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MiR-133a alleviates renal injury caused by sepsis by targeting BNIP3L

L.-Y. QIN[1], M.-X. WANG[1], H. ZHANG[2]

[1]Department of Emergency, Beijing Friendship Hospital, Capital Medical University, Beijing, China
[2]Department of Critical Medicine, Jining No. 1 People's Hospital, Jining, China

Abstract. – OBJECTIVE: Sepsis is an important cause of acute kidney injury (AKI), seriously jeopardizing the health of patients. This paper's aim was to investigate whether microRNA-133a had a protective effect on sepsis-induced kidney injury.

MATERIALS AND METHODS: We established a kidney injury model with lipopolysaccharide (LPS) and divided TCMK-1 cells into 4 groups: control group (con); LPS treatment group; LPS + negative control (NC) treatment group; LPS + miR-133a mimic (mim) group. The expressions of miR-133a, TNF-α mRNA, IL-6 mRNA, Bax mRNA, Bcl-2 mRNA, BNIP3L mRNA, IκKα Mrna and IκB-α mRNA were detected by PCR. Western blot was used to detect the protein expression of TNF-α, IL-6, Bax, Bcl-2, BNIP3L, IκKα and IκB-α. Cell viability was measured by cell counting kit-8 (CCK-8). Flow cytometry was utilized to detect apoptosis rate. IL-1β immunofluorescence and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining were used to observe the inflammation and apoptosis in TCMK-1 cells.

RESULTS: The miR-133a expression was decreased in TCMK-1 cells treated with LPS. In the LPS treatment group, the expression of TNF-α, IL-6, Bax, BNIP3L and IκKα increased, and the expression of Bcl-2 and IκB-α decreased. When overexpressing miR-133a, the protein and mRNA expression of TNF-α, IL-6, Bax, BNIP3L and IκKα decreased markedly, while the expression of Bcl-2 and IκB-α increased markedly. Compared with the LPS-treated group, the apoptotic rate, the number of TUNEL-positive cells, and the immunofluorescence intensity of IL-1β in LPS+mim group were greatly decreased.

CONCLUSIONS: The miR-133a expression was decreased in TCMK-1 cells treated by LPS and miR-133a can inhibit inflammation and apoptosis of TCMK-1 cells induced by LPS by targeting BNIP3L *via* inhibiting NF-κB pathway.

Key Words:
Renal injury, Sepsis, MiR-133a, Lipopolysaccharide, BNIP3L.

Introduction

Sepsis is a clinically critical disease with serious life-threatening and poor prognosis, with nearly one-third of short-term prognosis mortality[1]. In patients with sepsis, multiple organ dysfunction syndrome is induced by systemic inflammatory response syndrome, which eventually leads to multiple organ failure[2]. Among them, acute kidney injury caused by sepsis is the most common, and it is also the main cause of poor prognosis of patients[3]. Despite the continuous development of ICU treatment levels and blood purification technologies, the overall mortality rate of acute kidney injury (AKI) patients remains high, so there is an urgent need to develop better ways to treat AKI[4].

Lipopolysaccharide (LPS) plays an important role in the pathogenesis of sepsis[5]. In sepsis endotoxemia caused by Gram-negative bacteria, LPS induces the release of inflammatory factors and activation of the endothelial cell coagulation system, resulting in multiple organ damage such as shock and AKI[6]. LPS is often used as a foreign toxin to establish a model of acute kidney injury in sepsis.

MicroRNAs (miRNAs) are small RNAs of non-coding proteins produced by a class of organisms that inhibit gene expression[7]. MiRNAs are widely involved in life processes and disease development including AKI[8]. MiR-133a has been shown to have anti-apoptotic and anti-inflammatory effects. Chen et al[9] showed that miR-133a can reduce cardiomyocyte apoptosis after myocardial infarction. Zhang et al[10] indicated that miR-133a can reduce inflammatory response in atherosclerosis. However, the function of miR-133a in AKI caused by sepsis is rarely known. In our paper, changes in miR-133a expression after LPS-induced inflammatory response and apoptosis in mouse renal tubular epithelial cells

Corresponding Author: Hao Zhang, BM; e-mail: zhanghao3383@163.com

(TCMK-1 cells) were observed to explore the function of miR-133a in AKI caused by sepsis.

Materials and Methods

Cell Culture

TCMK-1 cells (Procell, Wuhan, China) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Life Technology, Wuhan, China) containing 10% fetal bovine serum (FBS; Life Technology, Wuhan, China) and 1% penicillin/streptomycin (Life Technology, Wuhan, China), in 37°C, 5% CO_2 incubator. When the cells fused 80% to 90%, the cells were subcultured. The new medium was replaced the next day after subculture, and the medium was replaced every 2 to 3 days after that. LPS treatment group: TCMK-1 cells were treated with 500 ng/mL LPS (Sigma-Aldrich, St. Louis, MO, USA). Control group (con): TCMK-1 cells received an equal amount of phosphate-buffered saline (PBS). LPS + negative control treatment group (NC) and LPS + miR-133a mimic group (mim) were transfected with negative control and miR-133a mimic (RiboBio, Guangzhou, China), respectively and then treated with LPS.

Western Blot

After the cultured cells were attached, the medium was aspirated, and 1 mL of cold PBS was added and washed twice, shaking each time several times, and the medium was removed as much as possible. Phosphatase inhibitor (10 μL), protease inhibitor (1 μL) and phenylmethylsulfonyl fluoride (PMSF) (5 μL) per milliliter of precooled Lysis Buffer (KeyGen, Shanghai, China) and mix. Add 50 to 100 μL of Lysis Buffer per Petri dish or 6-well plate, then scrape the adherent cells with a brush and transfer to a 1.5 mL Eppendorf (EP; Eppendorf, Hamburg, Germany) tube. All samples were shaken vigorously for 30 s and then placed on ice for 4 min, repeated 5 times. After centrifugation at 12000 g for 20 min at 4°C, the supernatant is the total protein. The concentration was measured in line with the instructions of bicinchoninic acid (BCA) kit (Thermo Fisher Scientific, Waltham, MA, USA). Add 1 μL of protein loading buffer (Beyotime, Shanghai, China) to each 4 μL protein sample, mix by shaking, cover the tube with a sealing membrane, and heat it in boiling water for 5 min to fully denature the protein.

Dispense sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Beyotime, Shanghai, China) first, then perform electrophoresis (loading volume 30 μg), transfer the protein to polyvinylidene difluoride (PVDF) membrane (EpiZyme, Shanghai, China), block the protein bands with bovine serum albumin (BSA) for 1.5 h, wash the bands 3 times, incubate the bands with primary antibody (Bax, Abcam, Cambridge, MA, USA, Rabbit, 1:2000; Bcl-2, Abcam, Cambridge, MA, USA, Rabbit, 1:2000; TNF-α, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; IL-6, Abcam, Cambridge, MA, USA, Rabbit, 1:5000; BNIP3L, Abcam, Cambridge, MA, USA, Rabbit, 1:2000; IκKα, Abcam, Cambridge, MA, USA, Rabbit, 1:2000; IκB-α, Abcam, Cambridge, MA, USA, Rabbit, 1:2000; GAPDH, Proteintech, Rosemont, IL, USA, 1:3000) overnight, and wash the bands for the next day. After 3 times, the bands were incubated with the secondary antibody for 1 h, then the bands were washed 3 times, and finally the image was obtained with an Odyssey two-color infrared fluorescence scanning imaging system. The image J software (NIH, Bethesda, MD, USA) was used to analyze the gray value of the electrophoresis bands of each protein.

Cell Counting Kit-8 (CCK-8) Assay

CCK-8 (MCE, Nanjing, China) was utilized to detect the cell viability following the standard protocol.

Flow Cytometry

We collected the supernatant and collected the adherent TCMK-1 cells after trypsinization. Then, we centrifuged the cell suspension with centrifugation at 200 g for 5 min, discarded the supernatant, washed with PBS, and centrifuged again in the same manner, repeating twice. After the last centrifugation, the supernatant was discarded, and the cells were resuspended with 150 μL of binding buffer. We add 5 μL of Annexin V-FITC (fluorescein isothiocyanate) (KeyGen, Shanghai, China) and 5 μL of Propidium Iodide (PI; KeyGen, Shanghai, China) per tube in the dark. Finally, the apoptosis rate of cells was detected by flow cytometry.

Real Time-PCR

TRIzol kit (MCE, Nanjing, China) was utilized to extract total RNA from the cells. The total RNA content was determined by ultraviolet spectrophotometer. mRNA reverse transcription was performed using reverse transcription kit (MCE, Nanjing, China) according to the proto-

cols. SYBR Green qRCR Mix (MCE, Nanjing, China) was used to perform PCR. Use 10 μL of reaction system (SYBR: 5 μL, forward primer: 0.2 μL, reverse primer: 0.2 μL, cDNA: 1 μL, ddH_2O: 3.6 μL). Prism 7900 System (Applied Biosystems, Foster City, CA, USA) was utilized to perform PCR. We used U6 to normalize the expression of miR-133a and GAPDH to normalize the expression of TNF-α, IL-6, Bax, Bcl-2, BNIP3L, IκKα and IκB-α. All the primers were listed in Table I.

IL-1β Immunofluorescence

TCMK-1 cells were treated with tissue fixative for 15-20 min. After washing three times with PBS, an appropriate amount of Triton (Service, Wuhan, China) was added. After 20 min, the cells were washed with PBS and then blocked with goat serum for 1 hour. Then, add primary antibody IL-1β overnight. After that, incubate with the secondary antibody for 1 hour. Add 4',6-diamidino-2-phenylindole (DAPI; KeyGen, Shanghai, China) after washing PBS three times. Finally, the immunofluorescence was observed by a Confocal Laser Scanning Microscope (CLSM; Olympus, Tokyo, Japan).

Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP Nick End Labeling (TUNEL) Staining

TUNEL kit (Roche, Basel, Switzerland) was used as instructed by the manufacturer to detect the apoptotic cells. The nucleus was stained with DAPI. TUNEL staining was showed by CLSM.

Luciferase Activity Assay

The plasmids which contained wild-type (WT) or mutant (MUT) 3′-UTR of BNIP3L were co-transfected with mim or NC into HEK293T cells. After two days, Luciferase reagent (Ribo-Bio, Guangzhou, China) was added according to the protocols and the Luciferase activity was detected using Dual-Glo® Luciferase Assay System.

Statistical Analysis

Data were expressed as $\bar{x} \pm s$ and were plotted using GraphPad Prism5 software (La Jolla, CA, USA). The *t*-test was utilized to compare the mean between two groups. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Test level α=0.05.

Results

MiR-133a Expression Decreased in LPS-Treated TCMK-1 Cells

To derive the optimal dose of LPS for induction of acute injury to TCMK-1 cells, we performed a CCK-8 assay. We treated TCMK-1 cells with different concentrations of LPS and tested cell viability after 24 hours. As can be seen from Figure 1A, when the concentration of LPS was 500 ng/mL, cell viability was inhibited by about half, so we chose a concentration of 500 ng/mL for subsequent experiments. Then, we used PCR to measure the level of miR-133a in LPS-treated TCMK-1 cells. We found that miR-133a was decreased in TCMK-1 cells of the LPS-treated group compared to the control group (Figure 1B). To investigate the function of miR-133a in LPS-induced acute tubular epithelial cell injury, we transfected TCMK-1 cells with miR-133a mimic or NC. After transfection of mimic, miR-133a was increased in the cells (Figure 1C).

Table I. Real time PCR primers.

Gene name	Forward (5′ > 3′)	Reverse (5′ > 3′)
Bax	CAGTTGAAGTTGCCATCAGC	CAGTTGAAGTTACCATCAGC
Bcl-2	GACTGAGTACCTGAACCGGCATC	CTGAGCAGCGTCTTCAGAGACA
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
IL-6	ACAGAAGGAGTGGCTAAGGA	AGGCATAACGCACTAGGTTT
BNIP3L	GAAAGCGGCACAGAGAAC	TGCTGATGAGACAGACGAA
Ikkα	GTCAGGACCGTGTTCTCAAGG	GCTTCTTTGATGTTACTGAGGGC
IκB-α	GGATCTAGCAGCTACGTACG	TTAGGACCTGACGTAACACG
miR-133a	CTGCAGCTGGAGAGTGTGCG	GTGCTCTGGAGGCTAGAGGT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

qRT-PCR, quantitative reverse-transcription polymerase chain reaction.

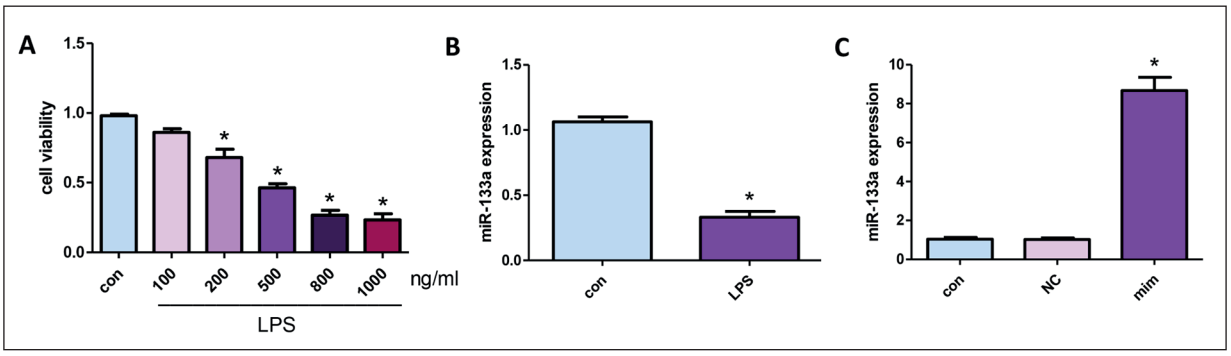

Figure 1. MiR-133a expression decreased in LPS-treated TCMK-1 cells. **A,** CCK-8 assay showed TCMK-1 cells viability at different concentrations of LPS ("*" $p<0.05$ *vs.* control, n = 3). **B,** Real-time PCR analysis showed the downregulation of miR-133a expression in TCMK-1 cells treated with 500 ng/mL LPS for 24 h ("*" $p<0.05$ *vs.* control, n = 3). **C,** PCR showed that miR-133a mimic increased the expression of miR-133a in TCMK-1 cells ("*" $p<0.05$ *vs.* NC, n = 3).

MiR-133a Inhibited LPS-Induced Inflammation of TCMK-1 Cells

We first examined TNF-α and IL-6 expression in different groups by Western blot. Induction of LPS markedly increased TNF-α and IL-6 expression in TCMK-1 cells while overexpression of miR-133a greatly inhibited the expression of both (Figure 2A-2C). Moreover, we detected the mRNA expression of them by PCR, and the results were similar to their protein expression (Figure 2D and 2E). At the same time, IL-1β immunofluorescence showed that the IL-1β fluorescence density of the LPS+mim group decreased markedly compared with the LPS+NC group (Figure 2F). These results indicated that LPS can promote the inflammatory response of TCMK-1 cells, while overexpression of miR-133a can inhibit inflammation.

MiR-133a Inhibited LPS-Induced Apoptosis of TCMK-1 Cells

First, Western blot was utilized to measure the expression of Bax and Bcl-2 proteins in the four groups. As can be seen from Figure 3A-3C, LPS can greatly reduce the expression of Bcl-2 and increase the Bax expression, but miR-133a overexpression could reverse the expression of both proteins. We then examined the expressions of Bax mRNA and Bcl-2 mRNA. Similarly, we can see that overexpression of miR-133a reversed the increase of Bax mRNA induced by LPS, and meanwhile reversed the decrease of Bcl-2 mRNA induced by LPS (Figure 3D and 3E). In addition, we assayed TUNEL-positive apoptotic TCMK-1 cells. Significant reduction of TUNEL-positive cells was noticed in LPS+mim group (Figure 3F). We also utilized flow cytometry to detect the apoptosis rate of the 4 groups. We can see that the overexpression of miR-133a can greatly reduce the increased apoptosis percentage caused by LPS treatment (Figure 3G and 3H). These showed that miR-133a could inhibit the apoptosis induced by LPS in TCMK-1 cells.

MiR-133a Inhibited Inflammation and Apoptosis in TCMK-1 Cells by Targeting BNIP3L Via Inhibiting NF-κB Pathway

To further explore the mechanism of action of miR-133a, we predicted the target of miR-133a using the StarBase database. And BNIP3L was predicted to have a binding site to miR-133a (Figure 4A). Western blot analysis showed that the expression of BNIP3L was markedly decreased in LPS+mim group compared to the LPS+NC group (Figure 4B and 4C). In the LPS treatment group, the expression of IκKα was greatly increased, and the expression of IκB-α was greatly decreased, while in the LPS+mim group, the expression of both proteins was reversed (Figure 4D and 4E). The mRNA levels of BNIP3L, IκKα and IκB-α detected by PCR were consistent with the results of Western blot (Figure 4F-H). To investigate whether miR-133a is directly linked to the 3′-UTR of BNIP3L, we performed Luciferase reporter gene experiment. We co-transfected miR-133a and the reporter plasmids into HEK293T cells. MiR-133a overexpression significantly inhibited Luciferase activity in the WT group but failed to inhibit activity in the mutant 3′-UTR (Figure 4I).

Discussion

Sepsis has the characteristics of high prevalence, high mortality, high treatment cost, etc. It

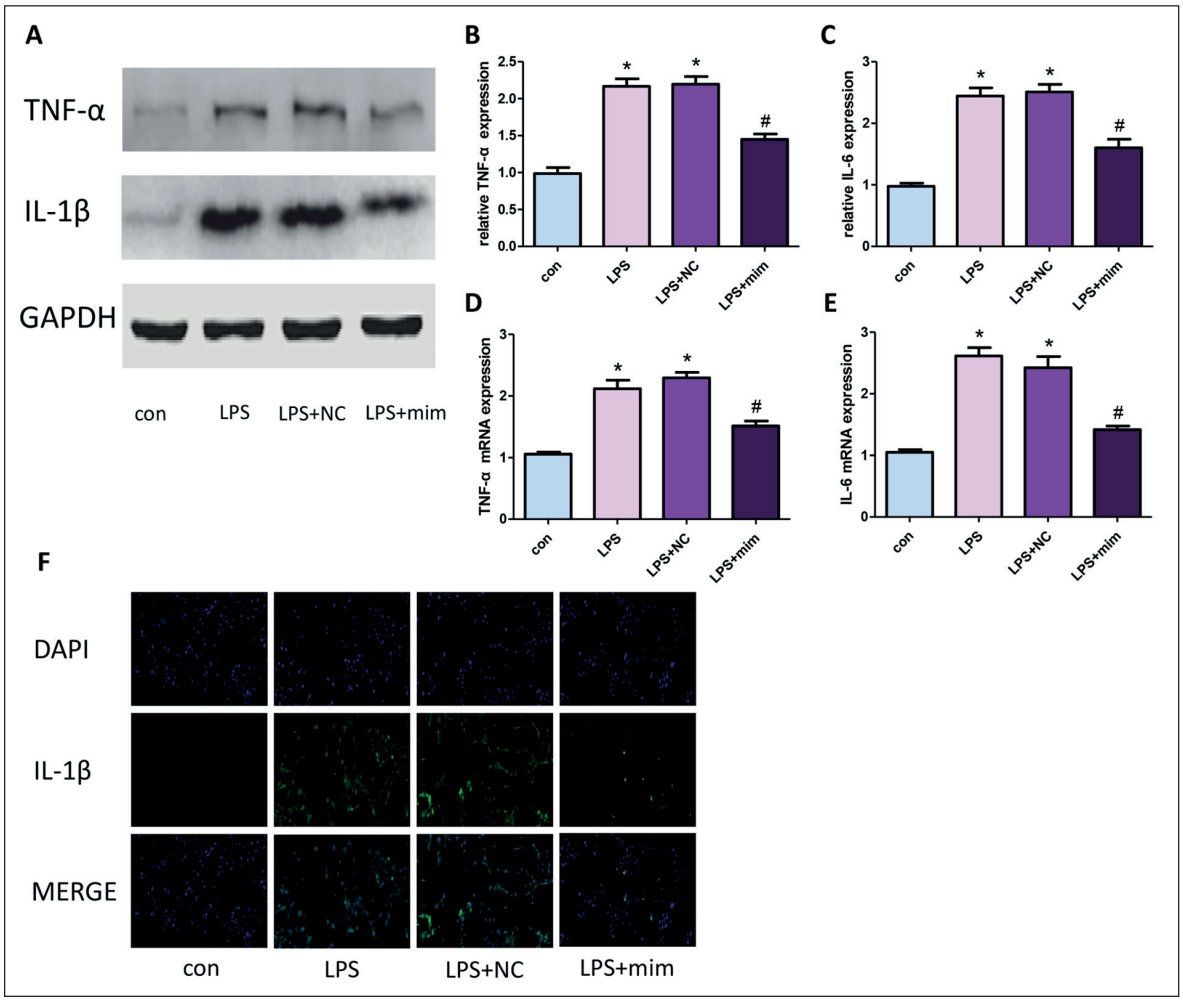

Figure 2. MiR-133a inhibited LPS-induced inflammation. **A,** The expression of IL-6 and TNF-α in LPS treatment group increased significantly, but decreased in LPS+mim group. **B,** Statistical results of expression of TNF-α ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **C,** Statistical results of expression of IL-6 ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **D,** TNF-α mRNA expression was similar to the results of Western blot ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **E,** IL-6 mRNA expression was also similar to the results of Western blot ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **F,** Immunofluorescence showed that miR-133a significantly reduced LPS-induced elevation of IL-1β (magnification: 400×).

is one of the most concerned clinical problems in the field of severe medicine, and it is also the main cause of death in ICU patients[11]. The occurrence of AKI in patients with sepsis is as high as 47.9%, and the mortality rate in 28 days is 32.7%[12]. In patients with sepsis, endotoxin and excessive inflammatory mediators destroy the balance of the clot-fibrinolysis system, promote the formation of microthrombus in renal microvessels, reduce glomerular perfusion pressure and filtration rate, and cause AKI[13].

MiRNAs not only function in many diseases such as tumor, diabetes mellitus, inflammatory bowel disease and kidney disease, but also act in immune inflammatory response[14]. MiRNA not only directly regulates the expression of pro-inflammatory and anti-inflammatory factors, but also indirectly by regulating other key molecules in the sepsis signaling pathway, maintaining a balance between the two[15]. Herein, we demonstrate that LPS can induce inflammation in TCMK-1 cells, and that miR-133a can inhibit inflammatory responses. At the same time, miRNA is also involved in the control of apoptosis[16]. We found that LPS induced apoptosis in TCMK-1 cells, whereas miR-133a greatly inhibited apoptosis.

BCL2/adenovirus E1B interacting protein 3-like (BNIP3L) belongs to the Bcl-2 family and is a BH3-only pro-apoptotic factor. The BNIP3L gene is

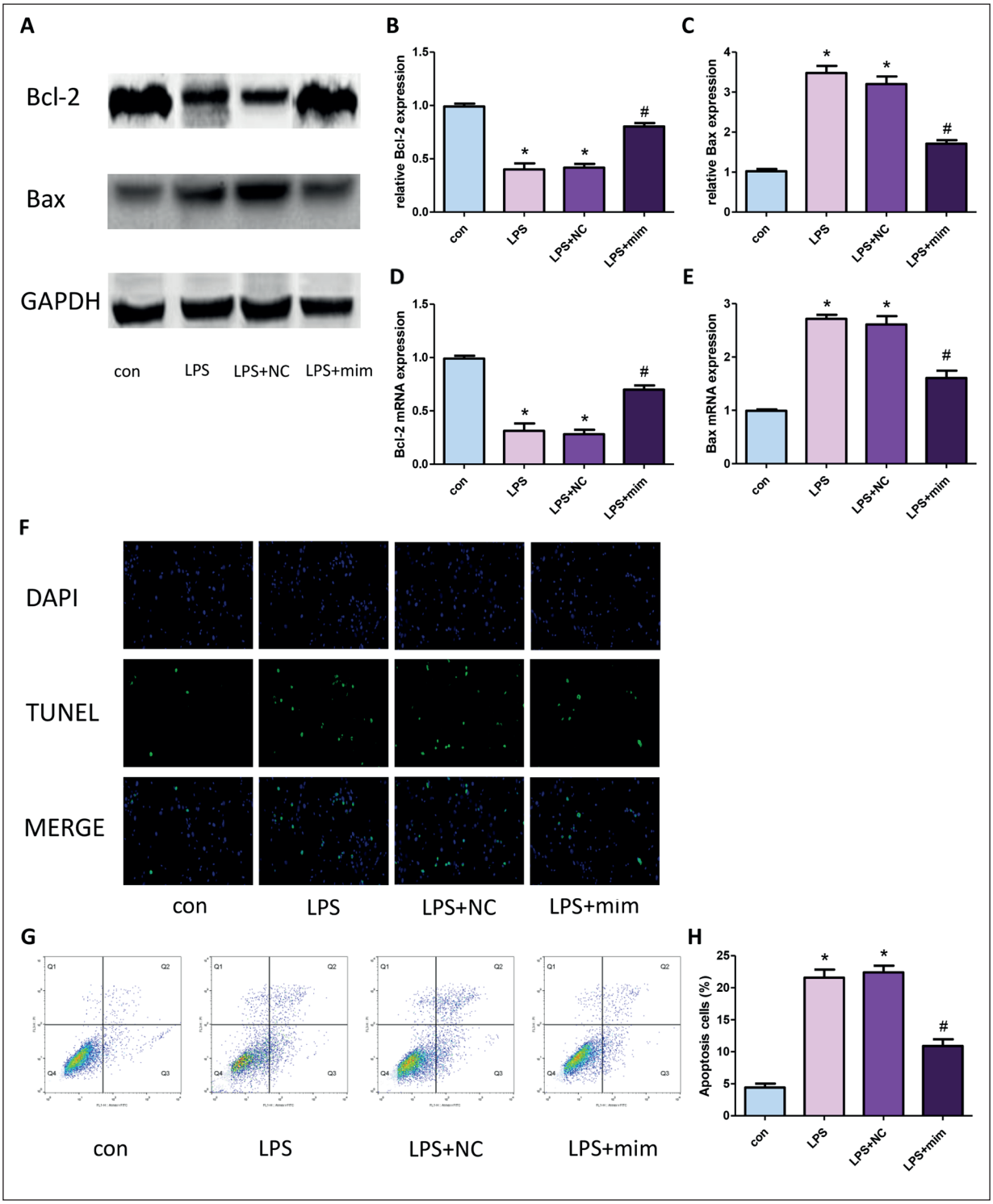

Figure 3. MiR-133a inhibited LPS-induced apoptosis. **A,** Expression of Bcl-2 in LPS treatment group decreased significantly but increased in the LPS+mim group. Bax expression was opposite to Bcl-2. **B,** Statistical results of protein level of Bcl-2 ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **C,** Statistical results of expression of Bax ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **D,** Expression of Bcl-2 mRNA was consistent with the Bcl-2 protein ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **E,** Expression of Bax mRNA was consistent with the Bax protein ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **F,** TUNEL staining showed that miR-133a mimic can remarkably reduce the increase of TCMK-1 cell apoptosis caused by LPS (magnification: 400×). **G,** Apoptotic rate of LPS treatment group increased, and decreased in LPS+mim group. **H,** Statistical results of apoptotic rate of TCMK-1 cells ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3).

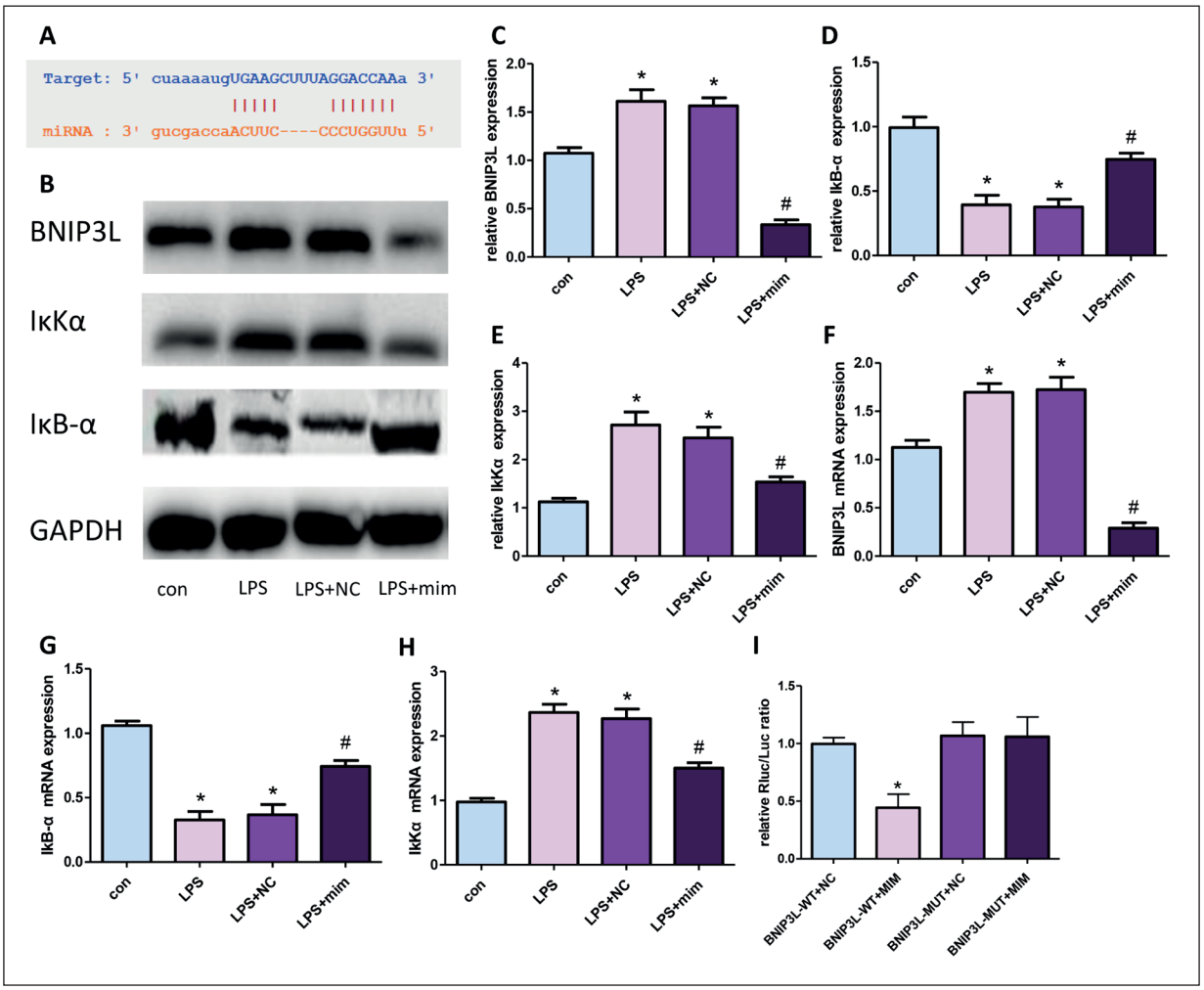

Figure 4. MiR-133a targeted BNIP3Land inhibited NF-κB pathway. **A,** Binding site predicted by the StarBase database. **B,** Western blot showed that the expression of BNIP3L, IκB-α and IκKα. **C,** Statistical results of protein level of BNIP3L ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **D,** Statistical results of protein level of IκB-α ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **E,** Statistical results of protein level of IκKα ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **F,** The expression of BNIP3L mRNA ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **G,** Expression of IκB-α mRNA ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **H,** Expression of IκKα mRNA ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* LPS+NC, n=3). **I,** MiR-133a overexpression significantly decreased the relative Luciferase activity in WT group but did not decrease the relative Luciferase activity in mutant group ("*" $p<0.05$ *vs.* WT+NC, n = 3).

a gene with tumor suppressor activity cloned from human fetal liver cDNA library and is located in the high frequency heterozygous loss region of 8p21 lung cancer[17]. BNIP3L protein can interact with anti-apoptotic proteins including Bcl-2 and E1B19K to promote apoptosis[18]. Moreover, BNIP3L mediates the intrinsic apoptotic pathway through the permeabilization of mitochondrial outer membrane. There have been many articles exploring the pro-apoptotic effects of BNIP3L. In this paper, we demonstrate that miR-133a can directly target BNIP3L, thereby inhibiting cell apoptosis.

NF-κB is an important transcription factor, located in the cytoplasm in an inactive state, consisting of the dimeric p50/p65 and another inhibitory subunit IκB[19]. The binding of the IκB subunit to the p65 subunit masks the nuclear transfer signal of NF-κB, making NF-κB unable to reach the receptor of the nuclear pore complex and not reach the nucleus[20]. When IκB is catalyzed by IκB kinase, it undergoes phosphorylation and degradation, which causes NF-κB to rapidly activate and transfer into the nucleus, and binds to numerous gene promoters to medi-

ate TNF-α, IL-6, Bcl-2, BCL-XL, COX-2, IL-1β and other gene expression, thereby regulating inflammation, apoptosis, proliferation and other pathophysiological processes[21]. In this paper, we found that miR-133a inhibited inflammation and apoptosis in TCMK-1 cells by targeting BNIP3L *via* inhibiting NF-κB pathway.

Conclusions

Summarily, miR-133a expression was decreased in LPS-treated TCMK-1 cells, while miR-133a overexpression inhibited TCMK-1 inflammation and apoptosis. This effect is achieved by targeting BNIP3L *via* inhibiting NF-κB pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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