimentin staining was positive, green fluorescence was seen in the cytoplasm (A), blue fluorescence was seen in the nucleus (B), and C was co-development of cytoplasm and nucleus. D-F: Keratin staining was negative, cytoplasm red fluorescence was not visible (D), the nucleus was blue fluorescence (E), and only the nucleus fluorescence could be seen in the cytoplasm co-development (F).

protein expression, while TAK-242 could significantly inhibit the increase of TLR4 protein expression induced by LPS. The expression levels of TGF-β1 protein in the LPS group were significantly higher than those in the normal control group and the TAK-242 group. We measured the relative gray value of each band and analyzed statistically (Figure 4). The results showed that LPS can increase the expression of TGF-β1 protein, while TAK-242 can significantly inhibit the expression of TGF-β1 protein induced by LPS, suggesting that TAK-242 can inhibit the release of inflammatory factors downstream of TLR4 signal path.

Enzyme Linked Immunosorbent Assay The content of IL-6 in each group was detected by ELISA. After LPS stimulation, the content of IL-6 in the supernatant was significantly higher than that in the normal control group. Compared with the normal control group, there was no significant change in IL-6 content in TAK-242 group (Figure 5). The results showed that TAK-242 could effectively block the TLR4 signal path in Tenon's capsule fibroblasts, thereby inhibiting the secretion of the inflammatory factor IL-6 induced by LPS stimulation.

Reverse Transcriptase-Polymerase Chain Reaction The relative transcription levels of IL-6 mRNA in three groups of cells were further detected. After grouping Tenon's capsule fibroblasts and total RNA was extracted, IL-6 mRNA expression was detected by RT-PCR in each group. The results showed that the relative expression of IL-6 mRNA in LPS group was significantly higher than that in normal group and TAK-242 group, and the relative expression level of lL-6 mRNA in TAK-242 group was significantly higher than that in normal control group (Figure 6).

Cell Counting Kit-8 Tenon's cystic fibroblasts were inoculated into 96-well plates, and the inoculated cells were divided into three groups: normal control group, LPS group, and TAK-242 group. After pre-culture, groups were treated differently. 1) The normal control group did not do any treatment; 2) The LPS group add LPS of 10 μg/mL; 3) The TAK-242 group add TAK-242 of 10 μg/mL. After a series of operations, such as CCK-8 administration and light-avoiding culture, OD values of the three groups were detected by microplate reader. CCK-8 is based on the principle that,

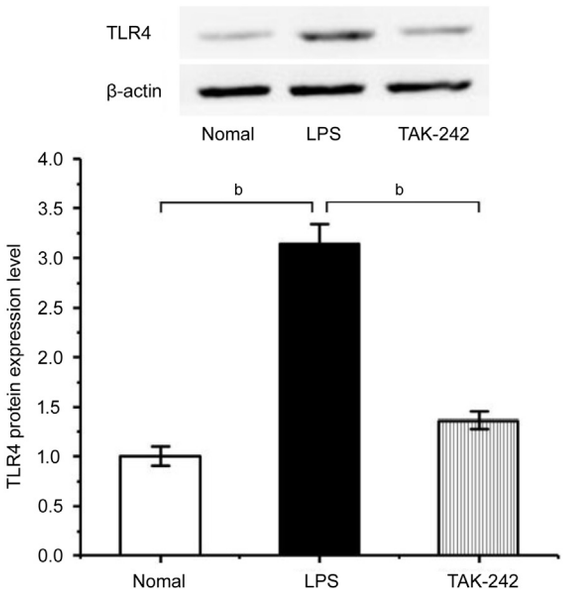

Figure 3 Expression of TLR4 protein in each group was detected by Western blot After LPS intervention, TLR4 protein expression level in the LPS group increased significantly compared with that in the normal control group (^bP<0.01). In the TAK-242 group, TLR4 protein expression was significantly lower than that in the LPS group due to the intervention of TAK-242 (^bP<0.01). There was no significant difference between TAK-242 group and normal control group (P>0.05).

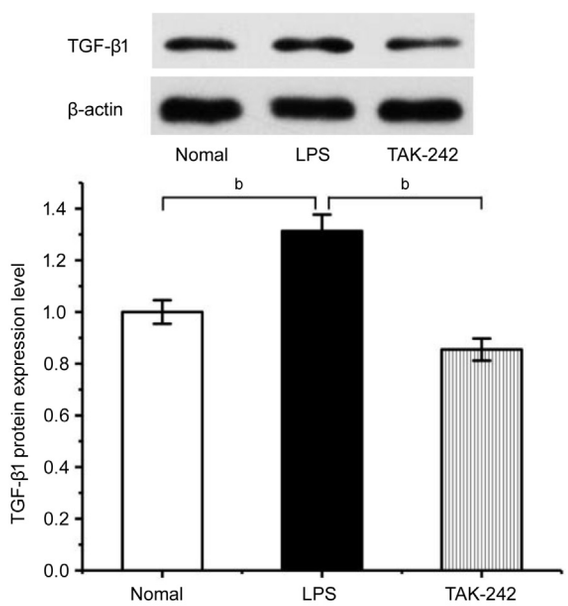

Figure 4 Western blot analysis of protein expression of TGF-β1 in each group After the intervention of LPS, the protein expression level of TGF-β1 in the LPS group was significantly increased compared with that of the normal control group (^bP<0.01). In the TAK-242 group, the protein expression of TGF-β1 was significantly lower than that of the LPS group due to the intervention of TAK-242 (^bP<0.01). However, compared with the normal control group, the expression level of TGF-β1 protein in TAK-242 group decreased slightly, and the difference have no statistics significance (P>0.05).

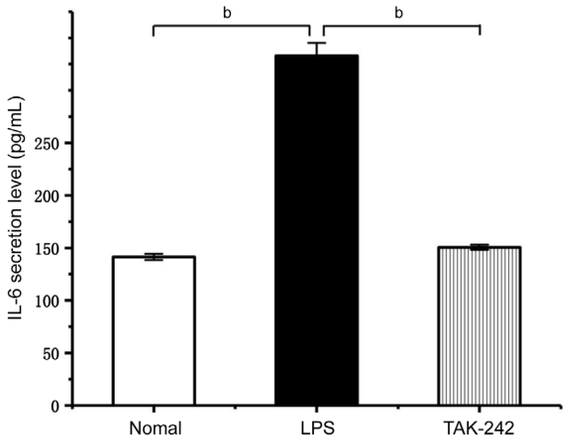

Figure 5 ELISA detect the secretion level of IL-6 in each group After LPS intervention, IL-6 secretion level in the LPS group was significantly increased compared with that in the normal control group (^bP<0.01). In the TAK-242 group, IL-6 secretion level was significantly lower than that in the LPS group due to the intervention of TAK-242 (^bP<0.01). There was no significant difference in IL-6 secretion level between the TAK-242 group and the normal control group (P>0.05).

under the action of the carrier, methyl thiazolyl tetrazolium-Water-Soluble Tetrazolium-8 (WST-8) is reduced to yellow formazan (Formazen dye) by cellular dehydrogenase in the mitochondria, and the number of formazan molecules generated, namely the gradation of color, is used to reflect the number of living cells. The results showed that OD value of LPS group was significantly higher than that of normal control group and TAK-242 group (P<0.05), while OD value of TAK-242 group was slightly higher than that of LPS group, but the difference was unobvious (P<0.05; Figure 7). This indicated that TAK-242 played a role in inhibiting cell proliferation induced by LPS.

DISCUSSION

Glaucoma is one of the leading causes of irreversible blindness worldwide. Glaucoma filtering operation remains the gold standard for glaucoma in patients who cannot control IOP with medication or laser therapy. The success rate of glaucoma filtering operation is often limited by postoperative scarring of the filtration tract[10]. The excessive synthesis of extracellular matrix (ECM) and the scar formed by the contraction of subconjunctival tissue block the filtration channel and prevent the outflow of aqueous humor, leading to the increase of IOP and the failure of surgery ultimately[11]. Mitomycin C, 5-fluorouracil and other antimetabolites can inhibit fibroblast proliferation and reduce postoperative scar formation to a certain extent. However, these drugs can cause more postoperative complications, such as long-term low IOP, corneal epithelial defect, conjunctival wound leakage, follicular infection, endophthalmitis and so on[12-13]. Therefore,

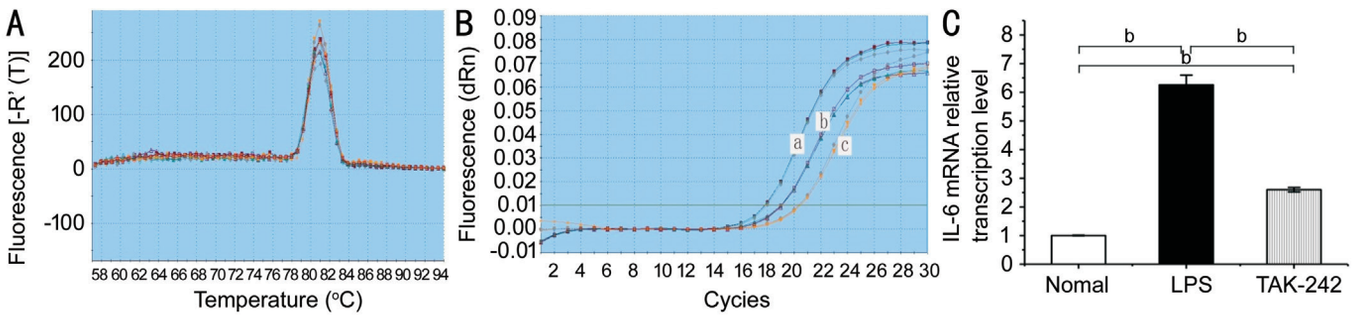

Figure 6 RT-PCR detect the relative transcription levels of IL-6 mRNA in each group A: Solubility curve. B: The amplification curve: a stands for LPS group; b stands for group TAK-242; c is the normal control group. All curves are unimodal distribution and the peak is concentrated. C: After the intervention of LPS, the relative transcription level of IL-6 mRNA in the LPS group was significantly raised compared with that in normal control group (^bP<0.01). In the TAK-242 group, the relative transcription level of Il-6 mRNA was significantly lower than that in the LPS group due to the intervention of TAK-242 (^bP<0.01). Compared with the normal control group, the relative transcription level of IL-6 mRNA increased in the TAK-242 group (^bP<0.01).

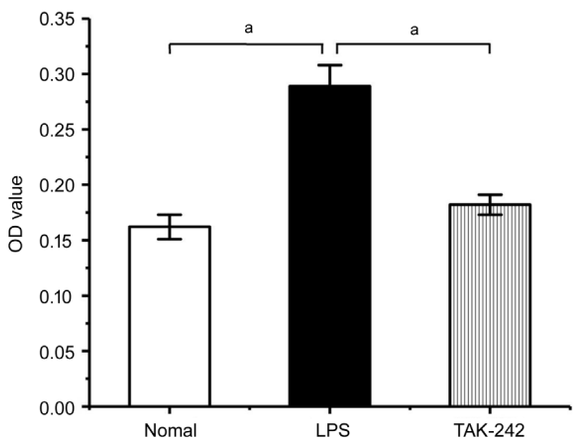

Figure 7 CCK-8 detect cell proliferation in each group OD value in the LPS group increased significantly compared with that in the normal control group after LPS intervention (^aP<0.05). In the TAK-242 group, OD value decreased significantly compared with LPS group due to the intervention of TAK-242 (^aP<0.05). However, there was no significant difference in OD value between the TAK-242 group and the normal control group (P>0.05).

we need a safer and more effective drug to fight the scarring after GFS.

TAK-242 is a cyclohexene compound that can specifically inhibit the TLR4 signal path. Although some synthetic lipid A analogues have been reported to act as LPS or TLR4 antagonists, TAK-242 is the first small molecule compound to inhibit the production of TLR4-mediated cytokines selectively[14-15]. The action principle is inhibiting TLR4 signal transduction by Cys747 binding to the toll-interleukin-1 receptor (TIR) domain of target protein TLR4 and disrupting the interaction between TLR4 and its downstream adaptor molecule TIRAP containing TIR domain and inducing the interaction between TLR4 and its downstream adaptor molecule TRIF-related adaptor protein (TRAM) containing interferon beta TIR domain[16]. The TLRs is a pattern recognition receptor that recognizes specific pathogen-associated molecular patterns (PAMPs) that are present in pathogens but not in mammalian cells. TLR is known to be highly expressed in innate immune cells in response to pathogens and environmental stress, to participate in the detection of foreign pathogens (bacteria, viruses or fungi) and to regulate innate and immunological adaptive response[17-18], thus becoming the first line of defense against infection. TLR4, as a member of the family, plays a key role in infection and fibrosis. It is a type I transmembrane protein, which is composed of three parts: extracellular domain, transmembrane domain and intracellular domain[19]. TLR4 signal pathway transduction mainly includes MyD88-dependent pathway and MyD88-non-dependent pathway, the former is not dependent on MyD88 protein activation, the latter is mainly dependent on MyD88 activation to induce the rapid production of inflammatory factors[19]. When the activation pathway of TLR4 is poorly regulated, it will lead to further development of the disease. For example, Astafurov *et al*[9] found in their study that TLR4 signal transduction and complement system up-regulation in glaucoma animal models would further lead to optic nerve atrophy, aggravating the degeneration of optic nerve in model animals. The level of TLR4 and its endogenous ligands is increased in systemic sclerosis (SSc), and it has a strong and effective stimulation effect on the expression of fibrosis genes. Genetic differences of TLR4 or its endogenous ligands can be targeted to improve the experimental fibrosis in the SSc model of mice[20-22]. In liver injury, blocking the TLR4 signaling pathway can inhibit the transformation of Hepatic stellate cell (HSC) into myofibroblasts and the production of type 1 gels, thereby inhibiting the formation of liver fibrosis. Studies have shown that TLR4 genetically deficient mice can also fight experimental liver fibrosis[9,23]. There is increasing evidence that TLR4 is an important player in the development

and progression of inflammatory and fibrotic diseases and an excellent therapeutic target for fibrotic diseases. We hypothesized that drugs targeting the inhibition of TLR4 may be of great significance in the prevention and treatment of glaucoma filtration tract scarring.

Fort *et al*[15] found that TAK-242 inhibited the production of LPS-induced inflammatory mediators such as tumor necrosis factor-α (TNF-α), IL-1β, IL-6 and nitric oxide (NO) at similar concentrations. In addition, the inhibitory effect of TAK-242 on cytokines was similar in both mouse and human macrophages, which may indicate that the difference in species does not have much impact on the efficacy of TAK-242. Zhang *et al*[20] found that TAK-242 was significantly effective against aldosterone-induced cardiac remodeling and renal fibrosis. In this study, we investigated the effect of TLR4 specific blocker TAK-242 on inhibiting the proliferation of rat eye Tenon's capsule fibroblasts.

Gram-negative bacteria LPS is a recognized ligand of TLR4, which can activate tlr4-mediated signaling pathways. Since Molteni *et al*[24] discovered the role of TLR4 as LPS sensor of bacteria, other endogenous and exogenous ligands have been discovered successively. PAMPs are isolated from bacteria, viruses, fungi, plants and cyanobacteria. Most of them are TLR4/(myeloid differential protein-2) MD2 complex agonists, and a few have blocking effect. There are two main types of damage-associated molecular patterns (DAMPs): 1) molecules originating from the ECM; 2) intracellular mediators are released or actively secreted by cells[24-27]. There is no doubt that endogenous molecules can induce pro-inflammatory responses through TLR4. Under normal conditions, hyaluronic acid exists in tissues in the form of a polymer. After tissue damage, it is decomposed into small fragments, which can activate macrophages in vivo and in vitro through TLR4[28]. Endogenous TLR4 intracellular triggers include: DNA binding protein high mobility group protein 1 (HMGB1) and cellular heat shock protein (HSP). After cell injury and necrosis, these molecules are released into the extracellular environment, thus causing a strong TLR4-mediated pro-inflammatory response[24]. In addition to its role in aseptic inflammation, HMGB1 is also actively released after exposure of immunoactive cells to pathogen products, so it is a common mediator between infectious and non-infectious inflammatory responses[24]. Studies have shown that Tenon's capsule fibroblasts are the main components of scar formation after glaucoma filtration, and the excessive proliferation and fibrosis main causes of scarring in the filter passage[29]. Therefore, the successful extraction and culture of SD rat Tenon's capsule fibroblasts are the basis of our follow-up study on the scar formation of the filter passage. In this study, we extracted Tenon's capsule subconjunctival tissue of SD rats and successfully isolated the primary cells with elongated spindle shape and fascicular arrangement by trypsin digestion. Combined with the morphology and arrangement of primary cells, these were identified by immunofluorescence method as Tenon's capsule fibroblasts. In the subsequent experiments, we selected cells with better vitality of 3-5 generations. The LPS was selected as the TLR4 signaling pathway agonist. Investigating the effect of TAK-242 on inhibition of mouse eye Tenon's capsule fibroblast proliferation, we combined action of LPS and TAK-242.

As mentioned above, considering the important role of TLR4 in fibrosis, this experiment detected the effect of LPS and TAK-242 on TLR4 protein expression in Tenon's capsule fibroblasts. As in previous studies, we found that TAK-242 significantly inhibited the expression of TLR4[30]. The results of this experiment showed that TLR4 protein expression level of Tenon's capsule fibroblasts were significantly increased after LPS stimulation, while TAK-242 could inhibit the increased protein expression caused by LPS. Combined with the above experimental results, we found that TAK-242 not only lower the TLR4 signaling pathway of Tenon's capsule fibroblasts, but also inhibited the expression of TLR4 protein itself increased by LPS.

The wound healing response at the subconjunctival follicular site is mediated in part by matrix metalloproteinases (MMPs) and is regulated by a variety of molecules including growth factors and inflammatory mediators. Cytokine TGF-β is a key regulator of wound healing and fibrosis and a major driver of conjunctival scar formation[31]. Both TGF-β1 and TGF-β2 can be detected on subconjunctival wounds after GFS[32]. Induction of collagen gel contraction by TGF-β1 and conversion of fibroblasts into myofibroblasts play an important role in scar formation and contraction of surgical incision[11]. Therefore, these factors have become an important target of the development of anti-fibrosis programs. Stifano *et al*[33] found in their experiments that TLR4 and its assistant receptors MD2 and CD14 were overexpressed in the dermal lesions of patients with diffuse skin SSc, and that inflammatory chemokines were overexpressed and TGF-β genes were raised in their chronic skin LPS exposure model. Stifano *et al*[33] found in their experiments that TLR4 and its assistant receptors MD2 and CD14 were overexpressed in the dermal lesions of patients with diffuse SSc, and that inflammatory chemokines were overexpressed and TGF-β genes were raise in their chronic skin LPS stimulation model. Seki *et al*[23] also suggested that LPS could enhance signal transduction at TGF-β by activating the TLR4-MyD88-NF-κB axis and thereby promote the development of inflammation and fibrosis.

Our results indicate that LPS induces increased secretion of TGF-β1 in Tenon's capsule fibroblasts, which can be inhibited

by TLR4 specific blocker TAK-242. This is consistent with the trend obtained by Stifano *et al*[33] and Seki *et al*[6] in the experimental study of fibrosis. Thus, TAK-242 can inhibit TGF-β1 mediated by TLR4 signaling pathway, and playing an important role in inhibiting the proliferation of Tenon's capsule fibroblasts.

IL-6 is a multifunctional monomolecular glycoprotein cytokine with extensive biological activities and plays an important role in the occurrence and development of multiple diseases. It has the function of promoting the proliferation of fibroblast B cells, T cells and other cells. Studies have found that IL-6 is closely related to the occurrence and development of periodontitis, oral lichen planus, renal fibrosis, liver fibrosis and other inflammation and fibrosis diseases[34-35]. For example, studies have mentioned that LPS directly acts on periodontal fibroblasts, which, as helper cells of immune response, produce inflammatory cytokines such as IL-6, TGF-β1, TNF-α and so on, mediate inflammatory reactions and participate in the destruction process of periodontal tissue, thus participating in the whole process of the occurrence and development of periodontitis. IL-6 plays an important role in the histopathological damage of periodontitis caused by LPS[35-36]. Other studies have reported that IL-6 can promote fibroblast proliferation and mRNA expression of collagen I and collagen III. IL-6 can also enhance the expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) mRNA, inhibit collagenase activity and promote the expression of α2-macroglobulin in monocytes, thereby reducing the decomposition of ECM. In addition, inflammatory cytokines IL-6 can also stimulate cells to release more cytokines such as TGF and PDGF, which can play a positive feedback amplification effect, produce more ECM and aggravate the course of fibrosis[37-38].

In this experiment, the cells were divided into normal control group, LPS group and TAK-242 group, and different treatments were taken respectively. Then, the secretion of IL-6 in the cell culture medium of each group was detected by ELISA kit. The results showed that IL-6 secretion of Tenon's cystic fibroblasts induced by LPS could be significantly inhibited by TAK-242. We also used RT-PCR to detect the secretion of IL-6 mRNA at the RNA molecular level, and the results showed that the expression level of IL-6 mRNA in Tenon's capsule fibroblasts induced by LPS increased and could be inhibited by TAK-242 similarly. This further confirmed the inhibitory effect of TLR4 specific blocker TAK-242 on the release of inflammatory factors induced by LPS activation of TLR4 pathway.

CCK-8, the principle of which is in carrier under the action of tetrazolium salt-WST-8, all referred to as: 2-(2-methoxy-4-(phenyl)-3-[4-(phenyl)-5-(2, 4-disulfonic acid benzene)-2h-tetrazolium monosodium salt)], by reduction of cells in mitochondria dehydrogenase yellow formazan (Formazen dye), which used to generate a formazans is the number of gradation of color to reflect the number of living cells. In recent years, CCK-8 kit, as a highly sensitive, fast, safe and effective method, is often used to detect the toxic effects of cell proliferation[39]. The absorbance value (OD value) detected by the enzyme marker in the experiment represents the lactate dehydrogenase (LDH) activity of lactate dehydrogenase in mitochondria, which can reflect the degree of cell proliferation, the rate of death and the metabolic rate. Compared with the traditional 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, CCK-8 kit method makes the experimental operation easier, the experimental process faster and the experimental results more accurate[40-41]. Therefore, CCK-8 kit was used in this experiment to measure the degree of cell proliferation in the three groups.

In this experiment, CCK-8 kit was used to detect the degree of proliferation of Tenon's capsule fibroblasts in three groups: normal control group, LPS group and TAK-242 group. The results showed that TAK-242 significantly inhibited cell proliferation induced by LPS. This indicated that in vitro exposure to LPS can cause significant proliferation of Tenon's capsule fibroblasts, and such a degree of cell proliferation can be inhibited by TLR4 specific blocker TAK-242.

In conclusion, TAK-242 can inhibit the proliferation and release of inflammatory cytokines in Tenon's capsule fibroblasts induced by proinflammatory factor LPS. TAK-242 acts as an effective specific TLR4 inhibitor, and this inhibition is accomplished by antagonistic to the up-regulated TLR4 signaling pathway. TAK-242 provides a new method to solve the scarring of glaucoma filter passage in the future.

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Conflicts of Interest: Liang L, None; **Zhu MN,** None; **Chen BJ,** None; **Wang Z,** None; **He LY,** None; **Zhang R,** None.

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