Research Article

**MicroRNA-322 inhibits inflammatory cytokine expression and promotes cell proliferation in LPS-stimulated murine macrophages by targeting NF-κB1 (p50)**

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Inflammation is the body’s normal self-protection mechanism to eliminate pathogens and resist pathogen invasion. The excessive inflammatory response may lead to inflammatory lesions. The mechanisms accounting for inflammation remain hazy. miRNAs have been proposed to have crucial effects on inflammation. In the present study, we reported that lipopolysaccharide (LPS)-stimulation increased the expression levels of inflammatory cytokines and the cell-cycle progression was suppressed in RAW264.7 cells. Meanwhile, the expression of miR-322 was significantly down-regulated after LPS treatment. Bioinformatics predictions revealed a potential binding site of miR-322 in 3′-UTR of NF-κB1 (p50) and it was further confirmed by luciferase assay. Moreover, both the mRNA and protein levels of NF-κB1 (p50) were down-regulated by miR-322 in RAW264.7 cells. Subsequently, we demonstrated that miR-322 mimics decrease in the expression levels of inflammatory cytokines and cell-cycle repression can be rescued following LPS treatment in RAW264.7 cells. The anti-inflammatory cytokines expression including IL-4 and IL-10 were significantly up-regulated. Furthermore, miR-322 could also promote RAW264.7 cells proliferation. These results demonstrate that miR-322 is a negative regulator of inflammatory response by targeting NF-κB1 (p50).

**Introduction**

Inflammation response plays a crucial role in eliminating pathogens and resisting pathogen invasion [1]. The excessive inflammatory response may lead to inflammatory lesions. Increasing evidence suggests that inflammatory response is related to many diseases and cancer [2]. The complex process of inflammatory response is initiated by innate immune system receptors, which recognize a lot of pathogens or damage signals and precisely regulate immune response. Cells expressing pathogen-recognition receptors (PRRs) are able to present pathogens, antigens and secrete cytokines and chemokines [3,4]. PRRs, such as toll-like receptors (TLRs), activate cellular signalling pathways that result in NF-κB translocating into nucleus, and lead to the production of inflammatory cytokines and chemokines, which directly contribute to inflammatory lesions and immune diseases [5].

NF-κB is an important transcription factor that regulates the transcription and expression of multiple genes. It is closely related to the cell activation, cell proliferation, immune response and inflammatory reaction process [6]. NF-κB comprises a family of proteins such as RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52). The protein p50, the transcript of NF-κB1, is an important subunit of NF-κB. It regulates gene transcription through combination with p65, which is another important subunit of NF-κB.
When the signalling pathway is activated, p50/p65 exposes nuclear localization signal and translocates into the nucleus, regulating the transcription of downstream genes. Therefore, p50 protein levels are critical for activation of NF-κB signalling pathway and body’s inflammatory reaction [5].

miRNAs are naturally occurring non-coding small RNA molecules capable of degrading the target mRNA or repressing its translation by targeting mRNA 3′-UTR region [7,8]. Overwhelming evidence shows that miRNA-mediated post-transcriptional regulation plays a critical role in inflammatory response and immune regulation [9,10]. miR-146 is probably one of the most studied miRNA that regulates inflammatory response by targeting TRAF6 and IRAK1 following lipopolysaccharide (LPS)-stimulation [11]. miR-125b can target TNF-α resulting in inhibition of inflammatory response [12]. miR-181a and miR-155 regulate inflammation responses by targeting IL-1α respecting TAB2 and SOCS1 [13,14]. However, it remains largely unknown as to how inflammation is regulated by miRNA in immune response.

miR-322 is the homologue of human miR-424, which is differentially expressed in a variety of disease conditions and is highly conserved in different cells [15]. miR-322/424 is a member of miR-15/107 family, also known as miR-16 family [16,17]. It is involved in the regulation of cell proliferation, cell differentiation, diabetes and male infertility [15,18-20]. In our preliminary study, miR-322 was predicted to target several sites of inflammatory factors using the software programs. Little is known about the involvement of miR-322 during inflammatory response. RAW264.7 was a mouse peritoneal macrophage cell line established from a tumour induced by Abelson murine leukaemia virus. It is one of the commonly used inflammatory cell models. Here, we found that the level of miR-322 was down-regulated in RAW264.7 cells by administration of LPS. We also showed that miR-322 mimic transfection resulted in an inhibition in pro-inflammatory cytokines mRNA expression, such as IL-1β, IL-6, TNF-α and increased anti-inflammatory cytokines IL-4 and IL-10 expression. Besides, NF-κB1 (p50) was identified as a functional target, through which miR-322 acted as a negative regulator in macrophage inflammatory response. Moreover, miR-322 may promote cell-cycle procession and cell proliferation. Our findings demonstrate that the level of miR-322 is down-regulated by LPS-stimulation and miR-322 is a negative regulator of the immune response.

**Materials and methods**

**RAW264.7 cells’ culture and treatment**

RAW264.7 was a mouse peritoneal macrophage cell line established from a tumour induced by Abelson murine leukaemia virus. It is one of the commonly used inflammatory cell models. Cells were cultured in DMEM (Hyclone) medium supplemented with 10% FBS at 37°C in 5% CO2. RAW264.7 cells were seeded in six-well plates at a density of 2 x 10^5 cells/well. Twenty four hours later, the cell medium was replaced with fresh medium. Cells were collected at 0, 2, 4, 8, 12 and 24 h after 1 µg/ml LPS (Sigma–Aldrich, U.S.A.) induction.

**miR-322 mimics transfection**

miR-322 mimics and miR-322 inhibitors were purchased from GenePharma (China). RAW264.7 cells were seeded into six-well plates for 12 h. The cells were replaced with fresh medium (DMEM + 10% FBS) and transfected with 50 nM miR-322 mimics and miR-322 inhibitors using Lipofectaime 2000 (Invitrogen TM, U.S.A.) according to the manufacturer’s instructions. After transfection for 24 h, the medium was replaced with fresh medium containing 1 µg/ml LPS. The cells were collected after LPS induction for 8 h.

**Quantitative real-time PCR**

Total RNA was extracted from treated cells with TRIzol (Invitrogen) according to the instructions of the manufacturer. For mRNA analysis, reverse transcription was performed using a first-strand cDNA synthesis kit (Toyobo, Japan). To quantify mature miR-322 expression, a commercial Bulge-Loop™ miRNA quantitative reverse transcription detection method was used with miR-322-specific RT primer (Table 1). All gene transcripts were measured by quantitative real-time PCR (qPCR) using a 7500 real-time PCR system (Applied Biosystems) and SYBR Green PCR master mix (Toyobo, Japan). qPCR primers of miR-322 and the endogenous control gene U6 were from RiboBio (China), whereas other primers were designed by the Primer Express software and synthesized from Invitrogen (Table 2). Fold change was calculated using the 2^-ΔΔCt method of relative quantification. All experiments were conducted in triplicate.

**Computational prediction of the miRNA targets**

To further analyse the functions of miR-322, we used three computational approaches, TargetScan (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/) and miRDB (http://mirdb.org/miRDB/), to predict the targets of miR-322 in the TLR signalling pathways. Then, the miRNA-binding sites in target genes and the
Table 1  Nucleic acid sequences for miRNAs

| Gene names                  | Sequence (5′ to 3′)                  |
|-----------------------------|-------------------------------------|
| miR-322 inhibitors          | UUCAAAACAUJGUGCGUGCG                |
| Inhibitors NC               | CAGUACUUUGUGUAGUAAAGCAAC            |
| miR-322 mimics              | Sense: CAGCGCAUUGCUGUGUGUAGGAA     |
|                             | Antisense: CUAACCUUGUGUGUGUGUGUUG   |
| Mimics NC                   | Sense: UUUIUUGUGUGUGUGUGUGUGUGUUG  |
|                             | Antisense: ACGUGACACUGUGUGUGUGUGUGU |
| miR-322 stem loop-primer    | GTCGTATTCAGTGCGGTCGGTATGGGCACTGG    |
|                             | ATACGACTTTCAAA                      |
| miR-322                     | Sense: CAGCGCAATTCTAGTTTGAAGA       |
|                             | Antisense: GTCGAAGGGTGGGAGTGGGT     |
| U6                          | Sense: CTCCTTCAGGCAAGCA             |
|                             | Antisense: AACGCTTCAAGGATTTG        |

Table 2 Primers for mRNA

| Primer                  | Sequence 5′→3′                |
|-------------------------|------------------------------|
| NF-κB1 UTR-3′-WT        | Sense: CCCTCGAGTTCAACACGATAAAACAAAGC |
|                         | Antisense: ATTTGCGGCCTGAATCTAGGCCTGG     |
| NF-κB1 UTR-3′-MUT       | Sense: CAGCGCAACCAACTAAGAAGCA        |
|                         | Antisense: CAAAACGUGAGCGCAAGCAGAAGC   |
| GAPDH                   | Sense: GGTGAAGGTCGGTGTGAACG         |
|                         | Antisense: GGTGAAGGTCGGTGTGAACG      |
| IL-1β                   | Sense: CTAAGATGAGGCGACTG           |
|                         | Antisense: CTCAGCTGGTAAATGGGT       |
| IL-6                    | Sense: CGGAGAGGAGACTACCAAGAGC       |
|                         | Antisense: CGGAGAGGAGACTACCAAGAGC   |
| TNF-α                   | Sense: TGAAAGGAAATGGGTGTCAT         |
|                         | Antisense: TGAAAGGAAATGGGTGTCAT     |
| IL-4                    | Sense: GGTTCACCCACCCAGCTG           |
|                         | Antisense: GGGAGGACTGATCTGTCAGTG    |
| IL-10                   | Sense: CGGGAAGACAAATAACTG           |
|                         | Antisense: CGGGAAGACAAATAACTG       |
| Cyclin D1               | Sense: TCTCAGAATCTCTGAAAGG          |
|                         | Antisense: TCTCAGAATCTCTGAAAGG      |
| Cyclin E                | Sense: CACCTCCAGAAACCACTGGA         |
|                         | Antisense: AACCCTCAACACACCGAGAGA    |
| P21                     | Sense: CAAAGTGTGCCTGTCCTCTCTCTCT   |
|                         | Antisense: CAAAGTGTGCCTGTCCTCTCTCT  |
| P27                     | Sense: GCAGATACGGGTGGGAGGA          |
|                         | Antisense: GCAGATACGGGTGGGAGGA      |

binding free energy were analysed and calculated on the website (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) [21].

Luciferase reporter assays

293T cells were cultured in DMEM medium and seeded in six-well plates at a density of 2 × 10^5 cells/well. The 3′-UTRs of mouse NF-κB1 (p50) and their corresponding mutated 3′-UTRs were amplified by PCR using the primers shown in Table 1 and cloned into psiCheck-2 dual-luciferase reporter vector (Promega). Co-transfection was performed with constructed plasmid, miRNA mimics or inhibitors using Lipofectamine 2000. Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions.
Western blotting
Protein concentrations were extracted from cells and measured with the BCA Protein Assay kit (Thermo Scientific), then subjected to SDS/PAGE (10%) gel and transferred to PVDF membranes. The membranes were blocked with 5% BSA. Protein was blotted with different antibodies following the published protocol. All antibodies were obtained from Cell Signaling Technology (U.S.A.). The results were used to visualize the proteins by the ECL reagents (Thermo Scientific) and quantified using Image J 1.44 software (National Institute of Health, Bethesda, Maryland) after densitometric scanning of the films.

Flow cytometry
RAW264.7 cells were transfected with miR-322 mimics or negative control for 24 h and stimulated with LPS for 24 h. Cells were harvested, washed twice in PBS and fixed in 75% ethanol at 4°C overnight. Staining for DNA content was performed with 50 mg/ml propidium iodide (Sigma, U.S.A.) and 1 mg/ml RNase A for 30 min. Analysis was performed on FACS flow cytometer (BD Co., U.S.A.). The percentage of cells in G0/G1-, S- and G2/M-phases was determined using the Modfit LT Cycle Analysis software.

MTT assay
RAW264.7 cells (2 × 10^5 per well) were seeded in 96-well plates in a final volume of 100 μl. After growing to 70% confluence, 50 nM miR-322 mimics, inhibitors or negative controls were transfected by lipofectamine 2000. After 24 h, the cells were stimulated with LPS at a dose-gradient of 0, 0.5, 1.0, 1.5 and 2.0 μg/ml. The MTT assay was performed according to the manufacturer’s instructions.

Statistical analysis
Each result represents the mean ± S.E.M. in at least three experiments with similar results. Analyses were conducted using paired two-tailed Student’s t test with Prism 5.0 (GraphPad Software, San Diego, California) or one-way ANOVA with SPSS 20.0.

Results
LPS stimulates the inflammatory cytokines expression and inhibits the cell-cycle progression in RAW264.7 cells
LPS-stimulation could activate TLR4/NF-κB signalling pathways and lead to inflammatory response, which could contribute to the cell-cycle procession [5]. The excessive inflammatory response may lead to cell lesions and affect cell proliferation, differentiation and other physiological states. To investigate the effect of LPS on inflammatory response and cell cycle, we detected the mRNA expression levels of inflammatory cytokines, cell-cycle gene in LPS-induced RAW264.7 cells. The results revealed that LPS could promote the mRNA expression level of IL-1β, IL-6 and TNF-α. The expression level of inflammatory cytokines was highest at 4 h or 8 h open with 1 μg/ml LPS; then the expression level decreased gradually (Figure 1). The mRNA expressions of cyclin D and cyclin E were significantly increased, and P21 and P27 were significantly suppressed with time-dependent manner. The results indicated that LPS could mediate inflammatory response and inhibit the proression of cell cycle (Figure 2).

miR-322 is down-regulated in LPS-treated RAW264.7 cells
miR-322 plays an important role in cell proliferation, cell differentiation, diabetes and male infertility [18-20]. The role in inflammatory response is not exactly reported. To explore the function of miR-322 in LPS-induced inflammatory response, the expression level of miR-322 in LPS-treated RAW264.7 cells was tested. miR-322 expression level was dramatically suppressed upon LPS-stimulation and showed a time-dependent manner (Figure 3A). Moreover, the decrease in miR-322 exhibited a dose-dependent manner with LPS stimulation (Figure 3B). These results suggest that LPS could down-regulate miR-322 expression. miR-322 may be involved in the regulation of LPS-mediated inflammatory response.

miR-322 inhibits LPS-stimulated inflammatory cytokine mRNA expression
To assess the involvement of miR-322 on inflammatory cytokine expression, miR-322 was overexpressed in RAW264.7 cells by transfection of miR-322 mimics. Cells were treated with 1 μg/ml LPS for 8 h. miR-322 levels and the mRNA expression levels of inflammatory cytokines including IL-1β, IL-6, TNF-α and anti-inflammatory cytokines including IL-4 and IL-10 were measured by qPCR. We found that the expression of miR-322 increased after transfection of miR-322 mimics (Figure 4A). Inflammatory cytokines (IL-1β, IL-6 and TNF-α) were suppressed...
Figure 1. LPS increases the mRNA expression of inflammatory cytokines in RAW264.7 cells
RAW264.7 cells were incubated with 1 μg/ml LPS for 0, 2, 4, 8, 12 and 24 h. Total RNA was extracted and the mRNA expression of IL-1β (A), IL-6 (B) and TNF-α (C) were detected with qPCR. Results are representative of three independent experiments performed in triplicate (**P<0.01).

Figure 2. LPS inhibits the process of cell cycle of RAW264.7 cells
RAW264.7 cells were incubated with 1 μg/ml LPS for 0, 2, 4, 8, 12 and 24 h. Total RNA was extracted and the mRNA expressions of cyclin D (A), cyclin E (B), P21 (C) and P27 (D) were detected with qPCR. Results are representative of three independent experiments performed in triplicate (*P<0.1, **P<0.01 and ***P<0.001).
Figure 3. Expression of miR-322 is down-regulated in LPS-treated RAW264.7 cells
(A) RAW264.7 cells were incubated with different doses of LPS for 24 h. Total RNA was extracted and miR-322 levels were detected with qPCR and normalized to endogenous U6. (B) RAW264.7 cells were treated with 1 μg/ml LPS at indicated times. miR-322 levels were detected with qPCR and normalized to endogenous U6. Results are representative of three independent experiments performed in triplicate (*P<0.1, **P<0.01 and ***P<0.001; the marked group was compared with the group without LPS treatment).

after LPS exposure compared with control while overexpression of miR-322 (Figure 4B–4D) and anti-inflammatory (IL-4 and IL-10) were significantly up-regulated (Figure 4E and 4F). These data demonstrated that miR-322 may negatively regulate LPS-stimulated inflammatory cytokine expression.

miR-322 rescues cell-cycle procession and promotes cell proliferation in RAW264.7 cells
To investigate the involvement of miR-322 on cell-cycle procession in RAW264.7 cells, miR-322 was overexpressed in RAW264.7 cells for 24 h following LPS-stimulation. Then, cell cycle-related genes cyclin D, cyclin E, P21 and P27 mRNA levels were detected by qPCR. The results showed that mRNA expressions of cyclin D and cyclin E were significantly increased, whereas those of P21 and P27 were significantly suppressed (Figure 5). These findings indicated that miR-322 appeared to promote cell-cycle procession by regulating expression of cell cycle-related genes. To further confirm the impact of miR-322 on cell-cycle procession, flow cytometry was applied. Cell cycle was arrested in G0/G1 in RAW264.7 cells following LPS-stimulation. Transfection of miR-322 increased S and G2/M and decreased the population of cells in G0/G1 (Figure 6). It indicated that LPS-induced cell-cycle repression can be rescued by miR-322.

As inflammatory response may lead to cell lesions and affect cell proliferation, we wished to further elucidate the effect of miR-322 on RAW264.7 cells proliferation. miR-322 mimics were transfected to RAW264.7 cells and cells were treated with LPS at a dose gradient of 0, 0.5, 1.0, 1.5 and 2.0 μg/ml for 24 h. MTT assay demonstrated that the cell viability was significantly reduced with a dose-dependent manner. miR-322 could promote RAW264.7 cells proliferation (Figure 7). All these findings suggested that miR-322 could promote procession of cell cycle and cell proliferation.

miR-322 directly targeted NF-κB1 (p50) through 3′-UTR interaction
To further elucidate the mechanisms of miR-322 regulating LPS-induced inflammatory response, Targetscan and miRanda were used to predict miR-322 targets that are related to NF-κB signalling. The analysis results revealed that an miR-322 seed sequence was predicted in the NF-κB1 3′-UTR. NF-κB1, also named p50, is an important subunit of NF-κB, which affects inflammatory reaction seriously. To test whether miR-322 can directly bind to NF-κB1 (p50) 3′-UTR, we generated a luciferase construct containing the NF-κB1 (p50) 3′-UTR with the predictive miR-322-binding site. In addition, a luciferase construct containing the NF-κB1 (p50) 3′-UTR with a mutation at the putative miR-322 seed sequence was generated as the control construct (Figure 8A). Two hundred and ninety three T-cells were cotransfected with a psiCheK-2 vector, the luciferase-wild-type NF-κB1 (p50)-3′-UTR (WT-3′-UTR) or the luciferase mutant (MUT-3′-UTR) report vector, as well as miR-322 mimics or inhibitors. miR-322 reduced the WT-3′-UTR but not MUT-3′-UTR luciferase levels. These results indicated that miR-322 could directly target NF-κB1 (p50) 3′-UTR (Figure 8B and 8C). In order to further confirm the regulatory function of miR-322 to NF-κB1 (p50), we used qPCR and Western blotting to detect mRNA and protein levels of NF-κB1 (p50) in cells transfected with miR-322 mimics or inhibitors. NF-κB1 (p50) mRNA and protein levels were significantly decreased in cells
Figure 4. *miR-322* inhibited macrophage inflammatory response

RAW264.7 cells were transfected with *miR-322* mimics or the negative control for 24 h and then incubated with LPS (1 μg/ml) for 8 h. (A) *miR-322* was measured by qPCR and normalized to endogenous U6. (B–D) mRNA levels of IL-1β, IL-6 and TNF-α were detected with qPCR. (E and F) mRNA levels of IL-4 and IL-10 were detected with qPCR. Results are representative of three independent experiments performed in triplicate (***P < 0.001).

transfected with *miR-322* mimics, not with *miR-322* inhibitors (Figure 8D–8G). These data suggested that *miR-322* repressed NF-κB1 (p50) expression by directly targeting 3′-UTR of NF-κB1 (p50) mRNA.

**Discussion**

Inflammation is the normal self-protection mechanism to eliminate pathogens and resist pathogen invasion [22,23]. The excessive inflammatory response and prolonged inflammation may lead to cell lesions or tissue damage [24]. Here, we found that *miR-322* expression is markedly suppressed in LPS-treated RAW264.7 cells. Subsequent finding suggested that *miR-322* targets 3′-UTR of NF-κB1 (p50) and negatively regulates LPS-modulated inflammatory cytokine expression.
Figure 5. miR-322 promotes cell cycle process of RAW264.7 cells
RAW264.7 cells were transfected with miR-322 mimics or the negative control for 24 h and then incubated with or without LPS (1 μg/ml) for another 24 h. Cyclin D, cyclin E, P21 and P27 mRNA levels were detected with qPCR. Results are representative of three independent experiments performed in triplicate (*\(P<0.1\) and **\(P<0.001\)).

Figure 6. miR-322 rescues cell-cycle procession in RAW264.7 cells
RAW264.7 cells were transfected with miR-322 mimics for 24 h, cell-cycle analysis was performed at 24 h after LPS-stimulation. The diagram above displays the percentage changes in G0/G1 and G2/M.
miR-322, a member of an evolutionarily conserved miR-16 family, contributes to promoting osteoblast and muscle cells proliferation, differentiation by directly targeting Tob2 and Cdc25A [18,25]. miR-322 also plays a crucial role in diabetes, male infertility and other biological processes [19,20]. However, until now, the role of miR-322 in inflammatory response remains to be further studied. Emerging results indicated that LPS activates NF-κB signalling pathways, causing the release of inflammatory factors such as IL-1β, IL-6 and TNF-α [26]. We found that the expression levels of inflammatory cytokines were significantly increased and cell-cycle procession was seriously inhibited following LPS-stimulation. Meanwhile, the expression level of miR-322 was down-regulated and was opposite to the inflammatory cytokine mRNA level. Moreover, the anti-inflammatory expression level was up-regulated. The data revealed that miR-322 may be involved in the regulation of LPS-mediated inflammatory response.

Surveying the predicted targets associated with inflammatory response of miR-322, one conserved 7-nt site in the NF-κB1 (p50) 3′-UTR is complementary to the miR-322 ‘seed’ region. NF-κB is an important transcription factor that regulates the transcription and expression of multiple genes. It is closely related to the cell activation, cell proliferation, immune response and inflammatory reaction process [6]. The transcription factor p50, the transcript of NF-κB1, is an important subunit of NF-κB. It regulates gene transcription through combination with p65, which is another important subunit of NF-κB. When the signalling pathway is activated, p50/p65 exposes nuclear localization signal and translocates into the nucleus, regulating the transcription of inflammatory cytokine [2,6]. In the present study, the interaction between NF-κB1 (p50) and miR-322 was analysed by luciferase assay. Luciferase expression level was significantly reduced when cotransfected with miR-322 and WT-3′-UTR plasmid, whereas no significant modulation was observed with mut-3′-UTR. Furthermore, we found that overexpression of miR-322 reduced expression of NF-κB1 (p50) mRNA and protein. Meanwhile, the expression level of inflammatory cytokines was inhibited by miR-322 following LPS-stimulation. LPS/TLR4/NF-κB signalling pathway plays a crucial role in cell inflammatory effect. miR-322 can target 3′-UTR of NF-κB1 (p50), which indicated that TLR4 receptor and its signalling pathway may be involved in the process. Taken together, these results indicated that miR-322 negatively regulated LPS-mediated inflammatory response by directly targeting NF-κB1 (p50).

NF-κB signalling cascade involved in macrophage inflammatory response. Macrophages initiate the innate immune response and deal with antigen to T-cells regulating the adaptive immunity [27]. Macrophages protect the body from infection and produce various inflammatory cytokines. Nevertheless, overproduced cytokines may cause cell damage and result in pathological condition. Previous studies reported that many miRNAs are useful modulators in maintaining cell homoeostasis by regulating cell-cycle proteins [28,29]. In the present study, the function of miR-322
in cell proliferation and cell cycle was investigated in macrophages. We found that RAW264.7 cell cycle was arrested in G0/G1 phase and cell proliferation was significantly inhibited following LPS stimulation. We also found a decrease in miR-322 expression level in LPS-treated RAW264.7 cells. It indicated that miR-322 is involved in RAW264.7 cell proliferation. In the subsequent research, overexpression of miR-322 increased cell cycle arrest at G2/M-phase and
the number of cells in $G_0/G_1$ was significantly decreased. Besides, LPS-induced cell proliferation repression was rescued by miR-322. These data suggested that miR-322 could promote cell proliferation and arrest cell cycle following LPS-stimulation.

In summary, we had proved for the first time that NF-κB1 (p50) was a novel target of miR-322. Our data provided evidence that LPS-induced down-regulation of miR-322 and its subsequent effects on NF-κB-mediated transcriptional activity are responsible for fine-tuning inflammatory responses in RAW264.7 cells. Besides, miR-322 plays a significant role in RAW264.7 cell proliferation. However, the specific mechanisms of miR-322 regulation of cell-cycle procession and cell proliferation remain to be further elucidated. Overall, miR-322 may provide a new perspective for the treatment of inflammatory diseases.

Author contribution
Kai Zhang, Fengling Song, Hanchuan Dai conceived and designed the research; Kai Zhang, Fengling Song, Xiaoxia Lu, Wenyun Chen, Chunxiao Huang, Lexing Li, Danyang Liang performed the experiments; Kai Zhang, Fengling Song analyzed the data; Kai Zhang, Fengling Song, Shengbo Cao, Hanchuan Dai wrote the paper.

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Competing interests
The authors declare that there are no competing interests associated with the manuscript.

Abbreviations
DMEM, Dulbecco’s modified eagle medium; IL, interleukin; LPS, lipopolysaccharide; PRR, cells expressing pathogen-recognition receptor; qPCR, quantitative real-time PCR; SOSC1, suppressor of cytokine signalling 1; TAB, TAK1-binding protein; TLR, toll-like receptor; TNF, tumor necrosis factor; TRAF6, tumor necrosis factor receptor-associated factor 6; WT-3′-UTR, luciferase-wild-type NF-κB1-3′-UTR.

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