Original Article

Inhibition of neddylation plays protective role in lipopolysaccharide-induced kidney damage through CRL-mediated NF-κB pathways

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Abstract: It has been shown that NF-κB signaling path is very effective pharmacological target for the treatment of various inflammatory diseases, including bacterial infection-associated acute kidney injury (AKI), which remains a main cause of disability and death in patients. Notably, IκB, the upstream molecular of NF-κB, plays an important role by inhibiting NF-κB activity, and IκB is regulated by cullin-RING E3 ligases (CRLs)-mediated proteasomal degradation. Therefore inhibition of CRLs-mediated neddylation and degradation of IκB would prevent NF-κB-mediated inflammation. MLN4924, a potent neddylation-inhibiting pharmacological agent, has been shown to have significant protective effects against lipopolysaccharide (LPS)-induced pro-inflammatory cytokine production through restriction of the CRL-mediated NF-κB pathway. However, it is still unclear whether MLN4924 plays a protective role through its anti-inflammatory properties in sepsis-induced AKI. In the current research, we explored whether MLN4924 have anti-inflammatory action in LPS-induced AKI mice. Our results show that MLN4924 dramatically decreased the cytotoxicity of LPS and inhibited LPS-induced synthesis and release of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, in HK2 cells, a renal tubular cell line. In addition, MLN4924 inhibited Nedd8-activating enzymes, which broke the process of cullin proteins neddylation and subsequent CRL target proteins degradation. The MLN4924-induced degradation of CRL attenuated the phosphorylation modification of IκB and IKK-α/β and blocked the nuclear translocation of P50-NF-κB and P65-NF-κB in HK2 cells under LPS stimulation. Finally, our in vivo results show that MLN4924 protected against LPS-induced AKI at relatively low doses. Collectively, these results suggest that pharmacologically blocking neddylation by MLN4924 results in the suppression of pro-inflammatory cytokines generation through the CRL/NF-κB pathway in LPS-stimulated HK2 cells, and attenuated renal inflammation in LPS-induced AKI.

Keywords: Neddylation, MLN4924, anti-inflammatory effect, NF-κB pathways, acute kidney injury

Introduction

Renal dysfunction and acute kidney injury (AKI) play a key role in the morbidity and mortality in sepsis patients [1]. The research and development of new agents for the treatment of AKI has great potential for clinical use [2]. However, up-to-date, there is still lack of effective drugs for septic AKI.

It has been shown that nuclear factor-κB (NF-κB), an omnipresent pro-inflammatory transcription factor in mammalian cells, plays a pivotal role in the regulation of pro-inflammatory factors production, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and other related genes response during lipopolysaccharide (LPS) stimulation [3, 4]. The IκB kinase enzyme complex is part of the upstream NF-κB signal transduction cascade, the IκBα protein inactivates the NF-κB transcription factor by masking the nuclear localization signals of NF-κB proteins and keeping them sequestered in an inactive state in the cytoplasm. It has been proved that the deterioration of the IκBα was dominated by ubiquitylation, and further this ubiquitylation relies on neddylation. Cullins are scaffold proteins that play a support role in the multiunit Cullin-RING E3 ligases (CRLs) [5]. Neddylation is the binding of target proteins with the ubiquitin-like protein neural precursor cell expressed developmentally.
down-regulated 8 (NEDD8) [6, 7]. Importantly, cullins are also well-characterized targets of neddylation, therefore CRLs-mediated neddylation and degradation of IkB would promote NF-κB-mediated inflammation.

The process of neddylation is similar to ubiquitination, while it relies on the reactions of its own enzymes, such as Nedd8-activating enzyme E1 (NAE1), Nedd8-conjugating enzyme E2 (Ubc12), and Nedd8-E3 ligases [8]. Pevonedistat (MLN4924) is a first-in-class inhibitor of NAE1 [6, 9, 10]. After their binding at the active site, the covalent Nedd8-MLN4924 will block the neddylation pathway [10]. Consequently, the Cullin activation is blocked, leading to the accumulation of many CRL substrates, including IkB-α, thereby inhibiting NF-κB activity [11, 12]. Indeed, MLN4924 has been studied to decay LPS-induced pro-inflammatory cytokines generation in macrophages and rebate IFN-β expression in response to TLR3/4 and retinoic-acid-inducible gene-I stimulation [13, 14]. These results suggest that the neddylation pathway plays a pivotal role in the immune regulation in hypercytokinemia and imply that the neddylation inhibitors may be used as effective anti-inflammatory agents. Based on these findings, this research work was aimed to investigate whether MLN4924 could attenuate LPS-induced AKI through the inhibition of the CRL/NF-κB pathway.

Materials and methods

Aki animal model

All animal experiments were approved by the Ethic Committee on Animal Care of Ruijin Hospital, School of Medicine, Shanghai Jiaotong University. Twenty-five male C57BL/6 mice (10 weeks old) were used including five treatments: vehicle (saline) (n=5), LPS (n=5), LPS+MLN4924 (0.05 mg/kg, n=5), LPS+MLN4924 (0.2 mg/kg, n=5) and LPS+MLN4924 (0.5 mg/kg, n=5). LPS (10 mg/kg; Sigma, USA) and MLN4924 (TargetMol, USA) were injected through the tail vein. For the LPS+MLN4924 group, LPS and MLN4924 were administered simultaneously. Mice treated with saline were used as control. After intervening for 24 hours, the animals were sacrificed by CO₂ asphyxiation followed by the blood samples and renal tissues collection, which were used for biochemical analysis and morphologic studies, respectively.

Cell culture

HK2 cell line was purchased from ATCC (CRL-2190, USA) and cultured in cell incubator under 5% CO₂ at 37°C in Dulbecco's modified Eagle medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA). LPS was dissolved using sterilized phosphate-buffered saline.

Cell viability test

Cell viability was examined by a CCK-8 test. Briefly, HK2 cells were seeded in 96-well cell culture plates, and treated with MLN4924 or/and 2 μg/ml LPS. After treating for 24 hours, the medium was discarded and changed with 100 μl fresh DMEM medium which contains 10 μl of CCK-8 solution (Cell Counting Kit-8, Dojindo, Japan) and incubated for 4 h at 37°C. Then the absorbance was measured at 450 nm with a microplate reader (Infinite F50, TECAN, Switzerland). After subtracting the blank absorbance values, the subsequent analyses were conducted. The growth inhibition of HK2 cells was calculated as the percent viability compared with the control group (100%). The effect of MLN4924 on cell viability at the selected concentration was also analyzed by using a Live/Dead Cell kit (Life Tech, USA). After a 72-h culture, cells were treated with 500 μL of prepared dye for 10 min in the incubator and detected by fluorescence microscopy (Zeiss, Axio M1, Germany).

Cytokine assays

The creatinine concentrations of mice blood were determined using an Auto Analyzer (Roche Diagnostics, Germany). The cell culture medium were collected and the concentration of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 were measured by using the enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Inc, USA). Briefly, HK2 cells were plated in 24-well plates, with or without treatment of MLN4924 (10, 50 and 100 nM) and 2 μg/ml LPS for 24 h. Then, a 100 μl aliquot of supernatant culture medium was collected to determine the cytokine concentrations by the ELISA kits.

Histology and immunohistochemistry analysis

For histologic analysis, the mice were sacrificed and kidney tissues were fixed with 4% buffered
paraformaldehyde for 24 hours. Then they were embedded by using paraffin, and 5 μm thick continuous sections were obtained. H&E staining was carried out and the histologic changes were scored with the criterion in previous studies. According to the percentage of tubules that displayed damaged change they were scored as follows: no damage scores 0, 10% of tubules were damaged scores 1, 11-25% damage scores 2, 26-45% damage scores 3, 46-75% damage scores 4, and over 76% of damage scores 5 [15, 16]. For immunohistochemistry analysis, a mouse monoclonal against the active form of the p65 subunit of NF-κB (specifically against the NLS) (Chemicon, Harrow, UK) was used to detect its nuclear localization at a dilution of 1:200.

Confocal microscopy

After intervention, the HK2 cells were collected fixed in 4% formaldehyde. Then cells were permeabilized with 0.3% Triton X-100 for 0.5 h and blocked with 2% BSA. Next, HK2 cells were first incubated with anti-p65-NF-κB antibody (1:100, Cell Signaling Technology, USA) at 4°C overnight and then incubated with the FITC-conjugated goat anti-mouse IgG secondary antibody (1:400, Cell Signaling Technology, USA). And DAPI was used to stain cell nuclei. Finally the immunofluorescence images were obtained by using confocal microscopy (Zeiss LSM800, Germany). And images were produced using Zeiss Microscopy (Axio Imager, Germany).

Quantitative real-time PCR

Total RNA of the HK2 cells was obtained using Trizol (Ambion) according to the manufacturer's specification. Complementary DNA was obtained from 1 μg of total RNA by using the special PrimeScript RT reagent kit (TaKaRa, Japan) according to the manufacturer's direction. Real-time PCR was performed using a SYBR Green qRT-PCR kit (TaKaRa, Japan) and an ABI Step One Plus Real-Time PCR System (Applied Biosystems, USA). β-actin was set as the internal control. Each test was performed in triplicate. The primers used were listed as follows: β-actin sense, GAAGGAAAAGAAGTGCTCATG; and IL-6, sense, CTGGTCTTTTGGAGTGGAG and antisense, GGTCAAGGGTTGTATTG. The relative mRNA expression changes were calculated by using the 2-ΔΔCt method.

Cytosolic and nuclear protein preparation

Whole proteins of HK2 cells were obtained basing on the routine procedure in our lab by using cell lysis buffer. The buffer is made of 20 mM Tris (pH 7.5), 135 mM NaCl, 2 mM EDTA, 2 mM DTT, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM NaF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM PMSF. And the cytosolic and nuclear extracts were obtained using a kit (Beyotime, China) according to the manufacturer's instruction.

Western blot

After treatment, the proteins of HK2 cells were obtained and their concentration were measured by using a BCA kit. The protein samples were first fractionated by SDS-PAGE. Then they were moved to a polyvinylidene difluoride membrane (Millipore, US). Next, they were blocked with 5% skim milk for 60 min and then they were incubated with primary antibodies overnight at 4°C. The primary antibodies used were at dilutions of 1:5000 for β-actin (Sigma), 1:1000 for Nedd8 (Cell Signaling), 1:1000 for IκB-α (Cell Signaling), 1:1000 for phosphorylated IκB-α (Cell Signaling), and 1:1000 for NF-κB p65 antibody (Abcam). After washing with PBST for three times the membranes were treated with HRP-conjugated secondary antibodies (Cell Signaling Technology) for 2 hours and then were visualized by using of an enhanced chemiluminescence detection system as suggested by the manufacturer. HDAC1 and β-actin (Santa Cruz, CA, USA) were set as internal controls. The expression of the proteins were quantified according to the gray value by using Image-J software.

Luciferase assays

293T cells were transfected with 2 μg of pNF-κB-MetLuc2-Reporter plasmid (Sangon, China) using an Amaxa Nucleofector II apparatus along with the Amaxa Cell Line Nucleofector V Kit (Lonza, Switzerland) in accordance with the
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manufacturer’s protocol. After transfection, cells were plated onto a 24-well dish (10⁵ cells/well) and stimulated with LPS (2 μg/ml) for 24 h, with or without MLN4924. The luciferase activity was measured using the Ready-To-Glow™ NFκB Secreted Luciferase Reporter System according to the manufacturer’s protocol. Luciferase activity in the culture media, which was detected and measured using a microplate reader (Infinite F50, TECAN, Switzerland), was normalized to the total protein concentration of the corresponding whole cell lysates.

Statistical analysis

The data were expressed as the means ± SEM, and each test was repeated at least for three times. Measurement of the statistical significance of the difference was taken by way of Student’s t-test for two group comparison or one-way ANOVA followed by Tukey’s multiple comparison test. The SPSS software was used to process statistical analysis. P < 0.05 was set as the statistical significance unless otherwise indicated.

Results

MLN4924 inhibits LPS-induced production of proinflammatory cytokine in HK2 cells

Previous studies have shown that treating HK2 cells with LPS provokes the production of pro-inflammatory cytokines, including IL-1β, TNF-α and IL-6 [11, 18, 19]. We then evaluated whether MLN4924 would exert anti-inflammatory effect. And to make it clear that whether MLN4924 inhibits LPS-induced cytokine expression and external secretion, we collected the culture media of LPS-induced HK2 cells and analyzed the concentration of IL-1β, TNF-α and IL-6 protein. It was found that MLN4924 dramatically reversed the increases of IL-1β, TNF-α and IL-6 induced by LPS when using at a concentration of 50 and 100 nM (Figure 2A-C), and no effect on the excretion of these factors when used at a concentration of 10 nM. Similarly, the result of western blot analysis also revealed that LPS-induced excretion of IL-1β, TNF-α and IL-6, and that the effect of LPS was significantly inhibited by MLN4924 (Figure 2G-K).

Because LPS could regulate both IL-6 and TNF-α expression at transcription level [20, 21], we then detected that whether MLN4924 could inhibit the transcriptional activation of these pro-inflammatory cytokines induced by LPS. As shown in Figure 2D-F, MLN4924 at 50 and 100 nM significantly prevented LPS-induced mRNA increase of these cytokines. These data suggest that MLN4924 at sublethal doses, simultaneously led to a significant decrease of multiple proinflammatory cytokines in LPS-induced HK2 cells at the protein and transcriptional levels.

MLN4924 affects neddylation and subsequent IκB degradation in HK2 cells

Then, we determined whether MLN4924 would inhibit LPS-induced neddylation. As shown in Figure 3, Nedd8 antibody detects a Nedd8-conjugated protein band at approximately 90 kDa, the right molecular weight for a neddylated Cullin protein (Figure 3A, top panel) [17, 22]. MLN4924 reduced the increased expression of Nedd8-conjugated protein induced by LPS. Furthermore, we also examined the expression of neddylated Cullin1 protein (Figure 3A, middle panel), and MLN4924 also prevented LPS-induced reduction [23].
It has been proved that treating HK2 cells with LPS leads to the increase of IκB at both mRNA and protein level [22]. Our results show that when treating with LPS, the IκB protein levels were significantly reduced within 15 min and recovered after 6 h (Figure 3C, middle panel). We then detected the change of IκB phosphorylation and found that it is coincide with the decrease of IκB, showing that the levels of phosphorylated IκB increased 1 hour later and returned to low levels 6 h later when treated with LPS (Figure 3C, top panel). We then determined whether MLN4924 would promote the degradation of IκB in HK2 cells treated with LPS, since IκB is a target of CRLs. However, when treating HK2 cells with MLN4924 at 100 nM at the same time, the LPS-induced decrease in IκB levels was restrained (Figure 3D, middle panel).
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Panel). In contrast, the levels of phosphorylated IkB increased within 15 min and went on elevating for the next 6 h when treating with LPS (Figure 3D, top panel). These data indicate that MLN4924 prevented the degradation of phosphorylated IkB in HK2 cells through inhibiting Cullin neddylation and Cullin-mediated of IkB degradation.

MLN4924 affects translocation of nuclear NF-κB in LPS-induced HK2 cells

To further reveal the mechanism of MLN4924 for the protective effect on LPS induced HK2 cells, we next assessed whether MLN4924 could prevent LPS-induced NF-κB transcription. After treating HK2 cells with LPS with or without MLN4924, p65 NF-κB nuclear translocation was determined by western-blots analysis and immunocytochemistry. The result revealed that p65 NF-κB was mainly in cytosolic fraction in non-treated cells, and its distribution was shifted to nuclear fraction after 6 h LPS treatment (Figure 4A, top panel). However, treatment with MLN4924 showed prevented LPS-induced shift of p65 NF-κB from cytosolic fraction to nuclear fraction (Figure 4A). The shift of p65 NF-κB was further evaluated by immunocy-

Figure 2. MLN4924 inhibited LPS-induced pro-inflammatory cytokines in HK2 cells. Summarized data showing the inhibitory effect of 0, 10, 50, 100 nM MLN4924 on LPS-induced extracellular cytokines (A-C) and mRNA (D-F) increase in TNF-α, IL-1β and IL-6 in HK2 cells. Representative western blot images (upper panel) and summarized data (lower panel) showing the inhibitory effect of 0, 10, 50, 100 nM MLN4924 on LPS-induced protein increase of TNF-α (G), IL-1β (H) and IL-6 (I). Data were shown are means ± S.E.M. (N=6). ###, P < 0.001 vs the group without LPS or MLN4924; *P < 0.05, **P < 0.01 vs LPS-treatment group without MLN4924; *, P < 0.05, **, P < 0.001 vs LPS-induced group.
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As shown in **Figure 5A**, the p65 NF-κB was exclusively stained in cytoplasmic and there was no obvious nuclear accumulation of the protein. However, the accumulation of p65 NF-κB in the nucleus was increased in immunocytochemistry after LPS treatment for 1 h (**Figure 5A**). In addition, there is no significantly difference of p65 NF-κB staining in HK2 cells treated with MLN4924 and non-treated control cells. These results suggest that MLN4924 damps the nuclear translocation of p65 NF-κB induced by LPS in HK2 cells.

Additionally, the effect of MLN4924 on LPS-induced NF-κB transcriptional activity was assessed in 293T cells using a luciferase reporter under control of a promoter containing NF-κB transcription factor binding sites (**Figure 5B**). Luciferase assays revealed that treating transfected cells with LPS caused a 6.2-fold increase in NF-κB transcriptional activity. Furthermore, this LPS-induced increase in NF-κB transcriptional activity was significantly inhibited by treatment of cells with either 100 nM MLN4924, suggesting that MLN4924 inhibits LPS-induced NF-κB transcriptional activity.

**MLN4924 inhibits renal damage and protect renal function through inhibiting NF-κB activation in LPS-treated mice**

When treating the mice with LPS alone, massive kidney damage was observed in the tubules. From the HE staining, cortex tubules manifested cellular necrosis and detachment, brush border destruction, cast formation, tubular vacuolization, and tubule dilation (**Figure 6A**). Then percentage of the damage was also scored (**Figure 6B**). The results revealed that the LPS-induced tubular injury was significantly inhibited by MLN4924 at the middle and high concentration (0.2 mg/kg and 0.5 mg/kg). However, treating with MLN4924 at low concentration (0.05 mg/kg) did not affect the LPS-induced tubular injury. Then we further analyzed the function of kidney by measuring...
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serum creatinine. As shown in Figure 6C, LPS dramatically increased the serum creatinine levels compared to control, which was significantly prevented by MLN4924. Finally, immunohistochemistry analysis was performed to detect the inhibitory effect of MLN4924 on LPS-induced NF-κB activation in vivo. As shown in Figure 6D, there were significant increases in nuclear NF-κB activity in kidney from LPS-treated mice. And this increase was significantly inhibited by high concentration of MLN4924 (0.05 mg/kg). These results suggest that MLN4924 inhibited renal damage and protect renal function through inhibiting NF-κB activation in LPS-treated mice.

Discussion

Septic AKI accounts for dramatic disability and mortality clinically. However, currently there are still no effective treatment therapies for this disease. In this research, we proved that MLN4924, a cancer therapy agent, plays a protective role in LPS-induced AKI. It’s well known that the inflammatory response is responsible for the initiation and development of renal injury, including sepsis-induced AKI and other chronic kidney disease such as lupus nephritis (LN) [24, 25]. The generation of numerous inflammatory molecules, including cytokines (TNF-α, IL-1β and IL-6), had been proved to accelerate the development of these diseases. In this study, we revealed that MLN4924 decreased the synthesis and production of these inflammatory cytokines in response to LPS stimulation, both at the protein and transcriptional levels.

The inflammatory response during the pathogenic process in sepsis-induced AKI is mainly
depended on the activation of the NF-κB pathway [1, 26, 27]. Previous studies have proved that NF-κB could upregulate the synthesis and secretion of various pro-inflammatory factors,
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A

Saline  LPS  LPS+MLN4924(L)  LPS+MLN4924(M)  LPS+MLN4924(H)

B

![Bar graph showing tubular damage score](image)

C

![Bar graph showing serum creatinine level](image)

D

Saline  LPS  LPS+MLN4924(H)
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Figure 6. MLN4924 protected LPS-induced kidney damage in mice. Mice were treated with 10 mg/kg LPS with or without MLN4924 at the low (0.05 mg/kg), middle (0.2 mg/kg) and high (0.5 mg/kg) dosages for 24 h. Mice treating with saline was set as control. A. Representative hematoxylin & eosin staining images showing the protective effect of low (0.05 mg/kg), middle (0.2 mg/kg) and high (0.5 mg/kg) dosages of MLN4924 on LPS-induced tubular injury. B. Summarized tubular damage scores showing the protective effect of low (0.05 mg/kg), middle (0.2 mg/kg) and high (0.5 mg/kg) dosages of MLN4924 on LPS-induced tubular injury. C. Summarized data showing the protective effect of low (0.05 mg/kg), middle (0.2 mg/kg) and high (0.5 mg/kg) dosages of MLN4924 on kidney function in the serum creatinine measurement. D. Representative immunohistochemistry images showing the inhibitory effect of MLN4924 on LPS-induced changes in P65-NF-κB protein in vivo. Data were shown are means ± S.E.M. (N=5). ###P < 0.001 vs the group without LPS or MLN4924 treatment; *P< 0.05 vs LPS-treated group without MLN4924.

Figure 7. MLN4924 treatment of LPS-induced AKI in a mouse model via inhibition of CRL-mediated activation of the NF-κB pathway.

such as TNF-α, IL-1β, and IL-6. When binding to its inhibitory protein, IκB, the P65/P50-NF-κB protein usually exists in cell cytoplasm in its inactive state. And when stimulating with LPS, the subunits of IKK-α, IKK-β, and IKK-γ will interact with each other to form the IKK complex protein and then phosphorylate the IκB. After phosphorylation, IκB became ubiquitylated and then degraded quickly, which permitted the P65/P50-NF-κB protein to translocate into the nucleus, thereby activating the inflammatory responses [28]. In this study, we have shown that MLN4924 significantly reversed the decreases of IκB protein level and the translo-
cation and transcriptional activation of NF-κB in
LPS induced HK2 cells. What is noteworthy is
that MLN4924 inhibits LPS-induced cytokine
generation through the regulation of neddy-
dylation and degradation of IκB. Furthermore,
our in vivo study shows that MLN4924 signifi-
cantly decreased LPS-induced renal damage
and improved kidney function in LPS-induced
mice. Therefore, MLN4924 might be used as
anti-inflammatory agents through inhibiting
IKK/IκB/NF-κB pathway.

Notably, the inhibitory effect of MLN4924 on
LPS-induced cytokine upregulation was differ-
et with dexamethasone in the molecular
mechanisms [19]. If this is the case, then
MLN4924 might serve as an alternative treat-
ment for diseases concomitant with a defective
immune response, including septic shock,
chronic liver disease, liver cancer, or metabolic
disorders. Furthermore, when using simultane-
ously, dexamethasone and MLN4924 might
exist synergistic effect with better result.
Finally, targeting neddylation may server as an
effective alternative for those who are resistant
to dexamethasone therapy [29-32].

Conclusion

In summary, the present research demonstrat-
ed that MLN4924 prevented synthesis and pro-
duction of inflammatory factors induced by LPS
through CRL/NF-κB signaling pathway via inhi-
bition of neddylation (Figure 7). The neddy-
dylation process might be a novel target for attenuating
proinflammatory cytokine production in septic
AKI.

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Disclosure of conflict of interest

None.

Abbreviations

AKI, acute kidney injury; NEDD8, neural precur-
sor cell expressed developmentally down-regu-
lated 8; ELISA, enzyme-linked immunosorbent
assay; IL-1β, interleukin-1β; IL-6, interleukin-6;
LPS, lipopolysaccharide; NF-κB, nuclear factor-
κB; TNF-α, tumor necrosis factor-α.

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