• Basic Research •

Protection of tight junction between RPE cells with tissue factor targeting peptide

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Abstract

• AIM: To investigate the effect of tissue factor targeting peptide (TF-TP) on retinal pigment epithelium (RPE) cells tight junctions.

• METHODS: Cell counting kit-8 (CCK-8) was used to measure the proliferation of ARPE-19 cells. Expression of tight junction, ZO-1 in ARPE-19 cells was measured by Western blot and immunofluorescent staining. Western blot was also used to detect the expression of tissue factor (TF). CEC Transmigration Assay was used to measure the migration of ARPE-19 cells. The transport of fluorescent markers [fluorescein isothiocyanate dextrans of 4, 10, 20 (FD4, FD10, FD20)] and the transepithelial electrical resistance (TEER) were used to measure in ARPE-19 cell.

• RESULTS: CCK-8 assay showed that 5 μmol/L TF-TP can inhibit ARPE-19 cells abnormally proliferation stimulated by lipopolysaccharide (LPS; *P*<0.05). LPS increased the transport of fluorescent markers (FD4, FD10, FD20) and decreased TEER levels in ARPE-19 cells, respectively, which were prevented by 5 μmol/L TF-TP pretreatment (*P*<0.05). Furthermore, LPS significantly up-regulated the expression of TF and downregulated the expression of ZO-1 (*P*<0.05) in ARPE-19 cell which was inhibited by the TF-TP (*P*<0.05). In addition, TF-TP inhibited the abnormal migration induced by LPS in ARPE-19 cell (*P*<0.05).

• CONCLUSION: Our findings suggest that TF-TP suppressed proliferation and migration of ARPE-19 cells induced by LPS, and maintained the RPE tight junctions through inhibition of TF expression and increased expression of ZO-1.

• KEYWORDS: tissue factor targeting peptide; lipopolysaccharide; tight junction; ZO-1; retinal pigment epithelium

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INTRODUCTION

Choroidal neovascularization (CNV) is the leading cause of irreversible blindness in industrialized countries[1]. CNV appears as a neovascular sprout growing under or through the retinal pigment epithelium (RPE) through breaks in Bruch's membrane[2]. It is known that vascular endothelial growth factor (VEGF), oxygen deprivation, inflammation, and other stimuli play pivotal roles in the development of CNV[2]. Although the intravitreal injection of anti-VEGF agents have been applied in patients with CNV, one-sixth of treated patients still progress to legal blindness[3-4]. Thus, more applicable therapies for CNV are desirable.

Tissue factor (TF), a 47-kDa transmembrane glycoprotein, is the main initiator of the blood coagulation cascade. TF, also known as coagulation factor III, is one of the key mediators of physiological and pathological angiogenesis. TF is expressed in pathological blood vessels, but not in the normal blood vessels[5]. In addition to hemostasis, the TF/VIIa complex could not only mediate intracellular signaling through protease-activated-receptor, but also promote inflammation[6-7], oxidative stress[8-10] and angiogenesis[11-14] which has been implicated in the pathogenesis of CNV. More enhanced expression of TF was observed in the age-related macular degeneration (AMD) with CNV compared to the non-CNV AMD retina[12]. Studies have found a link between abnormal TF expression and TNF-α, IL-1, IL-6, C5a, and many other inflammation factors stimulated by lipopolysaccharide (LPS)[6]. It was also reported that TF siRNA could inhibit the proliferation and migration of endothelial cell *in vitro* models of neovascularization induced by LPS[15]. The suppression of TF by intravitreal injection of anti-TF monoclonal antibody significantly ameliorated CNV in the mouse CNV model[16]. However, potential signaling pathways related to the effect of TF on the role of RPE cell

in the function of blood-retinal barrier (BRB) remain poorly defined.

Therefore, we hypothesis that tissue factor targeting peptide (TF-TP) will attenuate the deleterious effects induced by LPS in ARPE-19 cell. The present study demonstrated that TF-TP possibly through downregulating TF and enhancing the expression of ZO-1 in LPS-stimulated ARPE-19 cell to maintain the proper functions of blood-retinal barrier.

MATERIALS AND METHODS

Cell Culture ARPE-19 cells (ATCC, Manassas, VA, USA) were cultured in the cell medium supplemented with 10% fetal bovine serum (FBS; Gibco Invitrogen Corporation, Carlsbad, CA, USA) and maintained in a 95% air and 5% CO_2 humidified atmosphere at 37℃.

Stimulation of ARPE-19 Cells ARPE-19 cells grown in 25 cm^2 flasks were cultured in regular medium without FBS for 24h, then with or without TF-TP for 24h, and subsequently with 2 μg/mL LPS for 24h.

Cell Counting Kit-8 Assay The proliferation of ARPE-19 was assessed using cell Counting kit-8 (CCK-8; Sigma-Aldrich, St.Louis, MO, USA) assays. ARPE-19 cells were seeded into 96-well plates at a density of 5×10^3 cells/well for 24h. Of 10 μL CCK-8 mixture solution was added to each well, and the plates were incubated at 37℃ in a 5% CO_2 incubator for 1 to 1.5h until visual color conversion occurred after selected to drug treatment. The absorbance in each well at 450 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Transepithelial Electrical Resistance Following the previous studies[17-18], ARPE-19 cells were cultured in Transwell plates until the confluent monolayer achieved a transepithelial electrical resistance (TEER)>300 $\Omega\cdot cm^2$ (about 15-18d), demonstrating a tight monolayer. A voltmeter (Millicell-ERS; Millipore, USA) was used for TEER: TEER (ohms per square centimeter)=(Total resistance-Blank resistance) (ohms)×area (square centimeter).

Transport Studies in ARPE-19 Cells According to the previous studies[19], ARPE-19 cells were seeded in 24-well culture plates coated with poly-d-lysine at a density of 20 000 cells/insert with a medium change every other day. These cells were ashed three times with phosphate buffer saline (PBS) and pre-incubated with sodium transport buffer (142.9 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 1.8 mmol/L $CaCl_2$, and 20 mmol/L HEPES, adjusted to pH 7.4). PBS containing increasing molecular weight fluorescent dextrans (FDs; 25 mg/mL) were then added to the apical side: [fluorescein isothiocyanate dextrans of 4, 10, 20 (FD4, FD10, FD20)]. After 1h at 37℃, aliquot samples were detached without repeated handling. Different florescence of samples were determined using a fluoroskan ascent FL2.5 fluorometer. The following equation was to measure the apparent permeability coefficients (Papp) of different fluorescent agents: Papp (cm/s)=(dq/dt)×1/(A×C0), where dq is the amount of fluorescence in the basolateral side (milligrams per milliliter), dt is a function of time per second, A is the surface area of the inserts (0.64 cm^2), and C0 is the initial concentration of fluorescent applied in apical compartment (milligrams per milliliter).

Western Blot Analysis Treated ARPE-19 cells were lysed using a subcellualar protein fractionation kit for cultured cells (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as per manufacture's guidelines and proteins were collected. Sample preparation and the whole process was followed as described[20]. Of 20-50 μg samples were separated with SDS-PAGE, transferred to polyvinylidene fluoride (PDF) membrane and blocked with 5% defatted milk in PBS-Tween-20 for 1h at room temperature. Then the PDF membrane was incubated with primary antibody against ZO-1 and TF (Invitrogen, Carlsbad, CA, USA) at 4℃ overnight. After washed with PBS three times as described[20], the PDF membrane was incubated with second antibody conjugated horseradish peroxidase (Vector Laboratories, Inc., Burlingame, CA, USA) for 2h and scanned with the Odyssey infrared imaging system (LI-COR Bioscience).

Immunofluorescent Staining ARPE-19 cells were fixed in 4% paraformaldehyde for 10min and permeabilized with 0.3% Triton X-100 in PBS for 10min. After blocking with 3% albumin from bovine serum for 1h, cells were incubated with rabbit anti-ZO-1 antibodies (Invitrogen) overnight at 48℃. Then, cells were incubated with Alexa Fluor 488 goat anti-rabbit (1:200, Molecular Probes; Invitrogen) for 1h. In some experiments, cytoskeleton was labeled with Alexa Fluor 594 conjugated phalloidin (1:200; Invitrogen). Cell nuclei were stained with 40, 6-diamidino-2-phenylindole (DAPI)-containing VECTASHIELD antifade mounting medium (Vector Laboratories, Inc.) and digitally photographed using a LSM confocal microscope (Carl Zeiss, Jena, Germany).

CEC Transmigration Assay CEC Transmigration Assay across the ARPE-19 cell monolayer was measured as previously described[21]. ARPE-19 cells were plated onto the underside of 8 μm pore size Transwell (Corning), and treated with 250 μmol/L 8CPT-29O-Me-cAMP (Biolog) overnight. ARPE-19 cells were fluorescently-labeled (VybrantDio Cell-labeling solution; Invitrogen), and then plated in the top well of the inserts. After 48h, CECs which had migrated across the filter and the RPE, were counted per 20×field by fluorescence microscopy. All data present as mean 6 standard error of the mean (SEM) from 6 Transwells per condition.

Statistical Analysis All experiment data were expressed as the mean±SEM. Comparison between two groups was made using Student's *t*-test with the SPSS16.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance in every figure was as follows: $P<0.05$, significant; $P>0.05$, not significant.

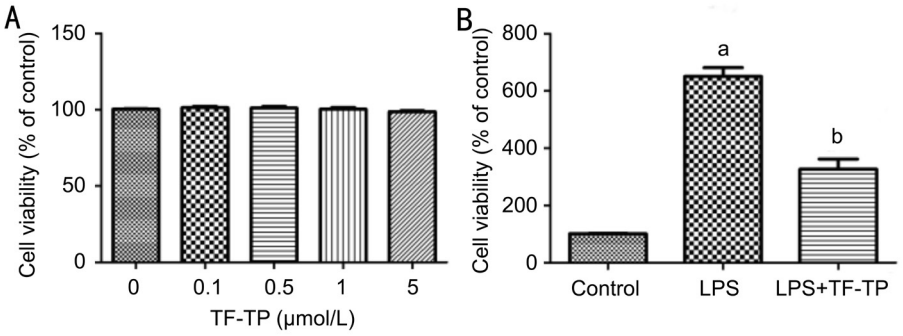

Figure 1 The cell proliferation was measured by CCK-8 A: ARPE-19 cells were treated with 0, 0.1, 0.5, 1 and 5 μmol/L of TF-TP for 24h; B: ARPE-19 cells were treated with 5 μmol/L of TF-TP for 24h and then stimulated with 2 μg/mL LPS for 24h. Data was shown as mean±SEM (n=3, ^aP<0.001 compared to control group; ^bP<0.01 compared to LPS group).

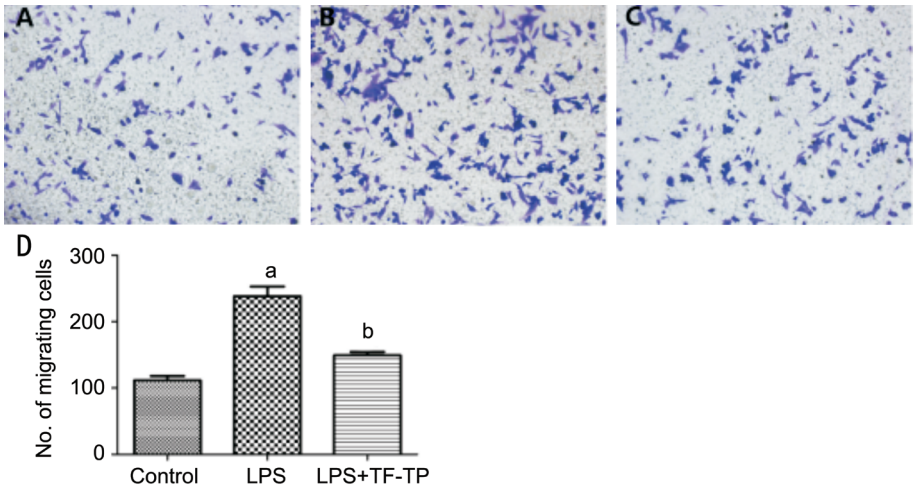

Figure 2 ARPE-19 cells were pre-treated with 5 μmol/L of TF-TP for 24h, following stimulated with 2 μg/mL LPS for 24h. The cell migration in the control group, TF-TP group and LPS group were compared by CEC Transmigration Assay A: The cell migration of the control group; B: The cell migration increasing was observed in ARPE-19 cells stimulated by LPS; C: The cell migration was significantly inhibited by TF-TP; D: Data was shown as mean±SEM (n=3, ^aP<0.001 compared to control group; ^bP<0.01 compared to LPS group).

RESULTS

Effect of TF-TP on LPS-induced ARPE-19 Cells Proliferation and Migration The cytotoxic test showed that no significant difference in cell viability was found after 24h of pre-treating with increasing concentration of TF-TP compared with the normal group (P>0.05; Figure 1A). CCK-8 assay was used to determine the inhibitory effects of 5 μmol/L TF-TP on LPS induced ARPE-19 cells proliferation. As shown in Figure 1B, the proliferation of ARPE-19 cells was significantly increased after stimulation with 2 μg/mL LPS for 24h, when compared to the control group. However, 5 μmol/L TF-TP inhibited LPS-induced ARPE-19 cells proliferation (P<0.05; Figure 1B). Therefore, 5 μmol/L TF-TP was used in the following experiments. Transwell assay was further used to determine the inhibitory effects of TF-TP on LPS-induced ARPE-19 cells migration. The ARPE-19 cells migration was notably increased after treatment with 2 μg/mL LPS, when compared to the control group. However, TF-TP significantly inhibited LPS-induced ARPE-19 cells migration. (P<0.05; Figure 2).

Effect of TF-TP on LPS-induced TEER Changes in ARPE-19 Cells We found that 5 μmol/L TF-TP produced strong effects on TEER (Figure 3). Compared with control group, TEER was reduced in ARPE-19 cells treated with LPS (P<0.001; Figure 3). Exposure to 5 μmol/L TF-TP significantly increased TEER in LPS-induced ARPE-19 cells, with the most substantial increase observed at 24h (P<0.05; Figure 3).

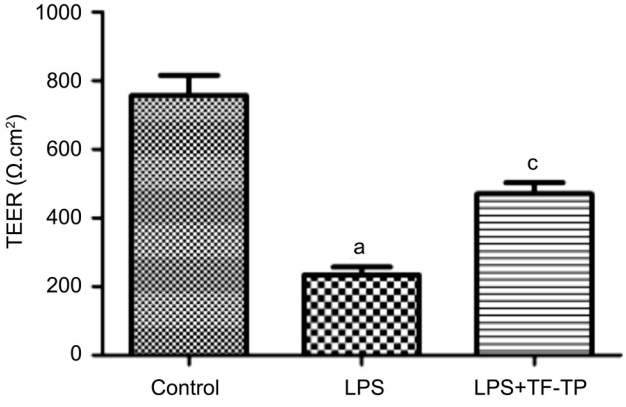

Figure 3 TEER changes induced in LPS group, control group and TF-TP group Data was shown as mean±SEM (n=3, ^aP<0.001 compared to control group; ^cP<0.05 compared to LPS group).

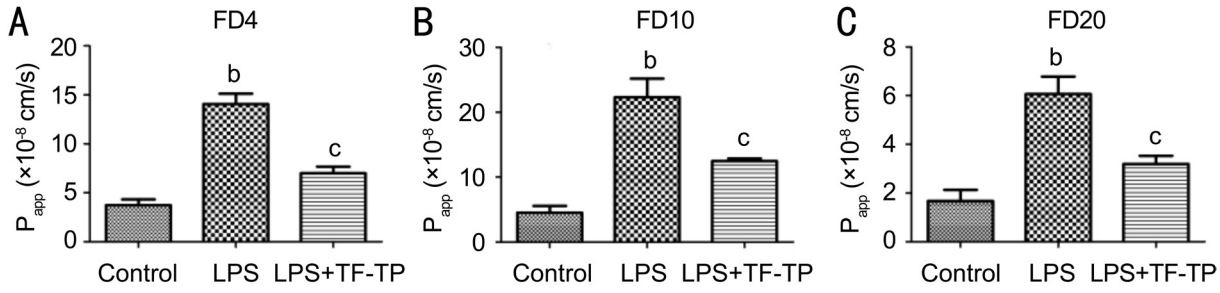

Figure 4 Effects of TF-TP on the paracellular transport (permeability coefficients) of FD4, FD10, FD20 Data was shown as mean±SEM (n=3, ^{b}P<0.01 compared to control group; ^{c}P<0.05 compared to LPS group).

Effect of TF-TP on LPS-induced Transport of Fluorescent Markers Changes in ARPE-19 Cells It was obvious that 5 μmol/L TF-TP significantly decreased transport of fluorescent markers: FD4 (Figure 4A), FD10 (Figure 4B) and FD20 (Figure 4C) compared with LPS group (P<0.05).

Effect of TF-TP on LPS-induced Up-regulation of Tissue Factor in ARPE-19 Cells Western-blot results demonstrated that TF expression level was significantly up-regulation in ARPE-19 cells treated with LPS, compared to control group (P<0.001; Figure 5). Figure 5 also showed the effects of various TF-TP concentrations (0.1, 0.5, 1 and 5 μmol/L) on the expression of TF by LPS-induced. TF-TP 0.1, 0.5 and 1 μmol/L did not alter the TF expression of the LPS group. While 5 μmol/L TF-TP significantly attenuated the up-regulation of TF protein after LPS-induced (P<0.01; Figure 5). Above all, the results of Figure 5 showed the obviously protection of TF-TP on the down-regulation of TF in the LPS-induced ARPE-19 cells.

Effect of TF-TP on LPS-induced Down-regulation of ZO-1 in ARPE-19 Cells It was obvious that ZO-1, tight junction protein level was reduced in ARPE-19 cells treated with LPS (P<0.01; Figure 6A, 6B). While pretreatment with 5 μmol/L TF-TP for 24h blocked the down-regulation of ZO-1 in the LPS-induced ARPE-19 cells (P<0.01; Figure 6A, 6B). Our immunofluorescent staining results (Figure 6C) also suggested significantly protective effects of TF-TP on the tight junction protein. This finding suggested the obviously protective effects of TF-TP on the intestinal barrier function in the LPS-induced ARPE-19 cells.

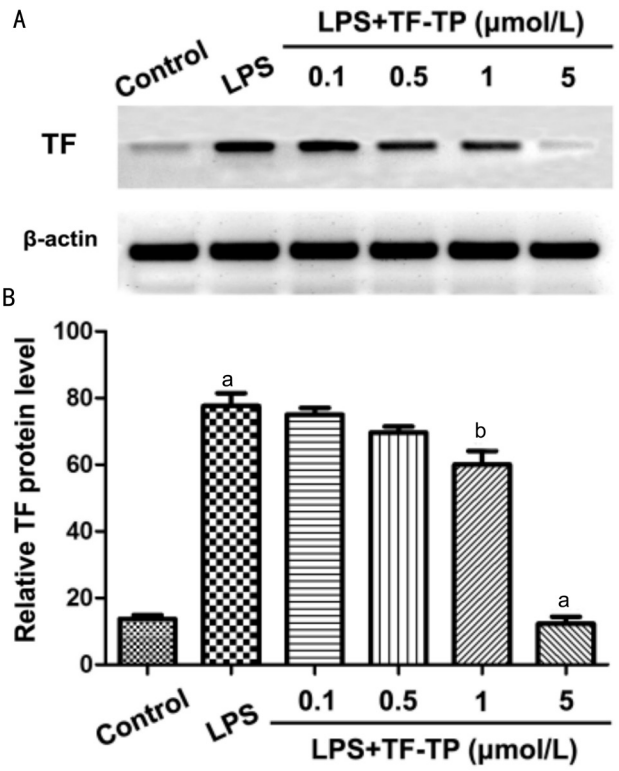

Figure 5 Protein levels of TF were evaluated by Western blot A: The effects of various TF-TP concentrations (0.1, 0.5, 1 and 5 μmol/L) on the expression of TF by LPS-induced; B: Data was shown as mean±SEM (n=3, ^{a}P<0.001 compared to control group; ^{b}P<0.01 compared to LPS group; ^{a}P<0.001 compared to LPS group and 0.1, 0.5, 1 μmol/L TF-TP group).

DISCUSSION

Tissue factor is not only a specific and high affinity receptor for factor VII/VIIa, but also promotes signaling activity and pleiotropic inflammatory responses *via* protease-activated receptors in concert with other coagulation factors exist in an encrypted form[22]. Many investigators have found the abnormal expression of TF in human CNV specimens and CNV models which reflects principally the neovascular component of AMD[12,23-25]. ARPE-19 cells are also used as an *in vitro* model to understand the microenvironment of AMD. Our results implicated that the TF expression level was significantly up-regulated and that TF-TP protected LPS-induced ARPE-19 cells. However, it remains unclear how TF-TP protected the ARPE-19 cells stimulated by LPS in relation with the expression of TF.

BRB consists of two components: the RPE forms the outer BRB, while the retinal vascular endothelium forms the inner BRB. Loss of barrier integrity precedes the development of BRB breakdown[26]. TF is expressed constitutively only on the surface of cells involved in the so-called “barrier functions”, such as smooth muscle cells, fibroblasts, pericytes, trophoblasts as well. TF is expressed in anatomic locations such as central nervous system, subendothelium, lung, placenta and in the eyes, *etc*[12,27]. Compared with the normal group, ARPE-19 cells subjected to 2 μg/mL LPS, as a chronic AMD model,

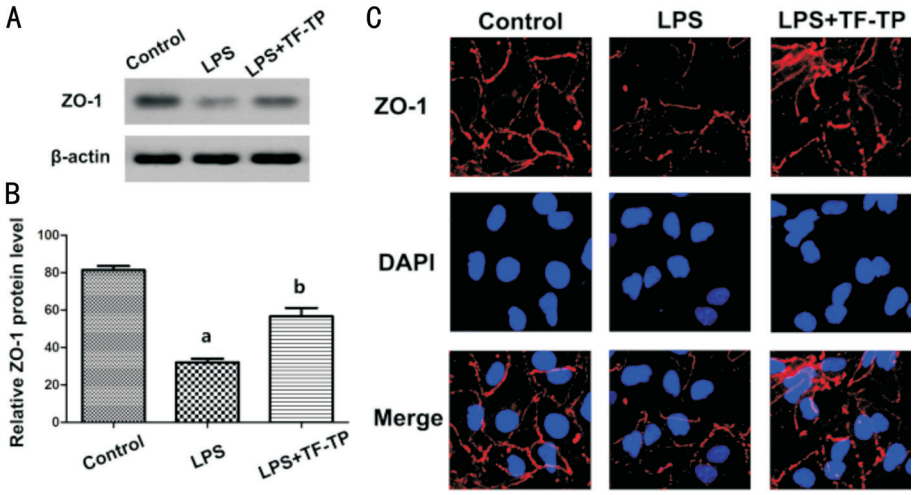

Figure 6 Expression of tight junction, ZO-1 in ARPE-19 cells was measured by Western blot and immunofluorescent staining A: Protein levels of ZO-1 were evaluated by Western blot; B: Data was shown as mean±SEM (n=3, ^aP<0.001 compared to control group; ^bP<0.01 compared to LPS group); C: Immunofluorescent staining showed the effects of TF-TP on the expression of ZO-1 exposed to LPS. The phenotype of nuclei were also investigated *via* DAPI staining.

exhibited a significant increase in viability and migration. While upregulated expression of fluorescent markers FD4, FD10, FD20 and reduction of TEER were observed in this chronic AMD model. Upon infection or inflammation, TF is readily induced on leukocytes that are usually devoid of surface TF. Stimuli triggering bloodborne TF expression during infection include pathogen associated molecular patterns, such as LPS and endogenous inflammatory cytokines[28]. Although the cellular viability of ARPE-19 cells was increased to this chronic AMD model by inflammatory mediator of LPS, the breakdown of tight junctions of RPE as reduction of TEER, increasing sizes of fluorescent markers and cell migration. Thus, it is necessary to maintain the PRE tight junctions and the integrity of BRB function, for to conserve the retina and choroid microenviroment. Polarity is an unusual property of ARPE cells that its apical surface was in close contact with a cellular layer of photoreceptors, whereas the apical sides of most other epithelial face a lumen devoid of neuronal cells. Polarized ARPE cells show apically located tight junctions and complex of perijunctional cytoskeleton composed of actin filaments with the appearance of a thick circumferential belt. ZO-1 is localized at an apical junction complex protein that participate in the development of the tight junctions and help to maintain interepithelial junctional integrity and permeability[29-31]. As we found a reduction of TEER, increasing sizes of fluorescent markers FD4, FD10, FD20 and cell migration in ARPE-19 cells stimulated to 2 μg/mL LPS. Then we discovered the significant downregulation of ZO-1 in ARPE-19 cell by LPS which was inhibited by the activation of TF-TP (Figure 6).

Accordingly, our findings suggested that TF-TP suppressed proliferation and migration of ARPE-19 cells induced by LPS, and maintained the RPE tight junctions through inhibition of TF expression and increased expression of ZO-1, further, maintained the proper functions of BRB. In the future, TF-TP may serve as a novel agent for the prevention and treatment of CNV. However, this study has some limitations. For instance, we have not investigated the effects of TF-TP on retinal vascular endothelial cells. Besides, the pathways of effects of TF-TP on ARPE-19 cells stimulated by LPS have not been explored. In summary, the detailed effects of TF-TP on the BRB still require further investigation.

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