

# miR-494-3p regulates lipopolysaccharide-induced inflammatory responses in RAW264.7 cells by targeting PTEN

SI ZHANG<sup>1</sup>, KANG HE<sup>2</sup>, WEIWEI ZHOU<sup>1</sup>, JUN CAO<sup>1</sup> and ZUOLIN JIN<sup>1</sup>

<sup>1</sup>State Key Laboratory of Military Stomatology and National Clinical Research Center for Oral Diseases and Shaanxi Clinical Research Center for Oral Diseases, Department of Orthodontics, School of Stomatology, The Fourth Military Medical University, Xi'an, Shaanxi 710032; <sup>2</sup>Department of Periodontology, College of Stomatology, Xi'an Jiaotong University, Xi'an, Shaanxi 710004, P.R. China

Received May 28, 2018; Accepted January 28, 2019

DOI: 10.3892/mmr.2019.10083

**Abstract.** MicroRNAs (miRNAs/miRs) serve important roles in regulating inflammatory responses at the post-transcriptional level. In the present study, the limma package was used to analyze the GSE43300 array dataset downloaded from the Gene Expression Omnibus database. It was identified that several miRNAs, including miR-494-3p, were upregulated in lipopolysaccharide (LPS)-treated RAW264.7 macrophages compared to control cells. Transfection experiments indicated that overexpressing miR-494-3p inhibited production of LPS-induced proinflammatory cytokines, including interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$ . Conversely, knockdown of miR-494-3p enhanced cytokine expression. Bioinformatics prediction and luciferase assay both revealed that miR-494-3p could directly target phosphatase and tensin homolog (PTEN) and upregulate protein kinase B activity. In addition, miR-494-3p mimics suppressed p65 translocation to the nucleus. Similar effects were observed following PTEN silencing. In conclusion, the results of the present study revealed that miR-494-3p may act as an important immune regulator in LPS-stimulated macrophages, and be an effective therapeutic target for treating infections in the future.

## Introduction

Toll-like receptor (TLR)-mediated production of proinflammatory cytokines is necessary to eliminate invading

pathogens. However, uncontrolled TLR activation can result in many diseases, including diabetes (1), obesity (2), cancer (3) and root resorption (4). Lipopolysaccharide (LPS) triggers innate immunity and activates multiple signaling pathways in macrophages, which results in the production of inflammatory cytokines (5,6). These activities are essential for the control and maintenance of balanced inflammatory reactions.

MicroRNAs (miRNAs/miRs), a class of short non-coding RNAs that are 19-24 nucleotides in length, can bind to the 3'-untranslated region (UTR) of target genes, resulting in target mRNA degradation and translation inhibition (7). A number of miRNAs associated with LPS-induced inflammatory responses have been identified in macrophages, including miR-146a (8), miR-71 (9), miR-709 (10), miR-181a (11) and miR-27a (12). However, there are many uncharacterized miRNAs that need to be explored with respect to their function in the inflammatory response.

Phosphatase and tensin homolog (PTEN) is involved in the tumorigenesis of many malignancies (13). PTEN is a lipid phosphatase that can dephosphorylate phosphoinositol (3-5)-trisphosphate at position 3, thus antagonizing phosphoinositide 3-kinase (PI3K) signaling. PTEN is involved in various cellular processes, including proliferation, cellular architecture and survival (14). A previous study suggested that this protein may have an important role in inflammatory responses (15). Agrawal *et al* (16) reported that overexpression of PTEN in dendritic cells from elderly individuals significantly increases LPS-induced secretion of tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-6. In addition, Cao *et al* (17) demonstrated that PTEN was a positive mediator of macrophage inflammatory responses.

The aim of the present study was to investigate and characterize miRNAs that are differentially expressed in macrophages in response to LPS stimulation. The results indicated that miR-494-3p, which can target the 3'-UTR of the PTEN gene, was involved in LPS-induced immune responses. In addition, the mechanisms associated with this observation were determined. Exploring the role of miR-494-3p in LPS-stimulated macrophages, including RAW264.7 cells, may aid in the development of anti-miR-494-3p as novel therapeutic agents for inflammatory diseases.

**Correspondence to:** Professor Jun Cao or Professor Zuolin Jin, State Key Laboratory of Military Stomatology and National Clinical Research Center for Oral Diseases and Shaanxi Clinical Research Center for Oral Diseases, Department of Orthodontics, School of Stomatology, The Fourth Military Medical University, 145 West Changle Road, Xi'an, Shaanxi 710032, P.R. China  
E-mail: wykun@fmmu.edu.cn  
E-mail: zuolinj@163.com

**Key words:** microRNA-494-3p, phosphatase and tensin homolog, protein kinase B, lipopolysaccharide, RAW264.7

## Materials and methods

**Affymetrix microarray data.** The GSE43300 array dataset, based on the GPL7723 platform, was downloaded from the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>) database. These data were deposited by Chen *et al* (18). A total of two LPS-treated cell groups and two negative control groups were included in the present study. Raw data and annotation files were used for subsequent analysis.

**Data preprocessing.** The R statistical software (version 3.5.0) program in Bioconductor (version 3.8; <http://www.bioconductor.org/>) was used, as it provides a robust multiarray average algorithm to preprocess raw expression data. A total of 579 miRNA expression values were obtained and the limma package (19) in Bioconductor was used to analyze differential miRNA expression between the LPS-treated and control groups. The t-test in the limma package was used to calculate P-values for differential miRNA expression. Log<sub>2</sub>-fold change  $\geq 1.2$  and  $P < 0.05$  were used as the cut-off criteria. The pheatmap package (version 1.0.12) was used to generate the heat map presented in Fig. 1.

**Cell lines and transfection.** The RAW264.7 murine macrophage leukemia cell line and 293T cell line were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Biological Industries USA, Cromwell, CT, USA) and antibiotics (100 U/ml penicillin and 100  $\mu$ g/ml streptomycin) at 37°C with 5% CO<sub>2</sub>. Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to transfect cells with miR-494-3p mimics (5'-UGAAACAACACGGGAAA CCUC-3'; 20  $\mu$ M), miR-494-3p inhibitor (5'-GAGGUUCC CGUGUAUGUUUCA-3'; 20  $\mu$ M), negative control (5'-UUC UCCGAACGUGUCACGUTT-3'; 20  $\mu$ M), inhibitor negative control (5'-CAGUACUUUUGUGUAGUACAA-3'; 20  $\mu$ M), short interfering RNA (siRNA)-PTEN (sense, 5'-CAAUC CAGAGGCUAGCAGUU-3' and antisense, 5'-CUGCUA GCCUCUGGAUUUGTT-3'; 20  $\mu$ M), siRNA negative control (sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'; 20  $\mu$ M; all from Shanghai GenePharma Co., Ltd., Shanghai China) and pmiRGLO Dual-Luciferase miRNA Target Expression Vector plasmids (500 ng/ $\mu$ l; Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. Following transfection for 48 h, cells were continuously stimulated with LPS at a concentration of 1  $\mu$ g/ml for 24 h at 37°C in the presence of 5% CO<sub>2</sub>. Total RNA and protein was subsequently extracted for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting analysis.

**RNA extraction and RT-qPCR.** RAW264.7 cells were seeded in 6-well plates and incubated overnight, following which cells were treated with various concentrations of LPS (0, 1, 2, 3 and 6  $\mu$ g/ml) for 24 h at 37°C in the presence of 5% CO<sub>2</sub>. The expression levels of IL-1 $\beta$  and TNF were detected by RT-qPCR. TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) was used to isolate total RNA from RAW264.7 cells. cDNA was synthesized using a PrimeScript™ RT reagent kit (RR037A;

Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocols. qPCR was performed using the SYBR-Green qPCR Master Mix (2X RealStar Green Power Mixture; A311-05; GenStar, Beijing, China) on an FTC-3000 thermal cycler (Funglyn Biotech Inc., Richmond Hill, ON, Canada). qPCR was performed as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. U6 and GAPDH were used as the internal reference genes. The 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method was used to calculate the relative expression of genes (20). All primers used in the study are listed in Table I. The RNA extraction and RT-qPCR experiments were repeated three times.

**Western blot analysis.** Radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors (Beyotime Institute of Biotechnology, Shanghai, China) was used to lyse RAW264.7 cells. Protein concentration was determined using a bicinchoninic acid kit (E162-01; GenStar). The nuclear protein was obtained using a Cytoplasmic and Nuclear Extract kit (KGP1100; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Protein (20  $\mu$ g/lane) was then added to 5X loading buffer, separated by 15% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk at room temperature for 1 h and incubated with primary antibodies overnight at 4°C:  $\beta$ -actin (1:1,000; cat. no. 4970; Cell Signaling Technology, Inc., Danvers, MA, USA); lamina (1:1,000; 10298-1-AP; ProteinTech Group, Inc., Chicago, IL, USA); IL-1 $\beta$  (1:1,000; cat. no. 31202; Cell Signaling Technology, Inc.); TNF- $\alpha$  (1:2,000; 60291-1-Ig; ProteinTech Group, Inc.); PTEN (1:1,000; cat. no. 9552; Cell Signaling Technology, Inc.); AKT serine/threonine kinase 1 (Akt1; 1:1,000; cat. no. 2967; Cell Signaling Technology, Inc.), phosphorylated (p)-Akt1 (1:1,000; cat. no. 9018; Cell Signaling Technology, Inc.) and p65 (1:1,500; ab16502; Abcam, Cambridge, UK). The membranes were subsequently incubated for 1 h at room temperature with donkey anti-rabbit immunoglobulin G secondary antibodies (1:2000; cat. no. 711-005-152; Jackson ImmunoResearch, West Grove, PA, USA). The protein bands were visualized using Immobilon™ chemiluminescent substrate (WBKLS0050; EMD Millipore).  $\beta$ -actin and lamina were used as the loading controls. Blots were scanned using a Bio-Rad ChemiDoc™ XRS (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the band densities were quantified using Quantity One® analysis software version 4.6.2 (Bio-Rad Laboratories, Inc.).

**Construction of luciferase reporter plasmid and dual luciferase assay.** Putative miRNA target genes were predicted by application of TargetScan 7.2 ([http://www.targetscan.org/mmu\\_72/](http://www.targetscan.org/mmu_72/)), picTar (<https://pictar.mdc-berlin.de/>), PITA version 6 ([https://genie.weismann.ac.il/pubs/mir07/mir07\\_data.html](https://genie.weismann.ac.il/pubs/mir07/mir07_data.html)) and miRanda version 3.3a (<http://www.microrna.org/microrna/home.do>). Venn diagram analysis (Venny; version 2.1; <http://bioinfogp.cnb.csic.es/tools/venny/>) was used to identify the intersection between the four target predictions; PTEN was identified as a candidate mRNA associated with miR-494-3p. To validate that miR-494-3p can target PTEN, a reporter plasmid containing the 3'-UTR sequence of PTEN

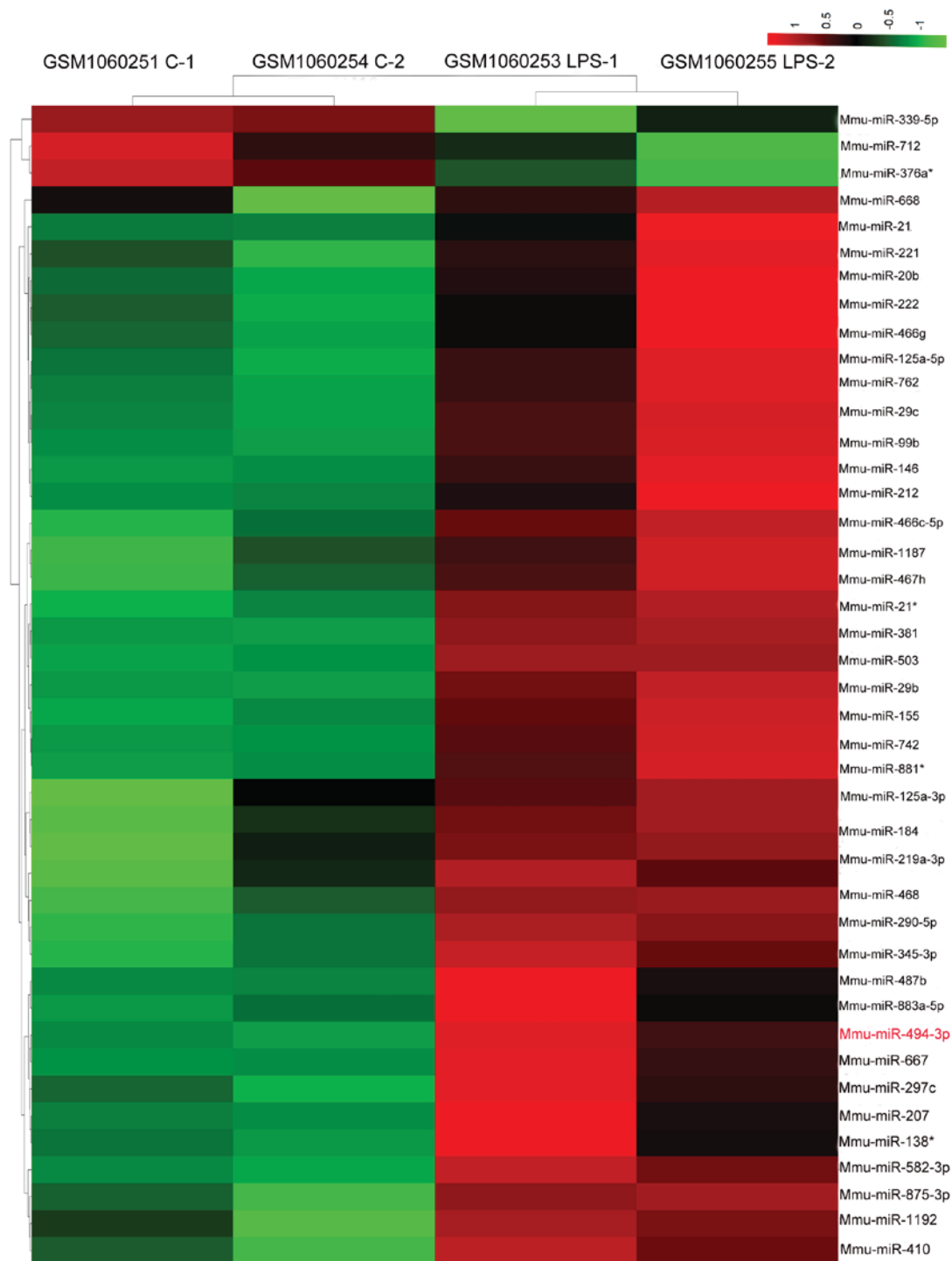


Figure 1. Heat map of differentially expressed miRNAs between LPS-treated and control RAW264.7 cells, based on the GSE43300 array dataset. Green represents low expression and red represents high expression. C, control; LPS, lipopolysaccharide; miR/miRNA, microRNA.

was generated. Primers were designed based on the National Center for Biotechnology Information reference sequence NM\_008960.2, and specific primers were used for the amplification of wild-type and mutant 3'-UTR sequences from PTEN mRNA (Table I). The wild-type and mutated 3'-UTR fragment were cloned downstream of the luciferase reporter gene of the pmirGLO-reporter vector, using the *XhoI* and *SacI* restriction sites. One day prior to transfection, 293T cells were seeded into a 96-well plate at a density of  $1 \times 10^4$  cells/well. Then, cells were co-transfected with miR-494-3p mimics and PTEN

luciferase reporter constructs using Lipofectamine 3000. Blank pmirGLO dual-luciferase was used as a positive control. After 24 h, a Dual-Luciferase® Reporter Assay system (Promega Corporation) was used to measure reporter activity. The ratio of the *Renilla* fluorescence value to the firefly fluorescence value was calculated.

**Confocal microscopy.** To determine the effect of miR-494-3p on the protein level of PTEN, immunocytochemistry was performed using the PTEN antibody. Cells were seeded 24 h

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction and luciferase reporter construction.

Gene	Sequence
U6	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTCACGAATTTGCGT-3'
miR-494-3p	F: 5'-ATCCAGTGCCTGTCGTG-3' R: 5'-TGCTTAGCTTATCAGACTG-3'
GAPDH	F: 5'-GGTTGTCTCCTGCGACTTCA-3' R: 5'-GGTGGTCCAGGGTTTCTTACT-3'
TNF- $\alpha$	F: 5'-TCTTCTCATTCTGCTTGTGG-3' R: 5'-GGTCTGGGCCATAGAACTGA-3'
IL-1 $\beta$	F: 5'-AGTTGACGGACCCCAAAAG-3' R: 5'-AGCTGGATGCTCTCATCAGG-3'
PTEN	F: 5'-AAATGCGTACCTACCTTGCC-3' R: 5'-TGTTGTTAGCCCACCAGAA-3'
pmiRGLO/PTEN-WT-3'UTR	F: 5'-CAGGGTTTTGATTTTGAATGTTTCAC-3' R: 5'-TCGAGTGAAACATTCAAATCAAACCCCTGAGCT-3'
pmiRGLO/PTEN-MUT-3'UTR	F: 5'-CAGCCTTTGATTTACAAACTTCAC-3' R: 5'-TCGAGTGAAGTTTGTAATCAAAGGCTGAGCT-3'

IL, interleukin; miR, microRNA; MUT, mutant; PTEN, phosphatase and tensin homolog; TNF, tumor necrosis factor; UTR, untranslated region; WT, wild-type.

prior to treatment. Following transfection with miR-494-3p mimics or negative controls for 48 h, RAW264.7 cells were stimulated with LPS (1  $\mu$ g/ml) for 24 h at 37°C with 5% CO<sub>2</sub>, and PTEN expression was determined. The transfected RAW264.7 cell lines were fixed with 4% formaldehyde for 30 min at room temperature, then incubated with 0.5% Triton X-100 for 10 min at room temperature. Cells were washed three times with PBS and blocked with goat serum (5%; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 1 h at room temperature. The cells were incubated with anti-PTEN antibody (1:100) at 4°C overnight, washed three times with PBS, and then incubated with goat anti-rabbit secondary antibody (Cy3 AffiniPure Goat Anti-Rabbit Immunoglobulin G; 1:1,000; E031640-01, EarthOx Life Sciences, Milbrae, CA, USA) for 1 h at 37°C in the dark. The cells were incubated with DAPI (1:10,000; cat. no. 4083; Cell Signaling Technology, Inc.) for 5 min at room temperature. Images of PTEN expression were acquired by confocal microscopy (magnification, x10; A1; Nikon Corporation, Tokyo, Japan).

**Statistical analysis.** SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analyses. Data are presented as the mean  $\pm$  standard deviation of at least three independent experiments. One-way analysis of variance followed by Bonferroni post hoc test was used to compare the means of multiple groups. Student's t-test was used to assess differences between two groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Data processing and analysis of differential miRNA expression.** Following preprocessing of the raw expression data

from the GSE43300 array dataset, 44 upregulated and three downregulated miRNAs were identified in the LPS-treated samples compared with in the control samples. The number of upregulated genes was higher than the number of downregulated genes. A hierarchical heatmap of the 47 differentially expressed miRNAs is shown in Fig. 1. Notably, miR-494-3p was upregulated by LPS in RAW264.7 cells.

**LPS stimulation regulates miR-494-3p expression in murine macrophages.** It was previously demonstrated that RAW264.7 cells can release inflammatory cytokines in response to LPS stimulation (21). To verify this, RAW264.7 cells were treated with different concentrations of LPS, and IL-1 $\beta$  and TNF- $\alpha$  expression were measured. The IL-1 $\beta$  and TNF- $\alpha$  mRNA and protein expression levels were increased following LPS stimulation, with peak expression occurring with 1  $\mu$ g/ml LPS (Fig. 2A and B). To determine whether LPS treatment would affect miR-494-3p expression in RAW264.7 cells, RT-qPCR was performed. As shown in Fig. 2C, peak expression of miR-494-3p occurred following stimulation with 1  $\mu$ g/ml LPS. A previous study also selected 1  $\mu$ g/ml LPS to induce inflammatory responses in RAW264.7 cells (22). The expression miR-494-3p, IL-1 $\beta$  and TNF- $\alpha$  following LPS treatment (1  $\mu$ g/ml) was upregulated; however, the expression of all the molecules notably decreased following stimulation with  $>2$   $\mu$ g/ml LPS compared with 1  $\mu$ g/ml LPS. This may be due to high concentrations of LPS inducing apoptosis in cells instead of inflammatory responses (23). Further investigation into the underlying mechanisms is required. The results indicated that miR-494-3p expression may be closely associated with LPS-induced inflammatory responses in RAW264.7 cells.



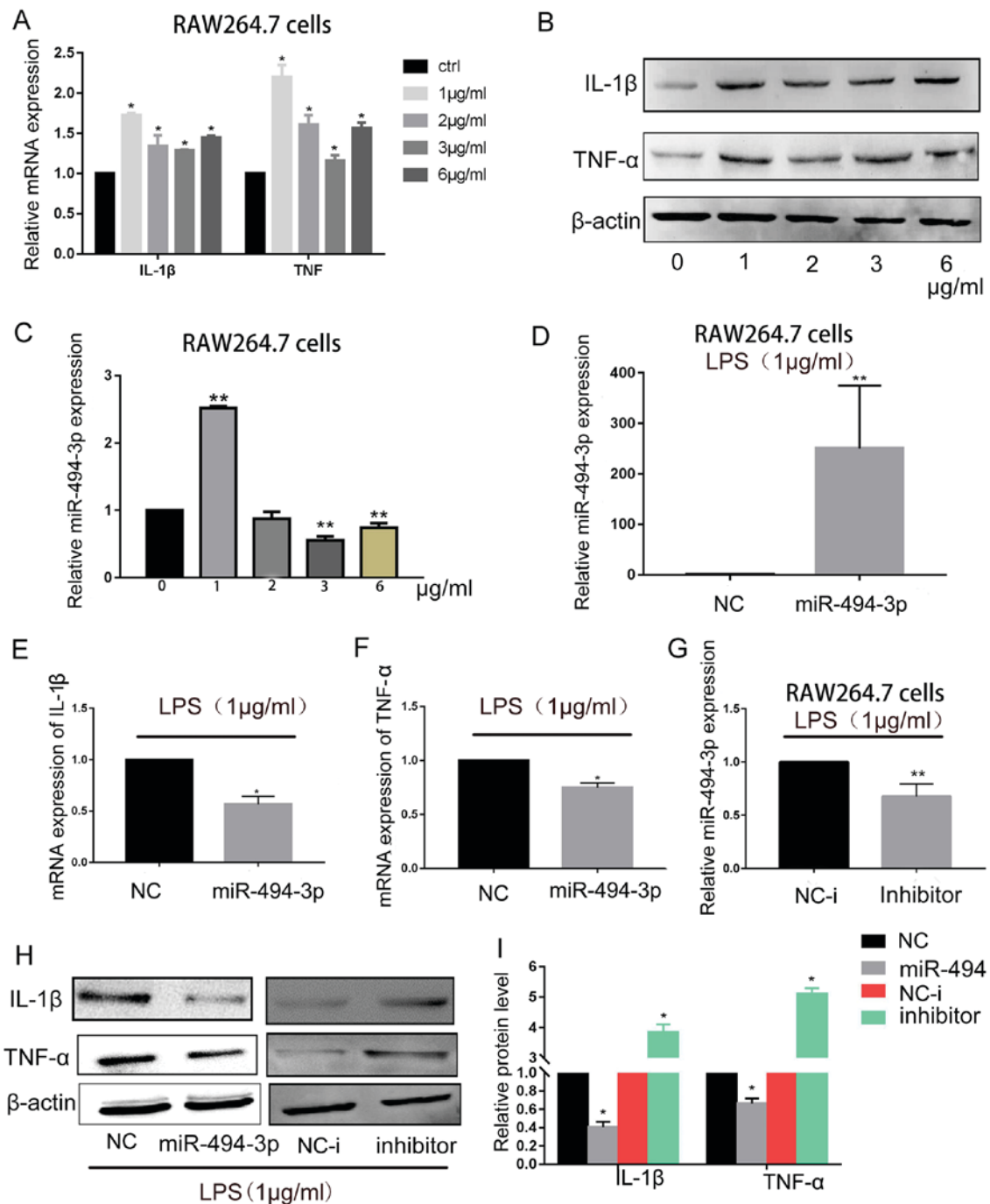


Figure 2. miR-494-3p inhibits inflammatory responses in LPS-treated RAW264.7 cells. RAW264.7 cells were treated with 1-6 μg/ml LPS for 24 h. (A) RT-qPCR and (B) western blotting of IL-1β and TNF-α were performed. (C) RT-qPCR was used to determine miR-494-3p expression levels. \*P<0.05, \*\*P<0.01 vs. 0 μg/ml LPS. (D) Next, RAW264.7 cells were transfected with 50 nM miR-494-3p mimics or NC. After 24 h, cells were treated with 1 μg/ml LPS for 24 h. Transfection efficiency was confirmed by RT-qPCR. (E and F) IL-1β and TNF-α mRNA expression levels were detected using RT-qPCR. (G) RAW264.7 cells were transfected with 50 nM miR-494-3p inhibitor or inhibitor NC. After 24 h, cells were treated with 1 μg/ml LPS for 24 h. Transfection efficiency was confirmed by RT-qPCR. (H) IL-1β and TNF-α protein expression levels were detected using western blotting. (I) Quantification of protein expression. Data are presented as the mean ± standard deviation of at least three independent experiments. \*P<0.05, \*\*P<0.01 vs. NC. IL, interleukin; LPS, lipopolysaccharide; miR, microRNA; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TNF, tumor necrosis factor.

**miR-494-3p regulates LPS-induced inflammation.** To explore the regulatory function of miR-494-3p during LPS-induced inflammation, RAW264.7 cells were transfected with miR-494-3p mimics or negative control (Fig. 2D), and RT-qPCR was performed to measure IL-1β and TNF-α mRNA levels. Overexpression of miR-494-3p downregulated IL-1β and TNF-α expression levels in LPS-treated RAW264.7 cells

(Fig. 2E and F). To further confirm these results, an inhibitor was used to suppress miR-494-3p expression (Fig. 2G), and the results demonstrated that the protein expression levels of IL-1β and TNF-α were increased in RAW264.7 cells (Fig. 2H and I). These results suggested that miR-494-3p may regulate proinflammatory cytokine production via a negative feedback loop in RAW264.7 cells.

*miR-494-3p targets the 3'-UTR of PTEN and regulates its expression.* Next, to explore the molecular function of miR-494-3p in suppressing inflammatory responses, bioinformatics analysis was used to identify potential target genes. Putative miRNA target genes were identified by employing TargetScan, picTar, PITA and miRanda. The miRNA databases predicted a seed match for miR-494-3p in the 3'-UTR region of PTEN (Fig. 3A and B). Sequences from the predicted miR-494-3p target site in the 3'-UTR of PTEN, and mutant variants, were successfully cloned into the pmiRGLO vector. RAW264.7 cells were co-transfected with miR-494-3p mimics and the pmiRGLO vector. Transfection with miR-494-3p mimics inhibited the luciferase activity of pmiRGLO-PTEN-wild-type, but not that of pmiRGLO-PTEN-mutant (Fig. 3C). This indicated that miR-494-3p bound specifically to the PTEN 3'-UTR. Confocal microscopy and western blotting was used to determine whether miR-494-3p inhibited the protein expression levels of PTEN in RAW264.7 cells. PTEN protein expression levels were decreased in RAW264.7 cells transfected with miR-494-3p mimics. Conversely, it was increased with a miR-494-3p inhibitor (Fig. 3D).

*miR-494-3p targets PTEN and suppresses LPS-induced nuclear factor (NF)- $\kappa$ B signaling.* TLR4, a member of the TLR family, interacts all four Toll-interleukin receptor domain-containing adaptor proteins to induce inflammatory responses; stimulation of TLR activates the NF- $\kappa$ B pathway (24). Based on a previous study, Akt1 activation is enhanced in PTEN<sup>-/-</sup> cells (25). This suggests that PTEN might augment TLR4-induced inflammatory responses by suppressing Akt1 activation (17). In addition, NF- $\kappa$ B pathways have been demonstrated to enhance LPS-induced inflammatory responses (26). Using miR-494-3p or negative control mimics to transfect RAW264.7 cells, it was revealed that miR-494-3p enhanced LPS-induced p-Akt expression, and suppressed the nuclear expression of its downstream molecule, p65 (Fig. 3E and G), which may induce decreased levels of the inflammatory mediators, IL-1 $\beta$  and TNF- $\alpha$ . Taken together, these results indicated that miR-494-3p enhanced Akt1 phosphorylation and suppressed the NF- $\kappa$ B pathway.

*PTEN silencing inhibits LPS-induced inflammatory responses.* To determine the role of PTEN in miR-494-3p-mediated inhibition of LPS-induced inflammatory responses, RAW264.7 cells were transfected with PTEN siRNA or control siRNA. Western blotting was performed to measure the siRNA efficiency; PTEN siRNA significantly reduced the expression of PTEN (Fig. 3F). Akt1 phosphorylation was upregulated in RAW264.7 cells following transfection with PTEN siRNA compared with control siRNA (Fig. 3F and H). The protein expression levels of proinflammatory cytokines and p65 were also measured (Fig. 3F). The results suggested that miR494-3p targets PTEN and suppresses LPS-induced inflammation via the Akt/NF- $\kappa$ B pathway.

## Discussion

LPS binds to TLR4, activates NF- $\kappa$ B signaling pathways, and induces the expression and release of inflammatory factors, including IL-1 $\beta$  and TNF- $\alpha$  (27,28). The production of these,

and other proinflammatory cytokines, results in a positive feedback loop to promote inflammation (29). In the present study, miRNA expression data from the GEO database were analyzed, and 44 upregulated miRNAs were identified in LPS-treated RAW264.7 cells compared with in control cells. RT-qPCR was used to validate the differential expression of miR-494-3p. The results indicated that miR-494-3p may have a role in regulation of inflammatory responses.

There is increasing research focusing on the critical role of miRNAs in innate immunity (30,31). For example, miR-718 was reported to repress proinflammatory cytokine production by targeting PTEN (32). In the present study, it was demonstrated that miR-494-3p may suppress LPS-induced inflammation in RAW264.7 cells. Notably, it was confirmed that PTEN is a target of miR-494-3p. Previous studies suggested that PTEN expression is tightly regulated and that several miRNAs, including miR-214, -21 and -217, downregulated the expression of PTEN (33-35). Another study demonstrated that miR-494-3p can target PTEN in human glioblastoma cells, and regulate cellular proliferation and apoptosis through the Akt signaling pathway (36).

PTEN is a phosphatidylinositol phosphate phosphatase that is specific for the 3-position of the inositol ring. Phosphatidylinositol (3,4,5)-trisphosphate can be dephosphorylated by PTEN, resulting in inactivation of Akt1 (37). Consistent with the results from other studies, the results in the present study demonstrated that inhibition of PTEN enhanced LPS-induced Akt1 activation (38,39).

In the early stage of immune responses, Akt1 suppresses NF- $\kappa$ B signaling and downregulates the expression of proinflammatory cytokines (40). Inhibition of PI3K/Akt can enhance TNF- $\alpha$  expression by increasing the transcriptional activity of p65 in LPS-treated human monocytes (39). The results in the present study indicated that inhibition of PTEN expression suppressed p65 translocation to the nucleus and decreased expression of NF- $\kappa$ B-dependent proinflammatory cytokines. The transcription factor, p65, is important for regulating inflammatory processes (41). It can translocate to the nucleus where it binds target DNA sequences to promote gene transcription. p65 activation triggers the transcription of many proinflammatory cytokines, including IL-1 $\beta$  and TNF- $\alpha$ , after LPS stimulation in RAW264.7 cells (42). It is also involved in many biological processes, and its function is tightly regulated by many proteins, including Akt1 (39). Taken together, the results demonstrated that PTEN inhibition may lead to the downregulation of p65 expression, leading to suppression of inflammatory pathways.

miR-494-3p is involved in many biological processes. It modulates the progression of Parkinson's disease in both *in vitro* and *in vivo* models by targeting Sirtuin 3 (43). miR-494-3p can also act as an oncogene by modulating NOTCH1 and PTEN/PI3K/Akt signaling, and stimulating growth and metastasis of A549 cells (44). However, to the best of our knowledge, there are no studies on the functional role of miR-494-3p in regulating inflammatory responses. In the present study, miR-494-3p overexpression reduced IL-1 $\beta$  and TNF- $\alpha$  production, whereas downregulation of miR-494-3p enhanced the production IL-1 $\beta$  and TNF- $\alpha$ . Further investigation revealed that miR-494-3p downregulated PTEN expression, and miR-494-3p-mediated suppression of IL-1 $\beta$

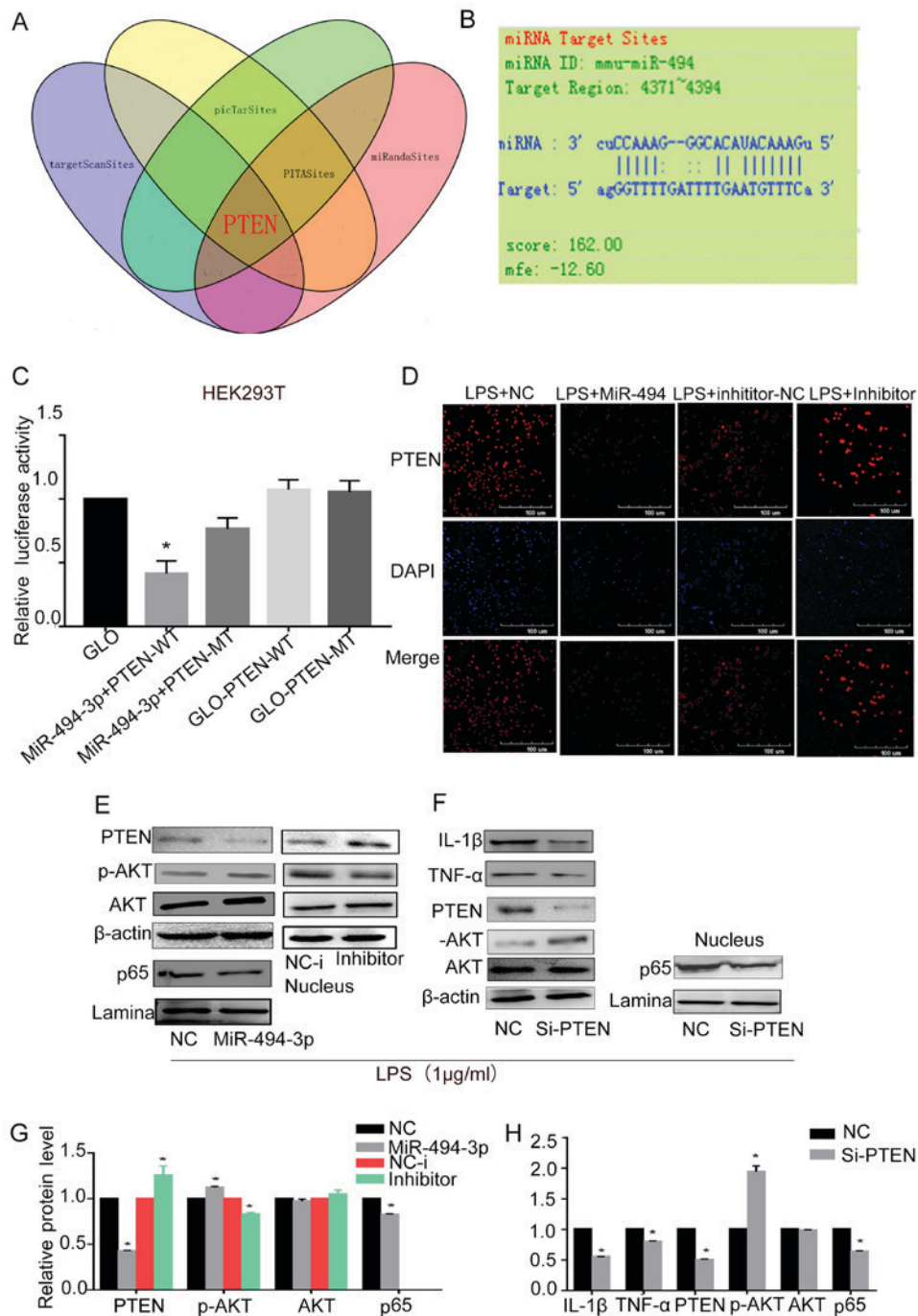


Figure 3. miR-494-3p regulates PTEN/Akt/NF-κB signaling. (A) miR-494-3p target prediction using four online tools. (B) Alignment of miR-494-3p and the 3'-UTR of PTEN. (C) Luciferase activity in 293T cells co-transfected with a reporter vector containing the 3'-UTR of PTEN and miR-494-3p mimics. RAW264.7 cells were transfected with miR-494-3p mimics, miR-494-3p inhibitor, NC or NC-i. After 24 h, cells were treated with 1 μg/ml LPS for 24 h. \*P<0.05 vs. GLO-PTEN-WT. (D) PTEN expression was detected by confocal microscopy. (E) Western blot analysis was used to determine the protein expression levels of PTEN, Akt1, p-Akt1 and p65. (F) RAW264.7 cells were transfected with PTEN siRNA or control siRNA. After 48 h, cells were treated with 1 μg/ml LPS for 24 h. Western blot analysis was used to determine the protein expression levels of IL-1β, TNF-α, PTEN, Akt1, p-Akt1 and p65. (G and H) Quantification of the protein expression presented in E and F. Data are presented as the mean ± standard deviation of at least three independent experiments. \*P<0.05 (miR-494-3p) vs. nc; \*P<0.05 (inhibitor) vs. NC-i; \*P<0.05 (si-PTEN) vs. NC. Akt1, AKT serine/threonine kinase 1; IL, interleukin; LPS, lipopolysaccharide; miR, microRNA; MT, mutant; NC, negative control; NC-i, inhibitor NC; p, phosphorylated; PTEN, phosphatase and tensin homolog; siRNA, small interfering RNA; TNF, tumor necrosis factor; UTR, untranslated region; WT, wild-type.

and TNF-α was associated with increased p-Akt1 expression and decreased p65 expression. These data indicated that miR-494-3p regulated inflammation by decreasing p65 translocation to the nucleus. This study also demonstrated that miR-494-3p was associated with suppressing immune responses by downregulating PTEN in RAW264.7 cells. In the

future, additional experiments will be conducted to explore the relationship between miR-494-3p and NF-κB signaling pathways. In addition, further studies are needed to explore the detailed mechanisms through which miR-494-3p regulates inflammatory responses. Thus, miR-494-3p may serve as a novel marker of inflammatory responses.



## Acknowledgements

Not applicable.

## Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 81070870).

## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

SZ performed the research and wrote the paper. KH completed the data preprocessing and heatmap generation. JC and ZJ made significant contributions towards the design of the present study. WZ made substantial contributions to the acquisition of data. All authors read and approved the final version of the 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.

## References

- Xie W and Du L: Diabetes is an inflammatory disease: Evidence from traditional Chinese medicines. *Diabetes Obes Metab* 13: 289-301, 2011.
- Johnson AR, Milner JJ and Makowski L: The inflammation highway: Metabolism accelerates inflammatory traffic in obesity. *Immunol Rev* 249: 218-238, 2012.
- Vendramini-Costa DB and Carvalho JE: Molecular link mechanisms between inflammation and cancer. *Curr Pharm Des* 18: 3831-3852, 2012.
- Lin YP, Love RM, Friedlander LT, Shang HF and Pai MH: Expression of Toll-like receptors 2 and 4 and the OPG-RANKL-RANK system in inflammatory external root resorption and external cervical resorption. *Int Endod J* 46: 971-981, 2013.
- Ingalls RR, Heine H, Lien E, Yoshimura A and Golenbock D: Lipopolysaccharide recognition, CD14, and lipopolysaccharide receptors. *Infect Dis Clin North Am* 13: 341-353, vii, 1999.
- Su GL, Simmons RL and Wang SC: Lipopolysaccharide binding protein participation in cellular activation by LPS. *Crit Rev Immunol* 15: 201-214, 1995.
- Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 116: 281-297, 2004.
- Huang C, Liu XJ, QunZhou, Xie J, Ma TT, Meng XM and Li J: MiR-146a modulates macrophage polarization by inhibiting Notch1 pathway in RAW264.7 macrophages. *Int Immunopharmacol* 32: 46-54, 2016.
- Zheng Y, Guo X, He W, Shao Z, Zhang X, Yang J, Shen Y, Luo X and Cao J: Effects of *Echinococcus multilocularis* miR-71 mimics on murine macrophage RAW264.7 cells. *Int Immunopharmacol* 34: 259-262, 2016.
- Li M, Chen H, Chen L, Chen Y, Liu X and Mo D: miR-709 modulates LPS-induced inflammatory response through targeting GSK-3 $\beta$ . *Int Immunopharmacol* 36: 333-338, 2016.
- Xie W, Li Z, Li M and Xu N, Zhang Y: miR-181a and inflammation: miRNA homeostasis response to inflammatory stimuli in vivo. *Biochem Biophys Res Commun* 430: 647-652, 2013.
- Cheng Y, Du L, Jiao H, Zhu H, Xu K, Guo S, Shi Q, Zhao T, Pang F, Jia X and Wang F: Mmu-miR-27a-5p-dependent upregulation of MCP1 inhibits the inflammatory response in LPS-induced RAW264.7 macrophage cells. *Biomed Res Int* 2015: 607692, 2015.
- Furnari FB, Lin H, Huang HS and Cavenee WK: Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain. *Proc Natl Acad Sci USA* 94: 12479-12484, 1997.
- Myers MP, Pass I, Batty IH, Van der Kaay J, Stolarov JP, Hemmings BA, Wigler MH, Downes CP and Tonks NK: The lipid phosphatase activity of PTEN is critical for its tumor suppressor function. *Proc Natl Acad Sci USA* 95: 13513-13518, 1998.
- Zhu D, Hattori H, Jo H, Jia Y, Subramanian KK, Loison F, You J, Le Y, Honczarenko M, Silberstein L and Luo HR: Deactivation of phosphatidylinositol 3,4,5-trisphosphate/Akt signaling mediates neutrophil spontaneous death. *Proc Natl Acad Sci USA* 103: 14836-14841, 2006.
- Agrawal A, Agrawal S, Cao JN, Su H, Osann K and Gupta S: Altered innate immune functioning of dendritic cells in elderly humans: A role of phosphoinositide 3-kinase-signaling pathway. *J Immunol* 178: 6912-6922, 2007.
- Cao X, Wei G, Fang H, Guo J, Weinstein M, Marsh CB, Ostrowski MC and Tridandapani S: The inositol 3-phosphatase PTEN negatively regulates Fc gamma receptor signaling, but supports Toll-like receptor 4 signaling in murine peritoneal macrophages. *J Immunol* 172: 4851-4857, 2004.
- Chen Y, Liu W, Sun T, Huang Y, Wang Y, Deb DK, Yoon D, Kong J, Thadhani R and Li YC: 1,25-Dihydroxyvitamin D promotes negative feedback regulation of TLR signaling via targeting microRNA-155-SOCS1 in macrophages. *J Immunol* 190: 3687-3695, 2013.
- Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W and Smyth GK: Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43: e47, 2015.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Zhu J, Zhang Y, Wu G, Xiao Z, Zhou H and Yu X: Inhibitory effects of oligochitosan on TNF- $\alpha$ , IL-1 $\beta$  and nitric oxide production in lipopolysaccharide-induced RAW264.7 cells. *Mol Med Rep* 11: 729-733, 2015.
- He Y, Sun X, Huang C, Long XR, Lin X, Zhang L, Lv XW and Li J: MiR-146a regulates IL-6 production in lipopolysaccharide-induced RAW264.7 macrophage cells by inhibiting Notch1. *Inflammation* 37: 71-82, 2014.
- Ding Y, Wang L, Zhao Q, Wu Z and Kong L: MicroRNA-93 inhibits chondrocyte apoptosis and inflammation in osteoarthritis by targeting the TLR4/NF- $\kappa$ B signaling pathway. *Int J Mol Med* 43: 779-790, 2019.
- Shi H, Wang XL, Quan HF, Yan L, Pei XY, Wang R and Peng XD: Effects of betaine on LPS-stimulated activation of microglial M1/M2 phenotypes by suppressing TLR4/NF- $\kappa$ B pathways in N9 cells. *Molecules* 24: E367, 2019.
- Liu YL, Yan Y, Webster C, Shao L, Lensing SY, Ni H, Feng W, Colorado N, Pathak R, Xiang X, *et al*: Timing of the loss of Pten protein determines disease severity in a mouse model of myeloid malignancy. *Blood* 127: 1912-1922, 2016.
- Ko W, Sohn JH, Jang JH, Ahn JS, Kang DG, Lee HS, Kim JS, Kim YC and Oh H: Inhibitory effects of alternanamide on inflammatory mediator expression through TLR4-MyD88-mediated inhibition of NF- $\kappa$ B and MAPK pathway signaling in lipopolysaccharide-stimulated RAW264.7 and BV2 cells. *Chem Biol Interact* 244: 16-26, 2016.
- da Silveira Cruz-Machado S, Carvalho-Sousa CE, Tamura EK, Pinato L, Cecon E, Fernandes PA, de Avellar MC, Ferreira ZS and Markus RP: TLR4 and CD14 receptors expressed in rat pineal gland trigger NFKB pathway. *J Pineal Res* 49: 183-192, 2010.
- He W, Qu T, Yu Q, Wang Z, Lv H, Zhang J, Zhao X and Wang P: LPS induces IL-8 expression through TLR4, MyD88, NF- $\kappa$ B and MAPK pathways in human dental pulp stem cells. *Int Endod J* 46: 128-136, 2013.



29. Sieve I, Ricke-Hoch M, Kasten M, Battmer K, Stapel B, Falk CS, Leisegang MS, Haverich A, Scherr M and Hilfiker-Kleiner D: A positive feedback loop between IL-1 $\beta$ , LPS and NEU1 may promote atherosclerosis by enhancing a pro-inflammatory state in monocytes and macrophages. *Vascul Pharmacol* 103-105: 16-28, 2018.
30. Niu Y, Mo D, Qin L, Wang C, Li A, Zhao X, Wang X, Xiao S, Wang Q, Xie Y, *et al*: Lipopolysaccharide-induced miR-1224 negatively regulates tumour necrosis factor- $\alpha$  gene expression by modulating Sp1. *Immunology* 133: 8-20, 2011.
31. O'Connell RM, Rao DS, Chaudhuri AA and Baltimore D: Physiological and pathological roles for microRNAs in the immune system. *Nat Rev Immunol* 10: 111-122, 2010.
32. Kalantari P, Harandi OF, Agarwal S, Rus F, Kurt-Jones EA, Fitzgerald KA, Caffrey DR and Golenbock DT: miR-718 represses proinflammatory cytokine production through targeting phosphatase and tensin homolog (PTEN). *J Biol Chem* 292: 5634-5644, 2017.
33. Fang Y, Qiu J, Jiang ZB, Xu SR, Zhou ZH and He RL: Increased serum levels of miR-214 in patients with PCa with bone metastasis may serve as a potential biomarker by targeting PTEN. *Oncol Lett* 17: 398-405, 2019.
34. Du G, Cao D and Meng L: miR-21 inhibitor suppresses cell proliferation and colony formation through regulating the PTEN/AKT pathway and improves paclitaxel sensitivity in cervical cancer cells. *Mol Med Rep* 15: 2713-2719, 2017.
35. Nie X, Fan J, Li H, Yin Z, Zhao Y, Dai B, Dong N, Chen C and Wang DW: miR-217 promotes cardiac hypertrophy and dysfunction by targeting PTEN. *Mol Ther Nucleic Acids* 12: 254-266, 2018.
36. Li XT, Wang HZ, Wu ZW, Yang TQ, Zhao ZH, Chen GL, Xie XS, Li B, Wei YX, Huang YL, *et al*: miR-494-3p regulates cellular proliferation, invasion, migration, and apoptosis by PTEN/AKT signaling in human glioblastoma cells. *Cell Mol Neurobiol* 35: 679-687, 2015.
37. Bayascas JR and Alessi DR: Regulation of Akt/PKB Ser473 phosphorylation. *Mol Cell* 18: 143-145, 2005.
38. Aksoy E, Vanden Berghe W, Detienne S, Amraoui Z, Fitzgerald KA, Haegeman G, Goldman M and Willems F: Inhibition of phosphoinositide 3-kinase enhances TRIF-dependent NF-kappa B activation and IFN-beta synthesis downstream of Toll-like receptor 3 and 4. *Eur J Immunol* 35: 2200-2209, 2005.
39. Guha M and Mackman N: The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J Biol Chem* 277: 32124-32132, 2002.
40. Lee YG, Lee J, Byeon SE, Yoo DS, Kim MH, Lee SY and Cho JY: Functional role of Akt in macrophage-mediated innate immunity. *Front Biosci (Landmark Ed)* 16: 517-530, 2011.
41. Tak PP and Firestein GS: NF-kappaB: A key role in inflammatory diseases. *J Clin Invest* 107: 7-11, 2001.
42. Shin JS, Im HT and Lee KT: Saikosaponin B2 suppresses inflammatory responses through IKK/IkBa/NF-kB signaling inactivation in LPS-induced RAW 264.7 macrophages. *Inflammation* 42: 342-353, 2019.
43. Geng L, Zhang T, Liu W and Chen Y: miR-494-3p modulates the progression of in vitro and in vivo Parkinson's disease models by targeting SIRT3. *Neurosci Lett* 675: 23-30, 2018.
44. Favarsani A, Amatori S, Augello C, Colombo F, Porretti L, Fanelli M, Ferrero S, Palleschi A, Pelicci PG, Belloni E, *et al*: miR-494-3p is a novel tumor driver of lung carcinogenesis. *Oncotarget* 8: 7231-7247, 2017.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.