LMTK2 regulates inflammation in lipopolysaccharide-stimulated BV2 cells

QIANYUN RUI[1], SHUGANG CAO[2], XIAOZHU WANG[1], XIAOYU DUAN[1], XINYI IAO[1], WANLI DONG[1], QI FANG[1,3], XUEGUANG ZHANG[4] and QUN XUE[1,3,4]

[1]Department of Neurology, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215006; [2]Department of Neurology, The Second People's Hospital of Hefei, Hefei, Anhui 230011; [3]Suzhou Clinical Medical Center of Neurology, Suzhou, Jiangsu 215004; [4]Institute of Clinical Immunology, Jiangsu Key Laboratory of Clinical Immunology, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215006 P.R. China

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Abstract. Microglia activation plays vital roles in neuroinflammatory pathologys. Lemurs tyrosine kinase 2 (LMTK2) was reported to regulate NF-κB signals. In the present study, the roles of LMTK2 were investigated in lipopolysaccharide (LPS)-treated BV-2 cells. Reverse transcription-quantitative (RT-q)PCR and western blotting (WB) were utilized to analyze LMTK2 levels in LPS-treated BV2 cells. MTT assay determined cell viabilities. Nitric oxide (NO) and prostaglandin E2 (PGE2) levels were assessed through Griess and enzyme-linked immunosorbent assay (ELISA), respectively. The expression level of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) were detected through RT-qPCR and WB. The release of inflammatory mediators under LPS stimulation, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6 and IL-10, were analyzed through ELISA. WB was used to analyze the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase 1 (HO-1)/NAD(P)H dehydrogenase quinone 1 (NQO1) signal pathway. The results showed that the levels of the inflammatory mediators, iNOS, NO, COX-2 and PGE2, along with pro-inflammatory factors, TNF-α, IL-1β and IL-6, were significantly decreased following the induction of exogenous LMTK2 expression by LMTK2 overexpression plasmids in LPS-induced BV2 microglia. In contrast, anti-inflammatory factor IL-10 showed obvious decrease. Additionally, LMTK2 overexpression induced the elevation of Nrf2 in the cytoplasm and nucleus, along with the upregulation of HO-1 and NQO1 expression. In conclusion, LMTK2 is postulated to regulate neuroinflammation possibly through Nrf2 pathway. The present study is essential to reveal the underlying function of LMTK2 and to identify novel therapeutic targets for drug development in treating neuroinflammation.

Correspondence to: Dr Qun Xue, Department of Neurology, The First Affiliated Hospital of Soochow University, 188 Shizi Street, Suzhou, Jiangsu 215006, P.R. China
E-mail: author@mail.com

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Introduction

Lemurs tyrosine kinase 2 (LMTK2) belongs to the trans-membrane serine/threonine protein kinase family anchoring membrane with unique structure (1,2). LMTK2, widely expressed in brain, is involved in regulating key cellular events, apoptosis and cell differentiation (3-7). According to a review, LMTK2 affects the sensitivities of cells to cytotoxicity depending on apoptotic and survival pathways (3). The latest research shows that LMTK2 can activate the NRF/ARE signaling pathway to reduce neurons injury induced by ischemia reperfusion (8). LMTK2 could be phosphorylated through CDK5/p35 in neurons, however, there is only a small amount of the phosphorylated forms of LMTK2 in non-neuron cells with inactive CDK5 (9-12). Furthermore, LMTK2 can exert vital roles through phosphorylating downstream targets in non-neuron cells (3). It has been proved that LMTK2 regulates NF-κB signals through the PP1/GSK3β/p65 pathway or PP1/IKK pathway in colon cancer cells (13).

Neuroinflammation is associated with the progression of multiple neurological diseases. Microglia, as the main immune effector cells of the central nervous system (CNS), play a substantial role in CNS diseases (14). Although microglia have essential neuroprotection functions, including sensing changes of the environment, maintaining normal neuronal function and defensing these changes, they can damage neurons in response to a particular stimulus or with neuroinflammation (15). Activated microglia are involved in the pathologic processes of CNS diseases, such as neurodegenerative disease, pain, infection and brain trauma (16-19). The activation of microglia could damage neurons in the brain by releasing inflammatory cytokines and generating oxidative stress, which further triggers neurological diseases (20,21). Therefore, the present study aims to explore the role of LMTK2 in lipopolysaccharide (LPS)-induced microglia inflammation and to explore whether it can activate Nrf2 signaling. LPS was used to activate

mouse microglia (BV2) cells to construct a cell model of neuroinflammation.

Materials and methods

Cells. Mouse microglial cells, BV2, were purchased (The Institute of Cell Biology, Chinese Academy of Sciences, China) and cultured with DMEM, a high-glucose medium containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), at a constant temperature of 37˚C in 5% CO_2. The microglia were used in the experiment when the cells reached the logarithmic growth stage.

Western blotting (WB). BV2 cell suspension was placed in a 6-well plate ($2x10^5$ cells/ml). The cells were stimulated with LPS at different concentrations (100, 200 and 500 ng/ml; Sigma-Aldrich; Merck KGaA) for 24 h. For LMTK2 overexpression, the cells were transfected with plasmids overexpressing LMTK2. After 24 h, the cells were stimulated with LPS (500 ng/ml) for 24 h. Subsequently, cells were collected to extract total protein using RIPA lysis solution (cat. no. R0278; Sigma-Aldrich; Merck KGaA). The protein concentrations were detected by BCA method. Then, 10% SDS-PAGE electrophoresis was performed to separate the proteins (40 μg protein in each well). Skim milk powder (5%) was utilized to block the PVDF membrane for 40 min at room temperature. Primary antibodies (LMTK2 (1:500; cat. no. DF3344; Affinity Biosciences), inducible nitric oxide synthase (1:1,000; iNOS; cat. no. ab178945; Abcam), cyclooxygenase 2 (1:1,000; COX2; cat. no. ab179800), nuclear factor erythroid 2-related factor 2 (1:1,000; NRF2; ab137550; Abcam), heme oxygenase-1 (1:2,000; HO-1; cat. no. ab189491; Abcam), NAD(P)H dehydrogenase quinone 1 (1:20,000; NQO1; cat. no. ab28947; Abcam), Histone H3 (1:2,000; cat. no. ab1791; Abcam), GAPDH (1:5,000; cat. no. ab8245; Abcam) were incubated with the membrane at 4˚C overnight. This was followed by incubation with the secondary antibodies goat anti-rabbit IgG (1:10,000; cat. no. ab6721; Abcam) and rabbit anti-mouse IgG (1:10,000; cat. no. ab6728; Abcam) at room temperature for 1 h. ECL was used to visualize the protein bands which then was quantified with ImageJ 1.52v software (National Institutes of Health).

Plasmids transfection. The plasmids overexpressing LMTK2 were constructed by Shanghai GenePharma Co., Ltd. BV2 cells were seeded into 6-well plates ($2x10^5$ cells/ml). LMTK2 overexpression plasmids (Oe-LM; 4 μg) and empty plasmids (Oe-NC; 4 μg) were respectively transfected into BV2 cells using Lipofectamine™3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. After transfection of 24 h at 37˚C, cells were activated by LPS (500 ng/ml).

Reverse transcription-quantitative (RT-q)PCR. BV2 cell suspension was placed in a 6-well plate ($2x10^5$ cells/ml). After experimental treatment, total RNA was extracted using TRIzol method and reverse transcribed into cDNA 42˚C for 1 h (RevertaidTM First Strand cDNA Synthesis kit, Fermentas; Thermo Fisher Scientific, Inc.). Relative determination of LMTK2 and GADPH mRNA was performed by SYBR-Green dye method (Clontech Laboratories, Inc.) and calculated using the $2^{-\Delta\Delta Cq}$ method (22). The primers of LMTK2 mRNA were as follows: Forward, 5'-TTGCCCGCCACAGTCTAAAC-3' and reverse, 5'-GATGACTCTTGCTACGCTAGT-3'; The primers of GADPH mRNA were as follows: Forward 5'-GCCTTCCGTGTTCCTACCC -3' and reverse 5'-TGCCTG CTTCACCACCTTC-3'. The target mRNA was amplified in the following thermocycling conditions: Predenaturation at 95˚C for 3 min; 30 cycles of 95˚C 30 sec, 58˚C for 30 sec and 72˚C for 1 min. The total extension at 72˚C for 10 min.

MTT assay. Cells viability was detected as per the manufacturer's protocol. BV2 cells were seeded into 96-well plates ($1x10^5$ cells/ml). After cells were treated, MTT solution of 5 g/l (APExBIO Technology LLC) was added and placed in an incubator at 37˚C and 5% CO_2 for 4 h. The dimethyl sulfoxide (DMSO) solution of 100 μl was added to each well. After shaking the mixture, the absorbance (A) value at the wavelength of 490 nm was detected by a microplate reader and the cell survival rate was calculated.

Griess assays. NO2 was formed from NO in aqueous and reacted with Griess reagent (Beyotime Institute of Biotechnology). Therefore, NO levels in the supernatant were indirectly detected. Cells were seeded into 24-well plates ($2x10^5$ cells/ml). After LPS treatment (500 ng/ml) for 24 h, the supernatant in the medium was collected and then 50 μl of Griess reagent was supplemented into the supernatant. After 10 min, the absorbance at 540 nm was detected.

Enzyme-linked immunosorbent assay (ELISA). BV2 cells were cultured in 6-well plates ($2x10^5$ cells/ml). After treatment, the supernatant in medium was collected. Interleukin (IL)-1β, IL-6 and IL-10 levels were analyzed through ELISA kits (Mouse IL-1β ELISA kit; cat. no. PI301; IL-6, Mouse IL-6 ELISA kit; cat. no. PI326; and IL-10; Mouse IL-10 ELISA kit, cat. no. PI522; all from Beyotime Institute of Biotechnology), along with the detection of tumor necrosis factor (Mouse TNF-α ELISA Standard Recombinant Protein; cat. no. 29-8321-65; Invitrogen; Thermo Fisher Scientific, Inc.) and prostaglandin E2 (PGE2) levels (Prostaglandin E2 ELISA; ab133021; Abcam).

Statistical analysis. GraphPad Prism 8.0 software (GraphPad Software, Inc.) was used for statistical analysis of data, and one-way ANOVA was used to perform the comparison among groups, followed by Turkey's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

LMTK2 was significantly decreased in LPS-induced BV2 cells. LPS of different concentrations (100, 200 and 500 ng/ml) was used to stimulate BV2 cells. The LMTK2 protein and mRNA levels presented a gradual decrease with the increasing dose of LPS (Fig. 1A and B). Therefore, 500 ng/ml LPS was utilized to perform the follow-up experiments. Subsequently, the plasmids Oe-LM and Oe-NC were utilized to pre-treat BV2 cells. A significant upregulation of LMTK2 mRNA levels was observed in cells transfected

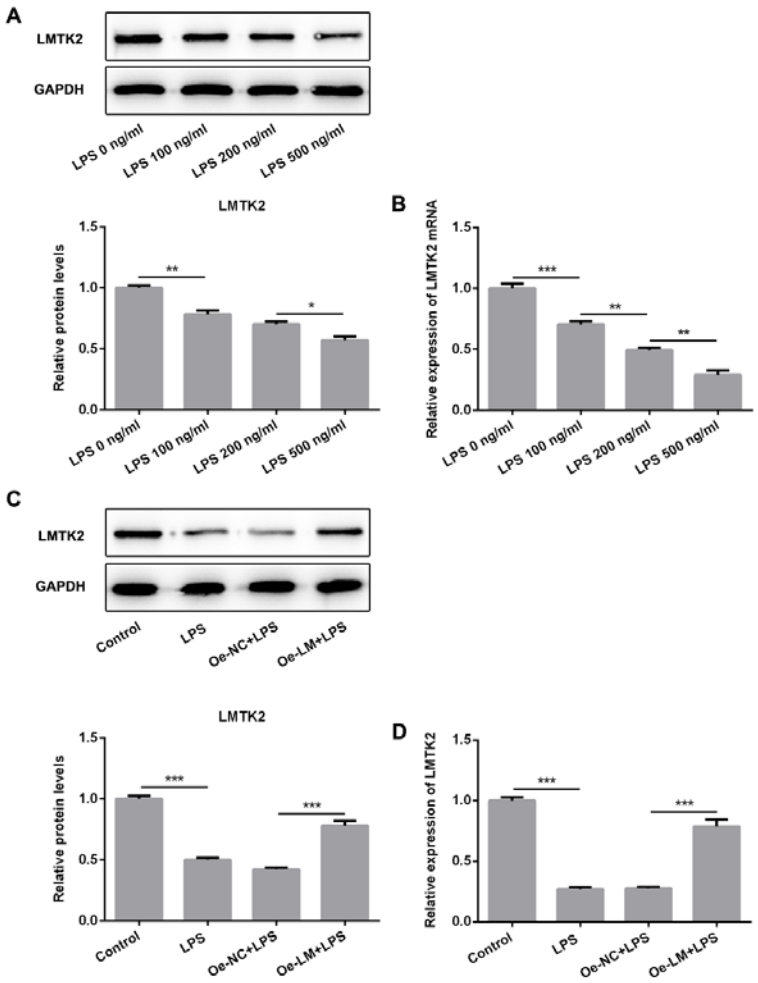

Figure 1. LPS stimulation decreased BV2 cell activities. (A and B) LMTK2 expression was analyzed through reverse transcription-quantitative PCR and western blot analysis in mouse BV2 cells stimulated by LPS at different concentrations (100, 200 and 500 ng/ml). (C and D) LMTK2 levels were markedly increased through exogenous induction of LMTK2 expression. Data are presented as mean ± SD of three independent experiments. *P<0.05; **P<0.01; ***P<0.001. LMTK2, Lemurs tyrosine kinase 2; LPS, lipopolysaccharide 2; Oe-NC, negative control plasmid; Oe-LM, LMTK2-overexpression plasmid.

with Oe-LM. (Fig. S1). Following plasmid transfection for 24 h, LPS was used to activate BV2 cells. A marked upregulation of LMTK2 protein and mRNA levels was observed in BV2 cells transfected with Oe-LM compared with Oe-NC (Fig. 1C and D).

LMTK2 overexpression notably decreased the levels of pro-inflammatory mediators in LPS-stimulated BV2 cells. In subsequent experiments, cell viability was evaluated through MTT assay in BV2 cells stimulated with LPS. Compared with the control group, LPS stimulation increased the cell viability of BV2 cells (Fig. 2A). As shown in a previous study (23), the levels of cytoskeletal protein α-tubulin and Iba1 were significantly increased in BV2 cells following LPS induction. Moreover, succinic acid dehydrogenase decreased exogenous MTT into water-insoluble blue-purple crystal formazan, contributing to the increase in OD value. In the present study, LMTK2 overexpression significantly decreased cell viability compared with cells treated with LPS alone (Fig. 2A). Subsequently, it was observed that the levels of proinflammatory mediators, consisting of NO generated by iNOS, PGE2 generated by COX-2), iNOS and COX-2, showed significant decreases in the presence of LMTK2 overexpression in LPS-activated BV2 cells (Fig. 2B-E).

The overexpression of LMTK2 regulated the release of inflammatory factors in LPS-induced BV2 cells. The levels of proinflammatory and anti-inflammatory factors were analyzed in LPS-treated BV2 cells with or without transfection of Oe-LM plasmids. As the result displayed, the proinflammatory mediators, TNF-α, IL-1β and IL-6, were markedly decreased in response to LMTK2 overexpression compared with LPS treatment alone (Fig. 3A). Subsequently, Nrf2 signals were analyzed by detecting the expression of Nrf2 and its downstream genes (HO-1 and NQO1). Nrf2 was upregulated in the cytoplasm and nucleus, as well as HO-1 and NQO1, following overexpression of LMTK2 in BV cells (Fig. 3B), implying that the activation of Nrf2 is dependent on LMTK2.

Discussion

The present study showed that exogenous LMTK2 significantly upregulated the expression of Nrf2 in the

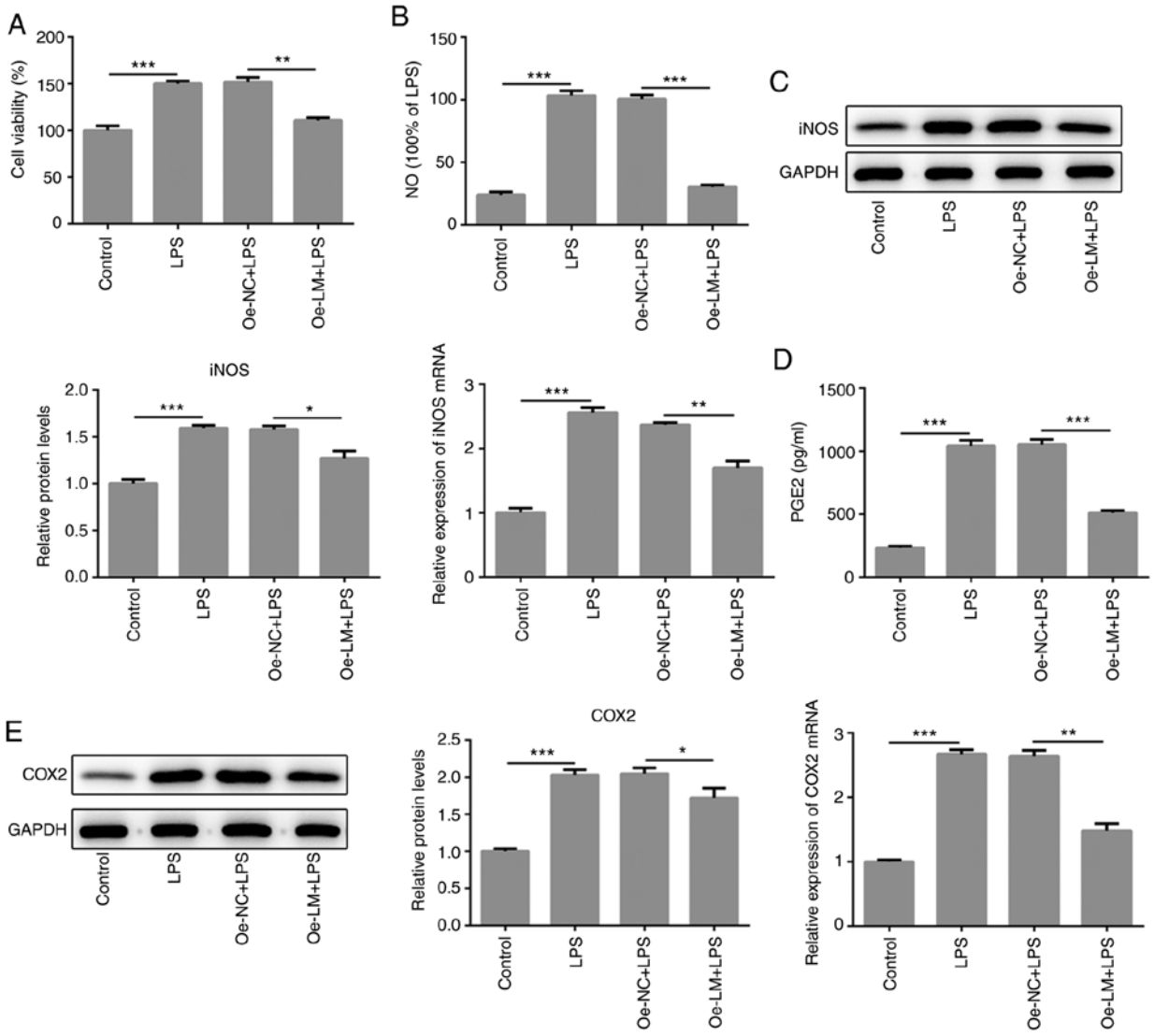

Figure 2. LMTK2 overexpression contributed to a reduction in the expression levels of pro-inflammatory mediators. (A) LMTK2 overexpression significantly decreased cell viability, as detected by MTT assay in LPS-induced BV2 cells. (B) Griess assays analyzed NO release in supernatant following LMTK2 overexpression in LPS-stimulated BV2 cells. (C) Reverse transcription-quantitative PCR and WB was used to evaluate the expression of iNOS in LPS-induced BV2 cells following LMTK2 overexpression. (D) PGE2 levels were markedly reduced, by WB analysis, in LPS-induced BV2 cells following LMTK2 overexpression. (E) LMTK2 overexpression significantly decreased COX2 expression in LPS-induced BV2 cells overexpressing LMTK2, by WB analysis. Data are shown as mean ± SD. Each experiment was repeated in triplicate. *P<0.05; **P<0.01; ***P<0.001. LMTK2, Lemurs tyrosine kinase 2; LPS, lipopolysaccharide 2; Oe-NC, negative control plasmid; Oe-LM, LMTK2-overexpression plasmid; WB, western blotting; NO, nitric oxide; iNOS, inducible nitric oxide synthase; PGE2, prostaglandin E2; COX2, cyclooxygenase 2.

nucleus and the levels of HO-1 and NQO1 proteins in BV2 cells stimulated with LPS; which implied that LMTK2 promoted the transcription of Nrf2-mediated downstream genes. A recent study demonstrated that LMTK2 regulates GSK-3β/Nrf2/ARE signaling to ameliorate neuronal injury induced by oxygen-glucose deprivation/reoxygenation (8).

There is a crosstalk between the Nrf and NF-κB pathways (24). Lack of Nrf2 is associated with enhanced production of cytokines (25), which could lead to the neurodegenerative changes in Nrf2 knockdown animals (26,27). Previously, Nrf2 was reported to show anti-inflammatory abilities through the downregulation of COX-2, TNFα and iNOS in LPS-induced peritoneal macrophages (28). HO-1, as a Nrf2-mediated downstream protein, has been demonstrated to inhibit Nrf2-modulated NF-κB (29). Collectively, the upregulation of HO-1 expression could suppress NF-κB activation and cause the decrease in pro-inflammatory factors in the present study. LPS treatment frequently induces the activation of NF-κB, along with the enhancement of iNOS, COX-2, PGE2 and pro-inflammatory factors in microglia cells (30,31). PGE2 produced by microglial cells are the main source for neuroinflammation, showing marked increase upon LPS stimulation in microglia (32,33).

The aforementioned studies imply that the Nrf2 pathway could negatively regulate the NF-κB pathway. Taken together, LMTK2 overexpression reduced the levels of iNOS, COX-2 and pro-inflammatory factors, TNF-α, IL-1β and IL-6, but increased IL-10 level; possibly due to the dependence of LPS-induced microglia on Nrf2 pathway. However, no significant changes were observed in IL-10 levels following LPS stimulation, which was consistent with a previous report (34). IL-10 is an important inflammatory suppressor *in vivo* and

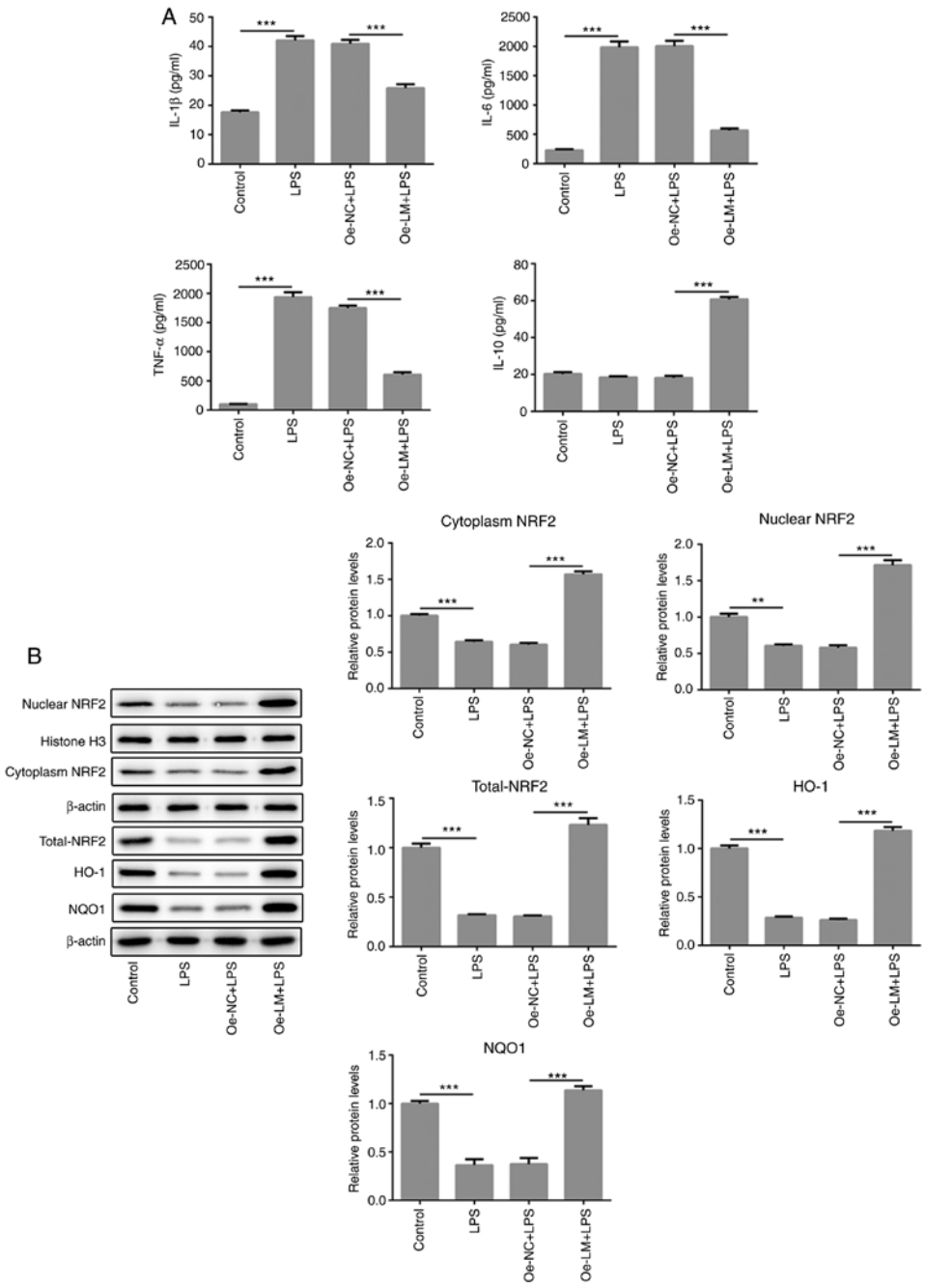

Figure 3. LMTK2 overexpression affected the release of inflammatory factors and Nrf2 pathway. (A) Inflammatory factors in cell supernatant were analyzed through enzyme-linked immunosorbent assay analysis in BV2 cells stimulated with LPS. (B) LMTK2 overexpression activated Nrf2 signal in LPS-stimulated microglia. Quantitation was performed based on three independent experiments and presented as the means ± SD. **P<0.01; ***P<0.001. LMTK2, Lemurs tyrosine kinase 2; LPS, lipopolysaccharide 2; Oe-NC, negative control plasmid; Oe-LM, LMTK2-overexpression plasmid; IL, interleukin; TNF, tumor necrosis factor; NRF2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase 1; NQO1, NAD(P)H dehydrogenase quinone 1.

can inhibit the release of pro-inflammatory cytokines in microglia cells in the central nervous system (35). Besides, LPS could induce the increase of NO, the production of iNOS, in microglia (36). In addition, NF-κB is considered upstream of NO and could initiate the synthesis of NO (37). A study has also shown that in lipoteichoic acid-induced microglia, matrix metalloprotease (MMP)-8 inhibitor regulates NF-κB and Nrf2 signals (38). Thus, LPS-mediated increase in pro-inflammatory factors were markedly reduced by inducement of exogenous LMTK2 expression, which implied the involvement of LMTK2 in regulating MMP-8 levels; however, this requires further study. In conclusion, the present study implies that LMTK2 regulates inflammation potentially by activating Nrf2 pathway.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QYR, and QX made substantial contributions to the conception and design of the study, acquired, analyzed and interpreted the data, and drafted and revised the manuscript for important intellectual content; QY, SGC, XZW, XYD, XI, WLD, QF and XGZ performed the experiments and interpreted the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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