



FULL LENGTH ARTICLE

Lipopolysaccharide-induced podocyte injury is regulated by calcineurin/NFAT and TLR4/MyD88/NF- κ B signaling pathways through angiopoietin-like protein 4

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Abstract Podocyte injury is an important cause of proteinuria. Angiopoietin-like protein 4 (Angptl4) is a secreted glycoprotein and has a role in proteinuria. However, the exact role of Angptl4 in podocyte injury and its upstream regulators has not been clarified. In this study, we used lipopolysaccharide (LPS)-induced mice and cultured podocytes as podocyte injury models. Our results indicated that LPS increased the expression of podocyte Angptl4 *in vivo* and *in vitro*. Furthermore, we showed that Angptl4 overexpression deteriorated LPS-induced

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Podocyte injury; TLR4/MyD88/NF- κ B

podocyte injury by inducing podocyte cytoskeleton rearrangement, reducing the expression of synaptopodin while Angptl4 knockdown alleviated LPS-induced podocyte injury. In addition, we found that inhibitors and siRNA targeting TLR4/MyD88/NF- κ B signaling inhibited the upregulation of Angptl4 in LPS-induced podocytes. Moreover, inhibitors and siRNA targeting calcineurin/NFAT signaling also relieved LPS-induced Angptl4 expression and podocyte injury *in vivo* and *in vitro*. Taken together, our study has elucidated that both of the TLR4/MyD88/NF- κ B and calcineurin/NFAT signaling mediate the upregulation of Angptl4 in LPS-induced podocytes, which has important implications for further understanding the molecular mechanism of LPS-induced podocyte injury.

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Introduction

Podocyte related nephropathy, including minimal change nephropathy (MCD), focal segmental glomerulosclerosis (FSGS), diabetic kidney disease (DKD) and membranous nephropathy (MN), are glomerular diseases characterized by structural and functional change of podocytes.^{1,2} Podocyte injury reduces the expression of podocyte-specific proteins synaptopodin and podocin, leading to the disorder of podocyte cytoskeleton and fusion of foot processes, resulting in proteinuria and ultimately renal dysfunction.³ The molecular mechanism of podocyte injury has been extensively studied. Recent studies indicated that molecules such as Angptl4, suPAR, B7-1 and ATF3 were implicated in podocyte injury.^{4–7} Ducasa GM et al demonstrated that reduced ATP-binding cassette A1 was involved in mitochondria dysfunction of podocytes, which was susceptible to the development of DKD.⁸ Wu J et al showed that decreased miR-30 in damaged podocytes, and therapies targeting miR-30 could alleviate podocyte injury in different levels.⁹ However, the molecular mechanisms are still complex, and need further exploration.

Angiopoietin-like protein 4 (Angptl4) is a member of the angiopoietin-like protein family, which mainly participates in lipid metabolism by suppressing the activity of lipoprotein lipase (LPL). Angptl4 plays a vital role in angiogenesis, cancer, and coronary heart disease.^{10–12} In addition, Angptl4 also participate in inflammatory diseases such as diabetes mellitus, rheumatoid arthritis, pneumonia, and atherosclerosis.^{13–16} It has been found that influenza viruses promoted the expression of Angptl4 in lung through the IL-6-STAT3 signaling pathway, thereby improved the leakage of lung tissue and accelerated lung injury.¹⁴ Nevertheless, Angptl4 played an anti-inflammatory role through macrophages in atherosclerosis, and they found that Angptl4 inhibited systemic inflammatory response induced by saturated fatty acids through inhibiting the absorption of fatty acids by macrophages of mesenteric lymph nodes.¹⁷ These results suggest that Angptl4 is closely related to inflammation and is an essential inflammatory regulator, but its role varied in different organs.

In the kidney, Clement LC et al firstly demonstrated that the upregulation of Angptl4 in podocytes was involved in the occurrence of proteinuria and hyperlipidemia,⁴ and therapies targeting podocyte Angptl4 reduced hyperlipidemia and proteinuria. In addition, podocyte Angptl4 also participated in membranous nephropathy and diabetic

nephropathy.^{18,19} Furthermore, there was also a significant correlation between Angptl4 gene polymorphism and proteinuria after renal transplantation.²⁰ However, the exact role of Angptl4 in podocyte injury and its upstream regulators has not been clarified.

To demonstrate the mechanism of Angptl4 in podocyte injury and its upstream regulators, we used LPS-induced model of podocyte injury *in vivo* and *in vitro*, which is expected to provide new ideas for understanding the mechanism of LPS-induced podocyte injury and treatment strategies.

Materials and methods

Animal experiments

Adult male BALB/c mice (20–25 g) were provided by the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). The procedures were accord with the Guidelines for the Care and Use of Laboratory Animals. Mice were randomly divided into four groups. For the podocyte injury model, mice were administrated with a single intraperitoneal injection of LPS (10 mg/kg body weight, L2880, Sigma). For the LPS plus FK506 treatment group, FK506 (Fujisawa Pharmaceutical, Japan) was intragastrically administrated at the dose of 2 mg/kg at the same time with LPS injection. For the LPS plus CsA treatment group, CsA (Novartis Pharmaceutical, Switzerland) was intraperitoneally injected at the dose of 20 mg/kg at the same time with LPS injection. The control mice were administrated the same volume of 0.9% saline. After 24 h, the mice were anesthetized with pentobarbital sodium, serum and urine were subsequently collected.

Transmission electron microscopy and measurement of foot process width

Renal cortex samples (1 mm³) were fixed in 2.5% glutaraldehyde, detailed steps for transmission electron microscopy, and the measurement of foot process width was referred to as previous literature.²¹

Isolation of mouse glomeruli

Adult male BALB/c mice were anesthetized and perfused with 3 mg/ml Fe₃O₄ diluted in PBS via the left ventricle.

The kidneys were decapsulated, cut into 1 mm³ pieces, and digested in 2 mg/ml collagenase A in water bath at 37 °C for about 40 min with gentle shake. The digested kidney tissues were re-suspended in 5 ml of cold HBSS and then pressed through a 100 µm cell strainer. The cell suspension was centrifuged at 800 rpm for 5 min. After that, the cell pellet was re-suspended in 1 ml of HBSS and gathered by a magnet. The gathered mouse glomeruli were examined with microscopy (Leica DMLB, Wetzlar, Germany) to observe the purity of the gathered glomeruli, and the pellet was used for RNA isolation.

Quantitative real-time PCR

Total RNA was extracted with an RNA extraction kit (ZYMO research, CA, USA) following the manufacturers' instructions. cDNA was reversely transcribed with a Prime-Script RT reagent kit (Takara Biotechnology, Dalian, China). PCR reaction was performed with SYBR Green Mix (Vazyme, Nanjing, China). Quantitative RT-PCR was performed using a CFX96 system (Bio-Rad, USA). The mRNA expression level was quantified using 2^{-ΔΔCT} methods. The primers in this experiment were listed in Table S1.

Enzyme-linked immunosorbent assay (ELISA)

The concentration of mouse Angptl4 from serum and urine samples was determined via ELISA (ab216789, Abcam, USA).

The protocol was achieved following the manufacture instructions. Absorbance was measured at 450 nm with an ELISA reader (Infinite® M1000, TECAN, Switzerland).

Immunofluorescence

Frozen kidney tissues were firstly cut into 4 µm thick sections and were incubated with anti-synaptopodin antibody (1:100, Santa Cruz, sc-21537), anti-podocin antibody (1:100, Sigma, P0372), anti-NFATc1 antibody (1:50, Santa Cruz, sc-13033) overnight at 4 °C, followed by incubation with fluorescein-conjugated secondary antibody. The results were examined with immunofluorescence microscopy (Leica DMLB, Wetzlar, Germany). For *in vitro* cultured podocytes, coverslips containing podocytes were firstly fixed with 4% paraformaldehyde and permeabilized in 0.3% Triton X-100 for 10 min. Podocytes were blocked with 10% FBS for 1 h and then incubated with FITC-phalloidin (1:100, Sigma, P5282), anti-synaptopodin, anti-podocin, and anti-NFATc1 antibody.

Cell culture and treatments

The conditionally immortalized mouse podocyte cell line was kindly donated by Professor Shengqiang Yu from Shanghai Changzheng Hospital. Briefly, podocytes were cultured as previously shown.²¹ Podocytes were starved overnight before experiments, and treated with LPS (20 µg/

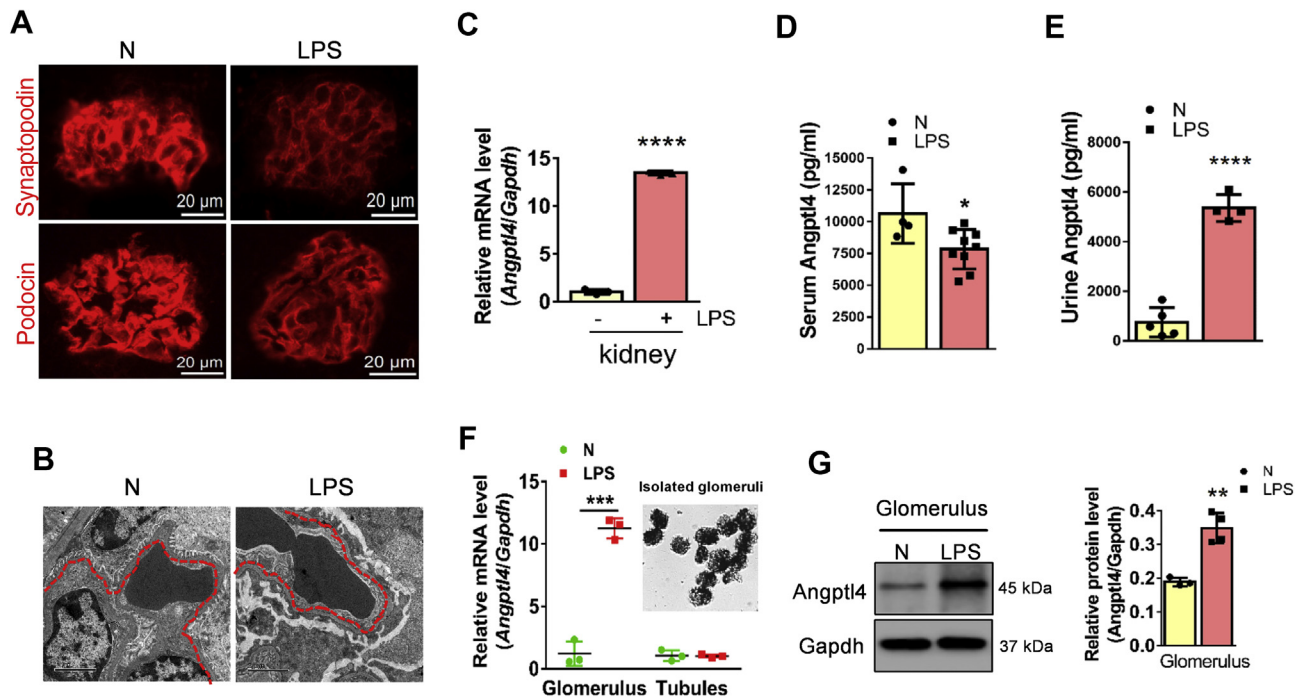


Figure 1 LPS induces the expression of Angptl4 in podocytes *in vivo*. (A) Immunofluorescence staining of synaptopodin (red) and podocin (red) in normal and LPS-treated mouse glomerulus. Scale bar = 20 µm. (B) Transmission electron microscopy analysis of foot process fusion in control and LPS-induced mice. Scale bar = 2 µm. (C) mRNA detection of kidney Angptl4 at 24 h after LPS induction. (D, E) ELISA detection of serum and urine Angptl4 in LPS-induced mice. (F) mRNA detection of Angptl4 in separated mouse glomerulus and tubules at 24 h after LPS induction. (G) Western blot analysis and quantification of Angptl4 expression in LPS-induced glomerulus. All values were presented as mean ± SD, $n \geq 3$. * $P < 0.05$ versus control group, ** $P < 0.01$ versus control group, *** $P < 0.001$ versus control group, **** $P < 0.0001$ versus control group using a two-tailed Student's *t* test.

ml, L4391, Sigma), CsA (5 μ g/ml, C1832, Sigma), FK506 (10 μ g/ml, F4679, Sigma), TAK-242 (1 μ M, HY-11109, MCE), Ionomycin (0.5 μ M, I0634, Sigma) or PDTc (20 μ M, S1808, Beyotime) for indicated time. Mouse Glomerular Endothelial cells (mGenC) was provided by Professor Chuanming

Hao at Ruijin Hospital, and were cultured in RPMI 1640 medium with 10% FBS in a humidified atmosphere of 5% CO₂. Human Mesangial Cells (HMC) was bought from ATCC, and was cultured in DMEM medium with 10% FBS in a humidified atmosphere of 5% CO₂.

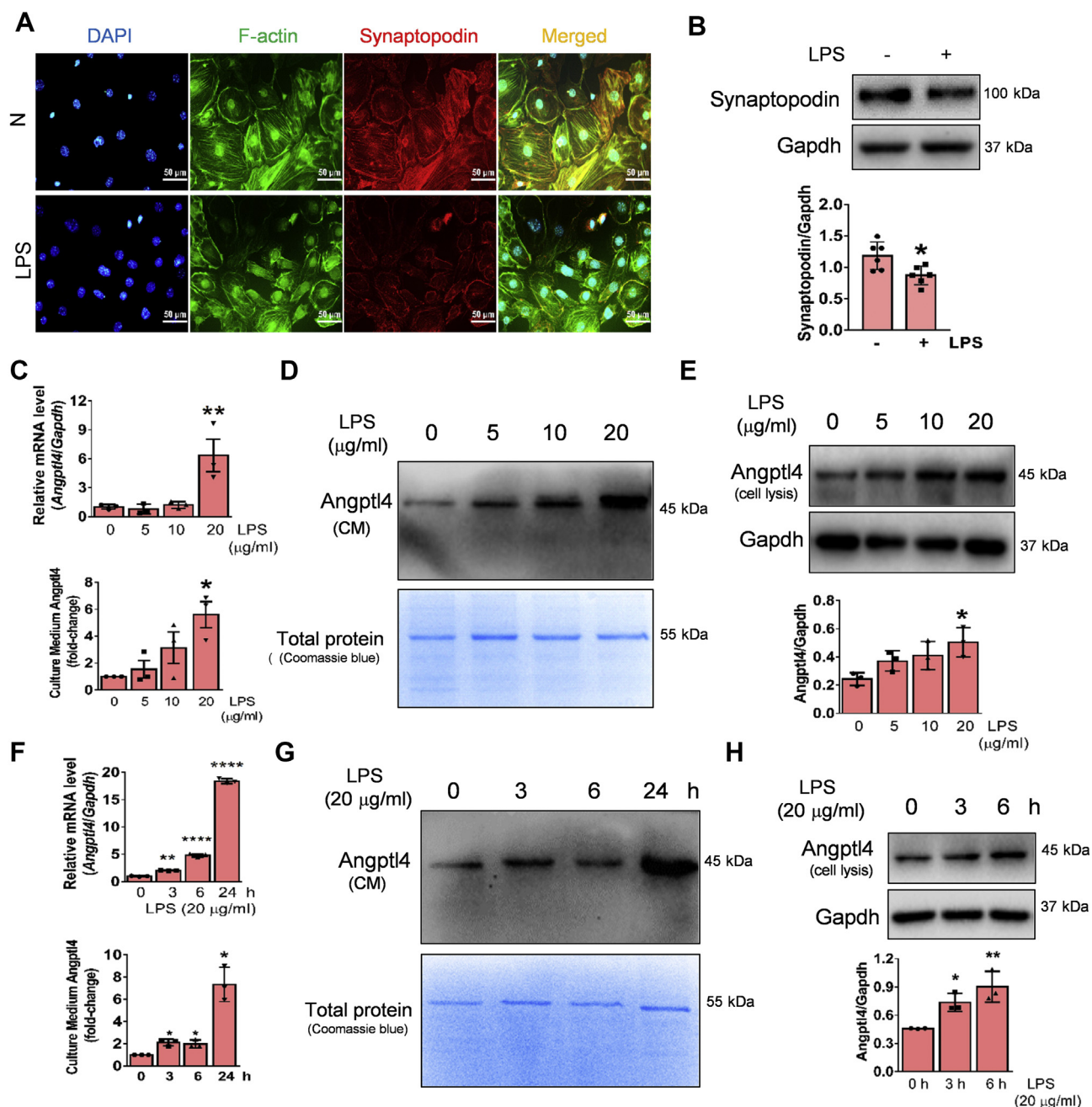


Figure 2 LPS increases podocyte *Angptl4* *in vitro*. (A) Immunofluorescence staining of F-actin (green) and synaptopodin (red) in control and LPS-treated podocytes. Scale bar = 50 μ m. (B) Western blot detection and quantification of synaptopodin in normal and LPS-treated podocytes. Comparison between control and LPS group was made using a two-tailed Student's t test. (C) mRNA detection of *Angptl4* at 24 h after 0, 5, 10 or 20 μ g/ml LPS induction. (D, E) Western blot detection and quantification of supernatant (CM: conditioned medium) and cellular (cell lysis) *Angptl4* at 24 h after 0, 5, 10 or 20 μ g/ml LPS induction. (F) mRNA detection of *Angptl4* at 0 h, 3 h, 6 h, and 24 h after 20 μ g/ml LPS induction. (G, H) Western blot detection and quantification of supernatant and cellular *Angptl4* at 0 h, 3 h, 6 h and 24 h after 20 μ g/ml LPS induction. All values were presented as mean $\pm$ SD, $n = 3$. Data from multiple groups were made using one-way ANOVA followed by Turkey's method. * $P < 0.05$ versus control group, ** $P < 0.01$ versus control group, **** $P < 0.0001$ versus control group.

Small interfering RNA (siRNA) transfection

The siRNAs were synthesized at the GenePharma company. The sequences of siRNAs in the experiments were shown in Table S2. The siRNA transfection was performed with lipofectamine RNAiMAX (life, USA) transfection protocols in differentiated podocytes. The specificity for siRNA mediated gene silencing was performed 48 h after siRNA transfection for RT-PCR and western blot analysis.

Lentiviral infection

PLKO.1-puro (EV) and PLKO.1-puro-Angptl4 (Angptl4-OE) lentivirus were purchased from Vigene biotechnology (Jinan, China). U6-puro (NC) and U6-puro-Angptl4 shRNA (Angptl4-KD) lentivirus were obtained from the Genechem company (Shanghai, China). Mouse podocytes at about 80% confluence were infected with Angptl4 overexpressed and Angptl4 shRNA lentivirus according to the manufacturer's

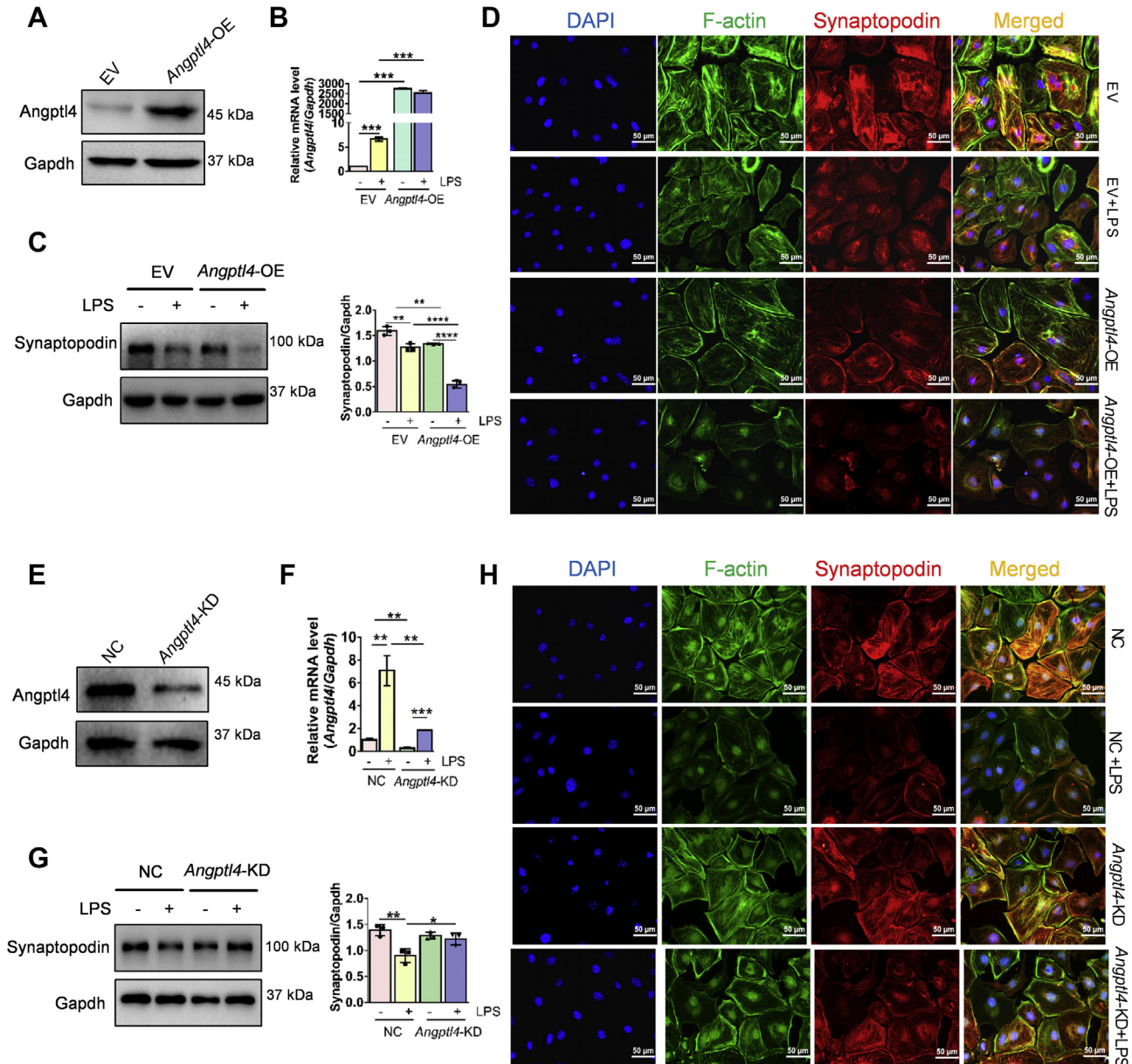


Figure 3 Angptl4 deteriorates LPS-induced podocyte injury. (A, B) mRNA and western blot quantification of Angptl4 in EV and Angptl4-OE podocytes. (C) Western blot quantification of synaptopodin in EV and Angptl4-OE podocytes before or after LPS induction. (D) Immunofluorescence staining of F-actin (green) and synaptopodin (red) in EV and Angptl4-OE podocytes before or after LPS induction. Scale bar = 50 μm. (E, F) mRNA and western blot quantification of Angptl4 in NC and Angptl4-KD podocytes. (G) Western blot quantification of synaptopodin in NC and Angptl4-KD podocytes before or after LPS induction. (H) Immunofluorescence staining of F-actin (green) and synaptopodin (red) in NC and Angptl4-KD podocytes before or after LPS induction. Scale bar = 50 μm. All values were presented as mean ± SD, $n = 3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ using one-way ANOVA followed by Turkey's method.

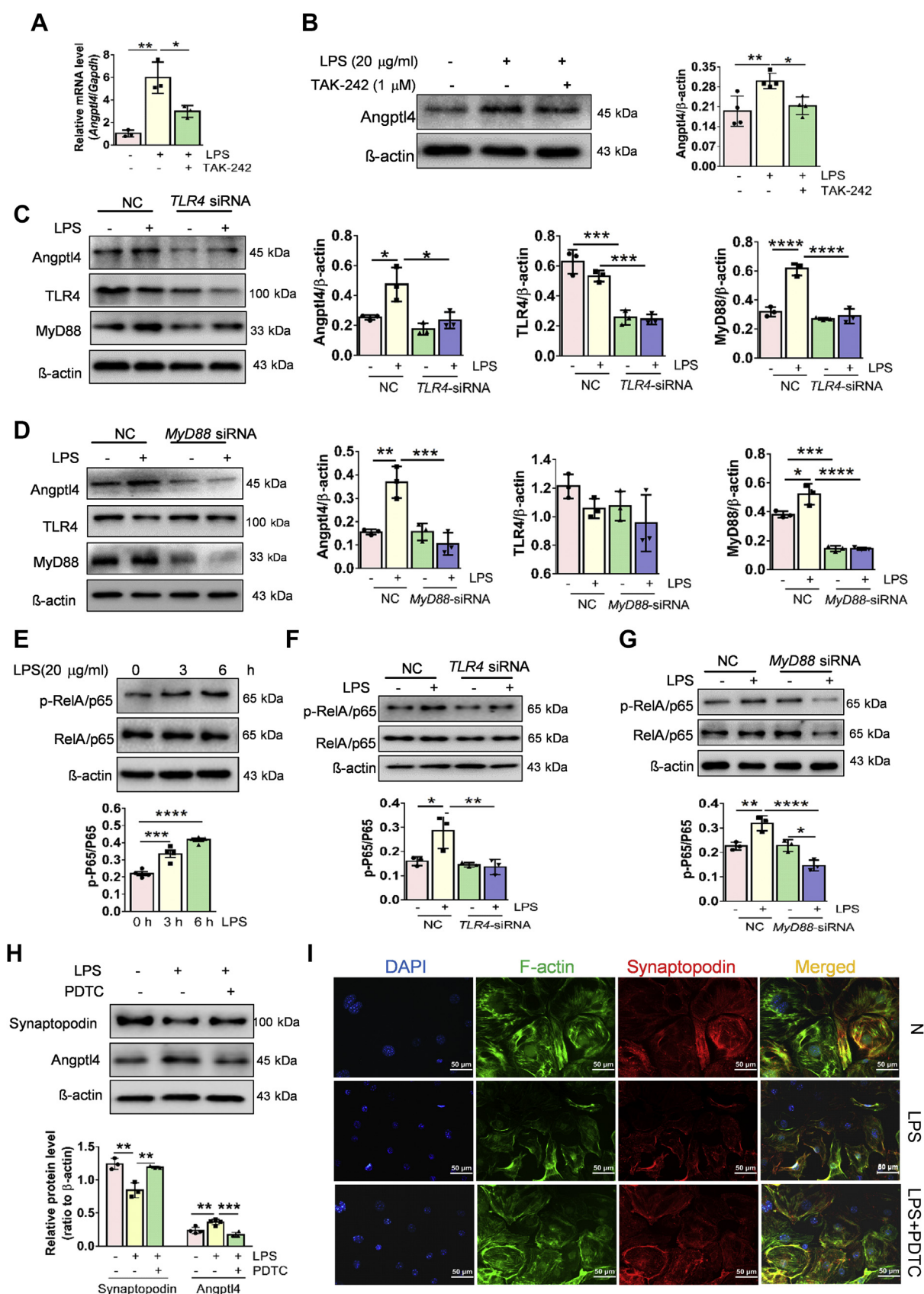


Figure 4 Effect of TLR4/MyD88/NF- κ B on the expression of Angptl4 in cultured podocytes. (A, B) RT-PCR and western blot quantification of Angptl4 in LPS-induced podocytes after TAK-242 treatment. (C) Western blot analysis of TLR4, MyD88, and Angptl4 in NC and *TLR4* siRNA-treated podocytes before or after LPS induction. (D) Western blot analysis of TLR4, MyD88, and Angptl4 in NC

protocol. Transfected podocytes were screened by puromycin selection. The specificity of Angptl4 was performed with RT-PCR and ELISA analysis.

Western blot analysis

Cultured mouse podocytes and isolated renal glomerulus were lysed with RIPA buffer to gain total proteins. For cell culture supernatants, 10 ml of culture medium was concentrated with 50 ml Amicon® Ultra Centrifugal Filters (Millipore, MA, USA) to about 200 µl for further analysis. The protein samples were separated on SDS-PAGE gels and transferred to PVDF membranes (Millipore, Temecula, CA). PVDF membranes were blocked with 5% non-fat milk for 2 h and incubated with different primary antibodies. The primary antibodies were used as follows: Angptl4(1:1000, Abcam); synaptopodin (1:1000, Santa Cruz); CaN (1:500, Santa Cruz); GAPDH(1:5000, Bioke); β -actin (1:5000, Bioke); TLR4 (1:500, Santa Cruz); CD14 (1:1000, Cell Signaling); MyD88(1:1000, Cell Signaling); p-p65 (1:1000, Cell Signaling); p65 (1:1000, Cell Signaling); NFATc1 (1:500, Santa Cruz); HIF-1a (1:1000, Cell Signaling). After incubation with HRP-conjugated secondary antibodies for 2 h, membranes were visualized with an enhanced chemiluminescence detection system ChemiDoc MP (Bio-RAD, California, USA).

Statistical analysis

Data are presented as mean $\pm$ SD. The quantitative results were analyzed with GraphPad Prism 7 software. Comparisons between two groups were made using a two-tailed Student's *t* test. Data from multiple groups were made using one-way ANOVA followed by Turkey's method. $P < 0.05$ was considered to indicate statistical significance.

Results

LPS induces the expression of Angptl4 in podocytes

First, we successfully established the LPS-induced podocyte injury model *in vivo*. As shown in Fig. 1A, LPS decreased the expression of synaptopodin and podocin compared to normal controls. The electron microscopy also indicated widely foot process fusion after LPS induction (Fig. 1B). Meanwhile, we found that LPS increased *Angptl4* mRNA expression (Fig. 1C). The ELISA detection showed that urine Angptl4 was increased while serum Angptl4 was decreased in LPS-induced mice compared to the controls (Fig. 1D, E). To determine the localization of Angptl4 in the kidney, we isolated glomerulus and tubules separately, the results showed that Angptl4 was significantly improved in the

glomerulus while not in the tubules (Fig. 1F, G). Besides the kidney, we also found increased Angptl4 in heart, lung, muscle, and adipose tissues in LPS-induced mice, while no change was found in the liver and spleen (Fig. S1a). These results indicated that Angptl4 might be involved in LPS-induced glomerulus injury.

To further verify the role of Angptl4 in the glomerulus, we cultured podocytes, glomerular endothelial cells (mGEnC) and mesangial cells (HMC) separately *in vitro*. Our results indicated that LPS only increased Angptl4 in podocytes while not in other cells of the glomerulus (Fig. S1b–e). Similar to others' results, we showed decreased expression of synaptopodin in LPS-induced podocytes (Fig. 2A, B). In cultured podocytes, we demonstrated that the mRNA expression of *Angptl4* in LPS-induced podocytes enhanced as time and concentration increased (Fig. 2C, F). Western blot analysis of Angptl4 in podocytes and cultured supernatant also showed the same tendency (Fig. 2D, E, G and H), indicating a time and dose-dependent manner of Angptl4 after LPS induction, especially at a concentration of 20 µg/ml LPS. Accordingly, we chose 20 µg/ml LPS to carry out the following *in vitro* experiments. These results demonstrated that LPS only induced the expression of Angptl4 in podocytes.

Angptl4 deteriorates LPS-induced podocyte injury

We next determined the effect of Angptl4 on podocyte injury. First, we successfully established *Angptl4* overexpressed (*Angptl4*-OE) podocytes (Fig. 3A, B). Moreover, synaptopodin was decreased in *Angptl4*-OE podocytes, especially after LPS induction (Fig. 3C, D; Fig. S2a). The immunofluorescence staining of F-actin showed decreased, disorganized and shortened actin stress fibers in *Angptl4*-OE podocytes compared to the control (EV) podocytes, which was further disorganized after LPS induction (Fig. 3D; Fig. S2a).

To further verify the role of Angptl4 in podocytes, we knocked down *Angptl4* in podocytes (Fig. 3E, F). Immunofluorescence staining of F-actin and synaptopodin indicated no apparent difference between normal (NC) and *Angptl4* knockdown (*Angptl4*-KD) podocytes (Fig. S2b). Furthermore, the *Angptl4* knockdown partially recovered the expression of synaptopodin after LPS induction compared to the control group (Fig. 3G, H). Moreover, we showed that *Angptl4*-KD podocytes restored LPS-induced F-actin rearrangement compared to the control podocytes (Fig. 3H). These results demonstrated that *Angptl4* overexpression aggravated LPS-induced podocyte cytoskeleton and decreased the expression of synaptopodin, implying the role of Angptl4 in LPS-induced podocyte injury.

and *MyD88* siRNA-treated podocytes before or after LPS induction. (E) Western blot analysis of p65 phosphorylation at 0 h, 3 h and 6 h after LPS induction. (F) Western blot analysis of p65 phosphorylation in NC and *TLR4* siRNA-treated podocytes before or after LPS induction. (G) Western blot analysis of p65 phosphorylation in NC and *MyD88* siRNA-treated podocytes before or after LPS induction. (H) Western blot quantification of Angptl4 and synaptopodin in LPS-induced mouse podocytes after PDTC treatment. (I) Immunofluorescence staining of F-actin (green) and synaptopodin (red) in PDTC pretreated cultured podocytes at 24 h after LPS induction. Scale bar = 50 µm. All values were presented as mean $\pm$ SD, $n = 3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ using one-way ANOVA followed by Turkey's method.

TLR4/MyD88/NF- κ B signaling is involved in the upregulation of Angptl4 in LPS-induced podocytes

LPS stimulation generally triggers the TLR4/MyD88/NF- κ B signaling pathway. We then detected the effect of TLR4 and the co-receptor CD14 on Angptl4 expression. As shown in Figure S3a, b, the expression of CD14 was promoted as LPS concentration increased. We then knocked down CD14 in podocytes (Fig. S3c), the results showed that CD14 knock-down inhibited CD14 and Angptl4 expression in LPS-induced podocytes (Fig. S3d, e). We next treated podocytes with TLR4 specific inhibitor TAK-242, the results showed that TAK-242 inhibited Angptl4 mRNA and protein in podocytes (Fig. 4A, B). Subsequently, we showed that both TLR4 and MyD88 siRNA decreased the expression of Angptl4 in LPS-induced podocytes. Moreover, TLR4 siRNA inhibited the downstream molecule MyD88 while MyD88 siRNA had no effect on TLR4 (Fig. 4C, D). We also found the phosphorylation of p65 in LPS-induced podocytes (Fig. 4E). TLR4 and MyD88 siRNA also decreased p65 phosphorylation after LPS induction in podocytes (Fig. 4F, G). In addition, we indicated that PDTTC, a NF- κ B inhibitor, promoted the recovery of synaptopodin and F-actin expression (Fig. 4H, I) and had a significant inhibitory effect on Angptl4 expression (Fig. 4H), indicating that TLR4/MyD88/NF- κ B signal pathway mediated LPS-induced Angptl4 in podocyte injury.

Calcineurin/NFAT signaling mediates LPS-induced Angptl4 expression

Previous studies have implied the role of the calcineurin/NFAT signaling pathway on Angptl4 regulation. Our results showed that LPS prompted the CaN expression and increased the percentage of nuclear NFATc1⁺ podocytes (Fig. 5A, B), which was inhibited by calcineurin inhibitors CsA or FK506 intervention (Fig. 5C, D), implicating a promoting effect of LPS on calcineurin/NFAT signaling pathway. Moreover, we indicated that CaN siRNA inhibited LPS-induced Angptl4 expression in podocytes (Fig. 5E). Our *in vivo* and *in vitro* results also indicated that CsA or FK506 treatment inhibited LPS-induced Angptl4 expression at mRNA and protein level (Fig. 5F, G, J, K), the ELISA results showed that urine Angptl4 was decreased in CsA or FK506 treatment group compared to the LPS-induced group (Fig. 5H, I). Subsequently, we stimulated podocytes with ionomycin, the results showed that ionomycin increased cellular and secreted Angptl4 in a dose-dependent manner, and the effect was inhibited by CsA or FK506 intervention (Fig. 5L, M).

Besides inhibiting the expression of Angptl4, our *in vivo* results indicated that CaN inhibitors CsA or FK506 treatment inhibited LPS-induced proteinuria (Fig. 6A, B). PAS staining showed no significant changes between groups (Fig. 6C). Electron microscopy indicated that CsA or FK506 treatment partially reversed foot process effacement (Fig. 6D). CsA or FK506 treatment also decreased synaptopodin and podocin in LPS-induced glomerulus and podocytes (Fig. 6E–G). These results imply that CaN inhibitors can be used to treat podocyte related kidney diseases partially through regulating Angptl4 expression.

Discussion

Here, this study shows for the first time that LPS increased podocyte Angptl4 expression in the *in vitro* and *in vivo* model. Furthermore, the Angptl4 overexpression and knockdown experiment indicated that Angptl4 promoted the disorder of podocyte cytoskeleton and decreased synaptopodin expression. In addition, our results implied that both of the TLR4/MyD88/NF- κ B and calcineurin/NFAT signaling mediated the upregulation of Angptl4 in LPS-induced podocytes.

Previous results have indicated that many molecules and pathways participated in LPS-induced podocyte injury.^{5,6,9,22} Zhang H et al showed ATF3 induction was involved in LPS-induced podocyte injury through NFAT signaling pathway.⁶ Another study of Senouthai S et al indicated that Fractalkine participated in LPS-induced podocyte injury through the Wnt/ β -catenin pathway.²² CD80 also participated in LPS-induced podocyte injury induced by LPS.⁵ It was reported that the down-regulation of miRNA-30 also facilitated LPS-induced podocyte injury.⁹ We are the first to indicated that Angptl4 also contributed to LPS-induced podocyte injury.

It was demonstrated that LPS differently regulated Angptl4. A study by Brown R et al firstly found that LPS increased Angptl4 expression in brain tissue.²³ Another study found that LPS increased Angptl4 in lung, heart, muscle, bone marrow, and adipose tissue.²⁴ Nevertheless, in a study of Feingold KR et al, they found LPS markedly decreased Angptl4 in mouse peritoneal macrophage.²⁵ Our results indicated that Angptl4 was increased in LPS-induced podocytes *in vivo* and *in vitro*. In addition, we also noticed that only podocytes showed an increase in Angptl4 expression in LPS-stimulated kidneys, other glomerular cells such as mesangial cells, glomerular endothelial cells, and tubular cells showed no significant changes, suggesting that there might be some particular pathway or protein regulating Angptl4 expression in podocytes, which needs further experiments to verify. Moreover, in the *in vivo* mouse model, we found LPS decreased serum Angptl4 while increased urine Angptl4, part of the explanation might be that most of Angptl4 secreted by podocytes leaked into renal tubules, and excreted from urine, resulting in an increase in urine Angptl4.

Previous reports showed that Angptl4 had a dual role in different diseases. In cancers, Zhu P et al found that Angptl4 stimulated tumor cells to escape anoikis.²⁶ However, in hepatocellular carcinoma, bladder cancer and cholangiocarcinoma,^{27–29} Angptl4 promoted cell apoptosis and played as a tumor suppressor gene. Concerning to Angptl4 in podocytes, Clement LC et al firstly found that PAN-induced rats showed low sialic acid Angptl4 secretion from podocytes into the basement membrane, which altered the charge barrier on GBM, causing foot process fusion, and eventually inducing proteinuria.⁴ In our experiment, we showed decreased, disorganized and shortened actin stress fibers in Angptl4-OE podocytes compared to the EV podocytes, especially after LPS induction, implying a direct effect of Angptl4 in inducing podocyte injury.

The mechanism of Angptl4 upstream regulation during podocyte injury is not clear. Previous reports have shown

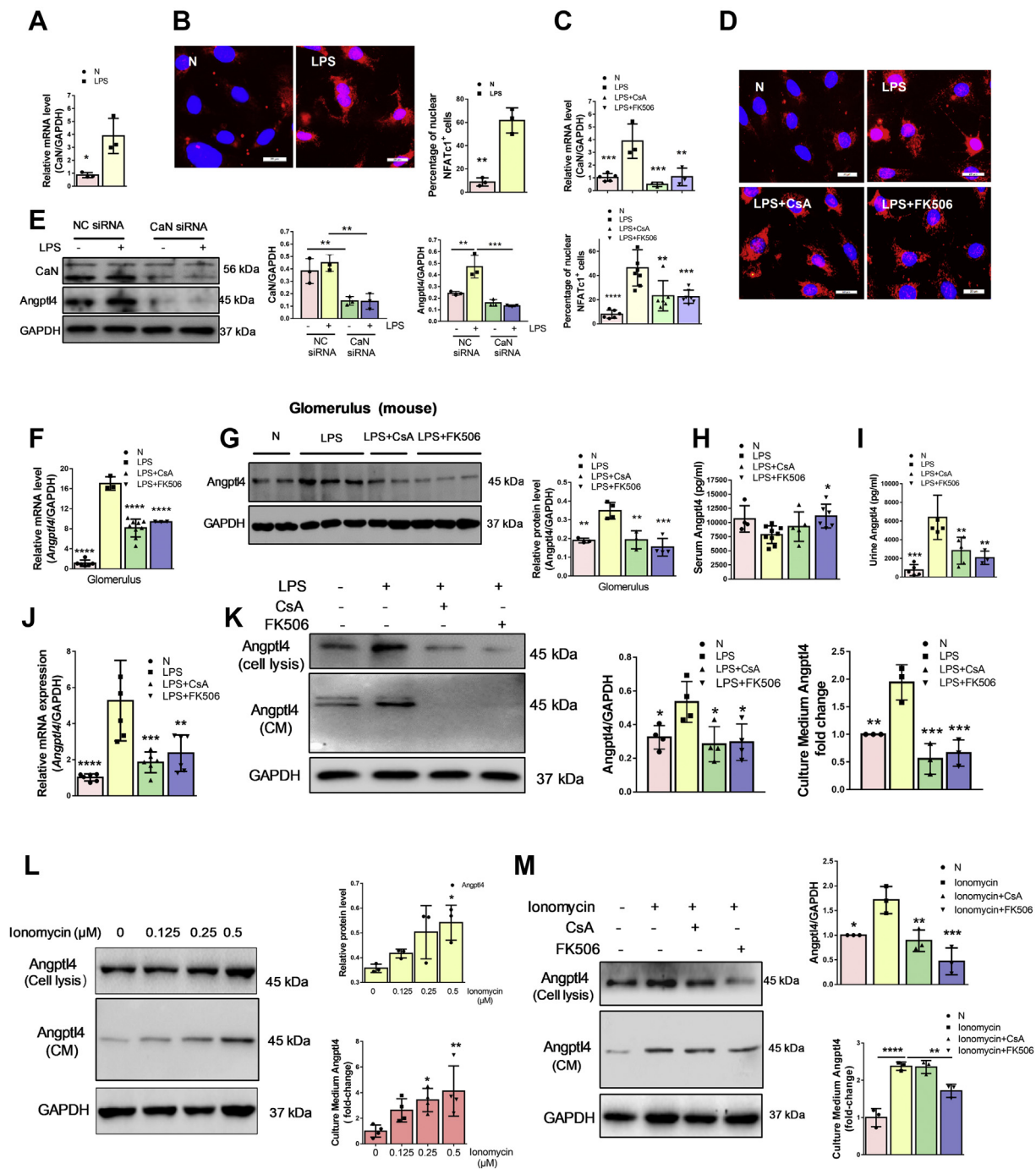


Figure 5 Effect of Calcineurin/NFAT on the expression of Angptl4 in cultured podocytes. (A) RT-PCR detection of *CaN* in control and LPS-induced podocytes. (B) Immunofluorescence staining and quantification of nuclear NFATc1 (red) in LPS-induced podocytes. Scale bar = 20 μ m. (C) RT-PCR quantification of *CaN* in LPS-induced podocytes after CsA or FK506 pre-treatment. (D) Immunofluorescence staining and quantification of nuclear NFATc1 (red) in LPS-induced podocytes after CsA or FK506 pre-treatment. Scale bar = 20 μ m. (E) Western blot analysis of *CaN* and Angptl4 in normal control and *CaN* siRNA podocytes before or after LPS induction. (F, G) RT-PCR and western blot quantification of Angptl4 in LPS-induced glomerulus after CsA or FK506 treatment. (H, I) ELISA detection of serum and urine Angptl4 in LPS-induced mice after CsA or FK506 treatment. (J) RT-PCR quantification of *Angptl4* in LPS-induced cultured mouse podocytes after CsA or FK506 treatment. (K) Western blot analysis and quantification of cellular and cultural supernatant Angptl4 in LPS-induced mouse podocytes after CsA or FK506 treatment. (L) Western blot analysis of cellular and cultural supernatant Angptl4 expression at different concentrations of ionomycin induction in podocytes. (M) Western blot analysis of podocyte cellular and cultural supernatant Angptl4 before or after CsA or FK506 treatment in ionomycin-induced cultured podocytes. All values were presented as mean $\pm$ SD, $n \geq 3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ using one-way ANOVA followed by Turkey's method.

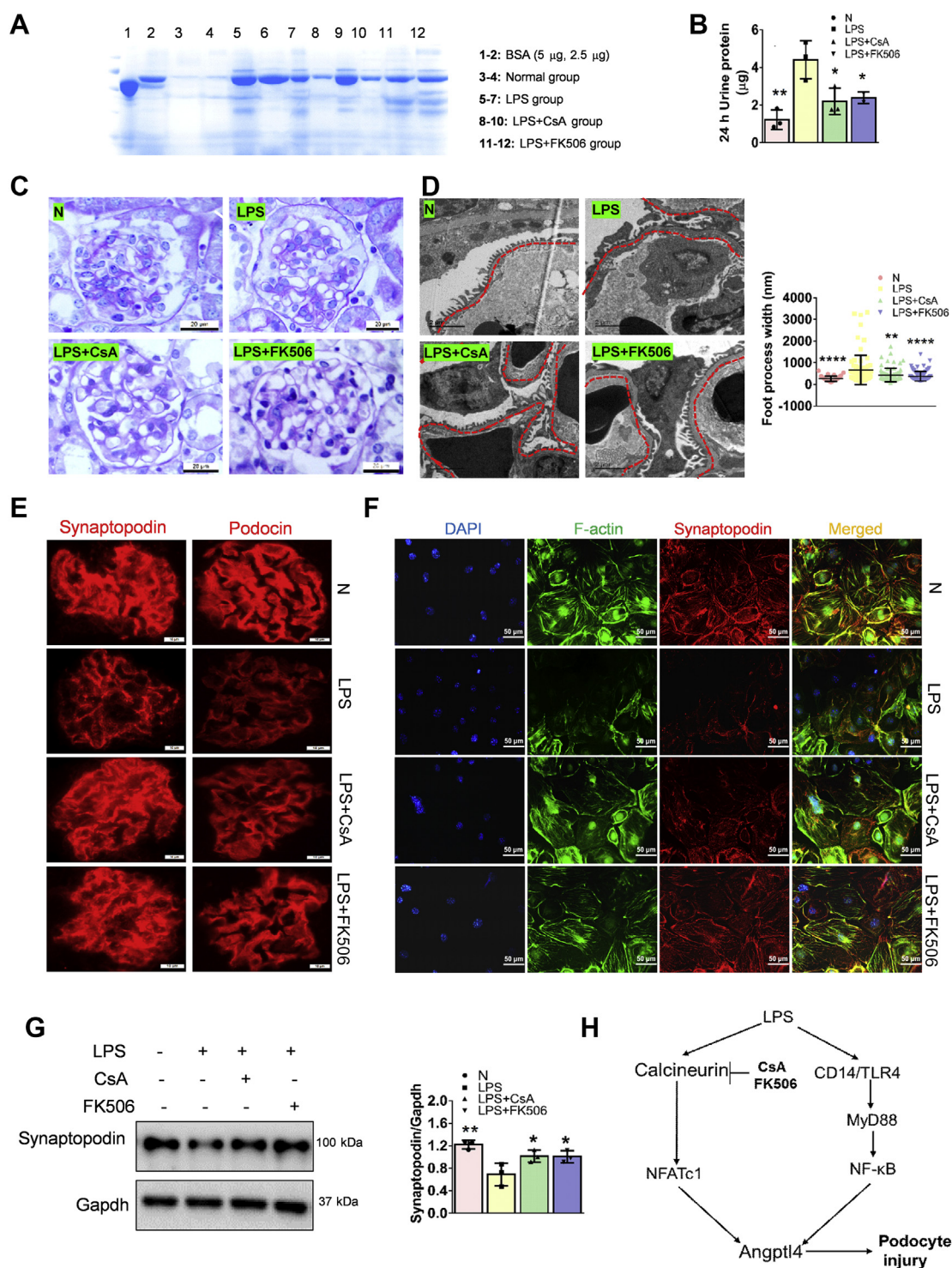


Figure 6 CaN inhibitors protect from LPS-induced podocyte injury. (A, B) CsA and FK506 decreased LPS-induced urinary protein in mice. (C) PAS staining of CsA or FK506 pretreatment in LPS-induced mice. Scale bar = 20 µm. (D) Transmission electron microscopy analysis of CsA or FK506 pretreatment in LPS-induced mice. Scale bar = 2 µm. (E) Immunofluorescence staining of synaptopodin (red) and podocin (red) in CsA or FK506 pretreated mice at 24 h after LPS induction. Scale bar = 10 µm. (F) Immunofluorescence staining of F-actin (green) and synaptopodin (red) in CsA or FK506 pretreated cultured podocytes at 24 h after LPS induction. Scale bar = 50 µm. (G) Western blot analysis of synaptopodin in CsA or FK506 pretreated cultured podocytes at 24 h after LPS induction. (H) Schematic diagram indicates LPS-induced podocyte Angptl4 is mediated by TLR4/MyD88/NF-κB and calcineurin/NFAT signal pathways. All values were presented as mean ± SD, $n \geq 3$. * $P < 0.05$ versus LPS group, ** $P < 0.01$ versus LPS group, *** $P < 0.001$ versus LPS group, **** $P < 0.0001$ versus LPS group using one-way ANOVA followed by Turkey's method.

that Angptl4 was mainly affected by HIF and PPAR signaling pathways.^{30–32} Our results showed that LPS had no effect on HIF-1 α , PPAR- γ , or PPAR- α in podocytes (Fig. S4a–c), implying that the HIF and PPAR signaling pathways might not participate in LPS-induced Angptl4 in podocytes. LPS played roles through the TLR4/MyD88/NF- κ B signaling pathway. As one of the co-receptors of LPS, CD14 was involved in podocyte related nephropathy. Poesen R et al showed that the urine sCD14 of nephrotic syndrome patients was significantly higher than healthy control and IgA nephropathy patients without nephrotic syndrome.³³ A study of Kang MJ et al found that soluble CD14 (sCD14) was positively correlated with the severity of proteinuria.³⁴ Our results indicated that LPS increased the expression of CD14 in podocytes. Meanwhile, CD14 knockdown experiment showed decreased expression of Angptl4, indicating that Angptl4 was partially regulated by CD14. TLR4 has been indicated to participate in glomerular injuries such as diabetic nephropathy and MCD.^{35–37} It was reported that Angptl4 might be regulated by TLR4 in bone marrow, muscle, and adipose tissue.^{24,38} We also found that TLR4, MyD88 siRNA and NF- κ B inhibitor treatment decreased the expression of Angptl4 in podocytes after LPS induction, implying that the TLR4/MyD88/NF- κ B signaling pathway regulated Angptl4 expression in podocytes.

Calcineurin/NFAT is a crucial signaling pathway for proteinuria production and podocyte injury.^{39–42} CaN inhibitors have been reported to inhibit Angptl4 expression in diabetic nephropathy and membrane nephropathy,^{18,19} implying that Angptl4 might be regulated by calcineurin-NFAT signaling pathway in podocytes. Our results found that CaN inhibitors and CaN siRNA markedly inhibited LPS-induced podocyte injury as well as Angptl4 expression. In addition, we also found a similar effect in heart, lung, and adipose tissues (Fig. S5), implying that the Angptl4 was not only the target in podocytes. Furthermore, we showed that the NFATc1 activator also promoted Angptl4 expression. A study by Ivan Zanoni et al demonstrated that the calcineurin/NFAT signaling pathway could also be affected by LPS, leading to apoptosis and inflammation of dendritic cells.⁴³ Combined with these findings, we speculated that LPS-induced podocyte Angptl4 was also regulated by the calcineurin/NFAT signaling pathway.

In addition to the above signaling pathways, we also noticed that some other signaling pathways also regulated Angptl4 expression. A study by Ma and colleagues showed that Angptl4 promoted Viral G protein-coupled receptor-mediated angiogenesis and vascular permeability through the Rho-ROCK pathway.⁴⁴ Angptl4 could also counteract hypoxia-induced vascular permeability by modulating the src signaling pathway.⁴⁵ Recently, it was found that TGF- β regulated the expression of Angptl4 via a smad3-signaling pathway to promote lung metastasis of breast tumors.⁴⁶ Li L et al also show that influenza infection increased Angptl4 through an IL-6-STAT3-mediated mechanism to enhance pulmonary tissue leakiness and exacerbate inflammation-induced lung damage.¹⁴ These results implied the complexity of Angptl4 upstream regulation.

In summary, our study revealed that Angptl4, induced by LPS induction, deteriorated LPS-induced podocyte cytoskeleton rearrangement and decreased the expression of synaptopodin. Furthermore, we showed that both of the

TLR4/MyD88/NF- κ B and calcineurin/NFAT signaling mediated the upregulation of Angptl4 in LPS-induced podocytes, which might be a therapeutic target for podocyte nephropathy treatment.

Author contributions

X.J.S and J.H.C conceived this study; X.J.S designed the project; X.J.S and H.B.W drafted the manuscript; X.J.S performed the experiments; H.B.W helped with the western blot experiment; Y.C.W and C.L.W performed the animal experiments; C.H.W offered valuable suggestions for the transfection experiments; H.J gave suggestions for drafting the manuscript; S.F helped to culture podocytes. X.Y.L and H.L offered valuable suggestions for the usage of calcineurin inhibitors.

Conflict of interests

All the authors declared no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gendis.2020.07.005>.

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