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Circ_VMA21 protects WI-38 cells against LPS-induced apoptotic and inflammatory injury by acting on the miR-409-3p/KLF4 axis

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Running title: circ_VMA21 inhibits the progression of pneumonia

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Highlights:

1. Circ_VMA21 ameliorates LPS-induced WI-38 cellular apoptosis and inflammation
2. Circ_VMA21 targets miR-409-3p and KLF4 is a target of miR-409-3p
3. Circ_VMA21 promotes the expression of KLF4 via sponging miR-409-3p

Abstract. Pneumonia is a common inflammatory lung disease. Circular RNA (circRNA) vacuolar ATPase assembly factor (circ_VMA21) has been reported to mitigate the inflammatory injury in WI-38 cells. This study was to investigate the functional mechanism of circ_VMA21. Cell model was established by lipopolysaccharide (LPS) treatment in WI-38 cells. Cell cycle and apoptosis were analyzed by flow cytometry. Cell proliferation was assessed by colony formation and MTT assays. The expression quantification of circ_VMA21, microRNA-409-3p (miR-409-3p) and Kruppel-like transcription factor 4 (KLF4) was performed by quantitative real-time polymerase chain reaction (qRT-PCR). The expression of relative protein was detected by western blot. Inflammatory response was evaluated by enzyme-linked immunosorbent assay (ELISA). The target binding was validated by dual-luciferase reporter assay. Cellular analysis indicated that LPS repressed cell cycle and proliferation but induced apoptosis. Circ_VMA21 was downregulated in

pneumonia samples and LPS-treated WI-38 cells. Functionally, circ_VMA21 assuaged the LPS-induced apoptotic and inflammatory damages. In addition, Circ_VMA21 directly targeted miR-409-3p and its function was dependent on the sponge effect on miR-409-3p. Also, KLF4 was a target of miR-409-3p and miR-409-3p inhibitor attenuated LPS-induced cell injury by upregulating KLF4. Moreover, KLF4 was upregulated by circ_VMA21/miR-409-3p axis. These findings suggested that circ_VMA21 relieved the LPS-induced apoptotic and inflammatory injury by the regulation of miR-409-3p/KLF4 axis.

Key words: circ_VMA21 — Pneumonia — miR-409-3p — KLF4

Introduction

Pneumonia is a severe lung disease with acute lower respiratory infection, which can diver acute and chronic comorbidities in other organs of the body(Quinton et al. 2018). Despite the great advancements have been achieved in prevention and treatment of pneumonia, it still remains one of the primary causes of morbidity and mortality in children and older adults (le Roux et al. 2017;Petrosillo et al. 2015;Wardlaw et al. 2006). Severe inflammation caused by microbial pathogens in pneumonia can lead to the systemic damage (Meijvis et al. 2012). Lipopolysaccharide (LPS) that is the main component of the cell wall of gram-negative bacteria has been considered as an effective stimulator of the inflammatory response in the immune system (Wyns et al. 2015). Genomic techniques have been supposed to improve the therapeutic management and poor prognosis of pneumonia (Gautam et al. 2018).

Circular RNAs (circRNAs) characterized by the covalently closed-loop structures are stable and conservative RNAs in almost all types of eukaryotic cells (Ebbesen et al. 2017). Emerging studies suggested that circRNAs have pivotal regulatory functions in various kinds of diseases, such as cancers (Zhao et al. 2017), cardiovascular diseases (Altesha et al. 2019), neurological diseases (Li et al. 2017) and inflammatory diseases (Ye et al. 2019). Cheng *et al.* reported that circRNA vacuolar ATPase assembly factor (circ_VMA21, hsa_circ_0091702) inhibited the inflammatory cytokines-induced intervertebral disc degeneration by regulating the miR-200c/XIAP pathway (Cheng et al. 2018). Yang *et al.* found circ_VMA21 protected against the LPS-induced pneumonia by targeting miR-142-3p (Yang et al. 2020).

CircRNAs can sponge microRNAs (miRNAs) to further affect the expression level of the downstream genes (Panda 2018). MiRNAs are a family of short non-coding RNAs and they can

bind to the 3'-untranslated regions (3'-UTRs) of mRNAs (Shahabi et al. 2019). MicroRNA-409-3p (miR-409-3p) was discovered to promote the production of inflammatory cytokines in encephalomyelitis via reducing the SOCS3 expression (Liu et al. 2019), which was one of the top 3 upregulated miRNAs in pneumonia children (Huang et al. 2018). Kruppel-like transcription factor 4 (KLF4) is an inflammatory palliative in many diseases, such as sepsis (Li et al. 2018), atherosclerosis (Hartmann et al. 2016) and cerebral ischemic stroke (Zhang et al. 2020). Zhang *et al.* have proved that miR-429 enhanced cell inflammation by downregulating KLF4 in neonatal pneumonia (Zhang et al. 2020). It remains unknown whether KLF4 is a target of miR-409-3p or circ_VMA21 can regulate the KLF4 expression as a sponge of miR-409-3p.

In the present study, we affirmed the target relation between circ_VMA21 or KLF4 and miR-409-3p. A novel mechanism underlying the function of circ_VMA21 in pneumonia injury is disclosed.

Materials and Methods

Cell culture and LPS treatment

Human lung fibroblast cell line WI-38 (American Type Culture Collection, ATCC; Manassas, VA, USA) was sustained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) added with heat-inactivated fetal bovine serum (FBS; Gibco, 10%) and penicillin/streptomycin solution (Gibco, 1%). Cell culture was conducted in an 37°C incubator with humidified air and 5% CO₂. To establish the *in vitro* model of pneumonia, WI-38 cells were treated with 10 µg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) for 24 h and 5 µg/mL LPS was used as a low concentration control group (Bai et al. 2018).

Cell cycle detection

Cell cycle was detected using Cell Cycle Assay Kit (Dojindo, Kumamoto, Japan). Cells were stained with propidium iodide (PI) in accordance with the specification of the manufacturer. Cell distribution was then determined on a flow cytometer (BD Biosciences, San Diego, CA, USA).

Colony formation assay

200 cells were transplanted into the 6-well plates and cultured in the 37°C incubator for two weeks. Subsequently, colonies were fixated in methanol (Sigma-Aldrich) and stained using crystal violet (Sigma-Aldrich) for 15 min. Then the colonies were photographed and counted.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay

Cell proliferation was examined by MTT assay after different treatments. Cells in each well was incubated with 10 μ L MTT (Roche, Basle, Switzerland) for 4 h. Formazan was dissolved by 100 μ L dimethylsulfoxide (DMSO; Invitrogen, Carlsbad, CA, USA), followed by the measurement of the absorbance by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 570 nm.

Cell apoptosis assay

The apoptotic cells were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI), in compliance with the instruction book of Annexin V/FITC Apoptosis Detection Kit (Dojindo). By performing the analysis of flow cytometer (BD Biosciences), cells labeled with Annexin V (+)/PI (-) and Annexin V (+)/PI (+) were indicated as the apoptotic cells. Cell apoptotic rate was calculated to evaluate cell apoptosis.

Serum specimens

31 infantile pneumonia (IP) patients (18 male and 13 female) in this study have not been diagnosed as other diseases, the age range was from 0-6 years old (<2 years old: n=5; 2-4 years old: n=17; 4-6 years old: n=9). Peripheral venous blood was collected from IP patients and healthy volunteers (n=31) at Jinan Maternity and Child Care Hospital. Blood samples were centrifuged at 1200 rpm for 10 min, and the supernatant serum was saved at -80°C. The written informed consent forms were signed by the participants or their guardians. This study was authorized by the Ethics Committee of Jinan Maternity and Child Care Hospital.

The quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol™ Reagent (Invitrogen) was used to extract the total RNA from cells. Reverse transcription was performed using First Strand cDNA Synthesis Kit (Roche). The expression level was quantified by KAPA SYBR® FAST One-Step qRT-PCR Master Mix (Roche) and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). In addition, RNA stability was assessed by qRT-PCR after treatment of 3 U/ μ g Ribonuclease R (RNase R; Epicentre Technologies, Madison, WI, USA) in total RNA or 2 mg/mL Actinomycin D (Sigma-Aldrich) in culture medium. The expression analysis was carried out by the $2^{-\Delta\Delta C_t}$ method. Circ_VMA21 (or mRNAs) and miR-409-3p levels were respectively normalized using glyceraldehyde-phosphate dehydrogenase (GAPDH) and U6. The sequences of specific primers were listed as below: circ_VMA21, forward

(F): 5'-ACCGTTTTCTGGATGGATTTTG-3' and reverse (R):
5'-GCGACCACTGCAACAATAGC-3'; VMA21, F: 5'-GCATCTACACTGAAGACGCTCC-3'
and R: 5'-GACCACTGCAACAATAGCAGCG-3'; miR-409-3p, F:
5'-GCGAATGTTGCTCGGTGA-3' and R: 5'-GTGCAGGGTCCGAGGT-3'; KLF4, F:
5'-ACAAAGAGTTCCCATCTCAA-3' and R: 5'-GTAGTGCCTGGTCAGTTC-3'; GAPDH, F:
5'-GACTCATGACCACAGTCCATGC-3' and R: 5'-AGAGGCAGGGATGATGTTCTG-3'; U6, F:
5'-CTCGCTTCGGCAGCACA-3' and R: 5'-AACGCTTCACGAATTTGCGT-3'.

Transient transfection

Circ_VMA21 sequence was inserted into the pCD5-ciR overexpression vector (GENESEEO, Guangzhou, China) and the positive vector was named as circ_VMA21. miR-409-3p mimic (miR-409-3p) and miRNA mimic (miR-NC), miR-409-3p inhibitor (anti-miR-409-3p) and miRNA inhibitor (anti-miR-NC), small interfering RNA (siRNA) targeting KLF4 (si-KLF4) and siRNA negative control (si-NC) were bought from RIBOBIO (Guangzhou, China). Transient transfection was conducted by Lipofectamine[™] 3000 Transfection Reagent (Invitrogen) following the users' manual.

Western blot

Protein extraction was performed by RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific). Protein concentration was determined using BCA kit (Thermo Fisher Scientific), and 30 µg proteins were applied for the western blot analysis (Zhang et al. 2017). The primary antibodies were purchased from Cell Signaling Technology (CST, Boston, MA, USA): Ki67 (#9129, 1:1000), B-cell lymphoma-2 (Bcl-2; #4223, 1:1000), Bcl-2-associated X (Bax; #5023, 1:1000), Cleaved-poly-ADP-ribose polymerase (Cleaved-PARP; #5625, 1:1000), KLF4 (#12173, 1:1000), Interleukin-6 (IL-6; #12153, 1:1000), tumor necrosis factor-alpha (TNF-α; #6945, 1:1000), GAPDH (#5174, 1:1000). The secondary antibody Anti-rabbit IgG, HRP (#7074, 1:2000) was also acquired from CST. Ultimately, the protein signals were emerged using SignalFire[™] Plus ECL Reagent (CST) followed by the protein analysis on the ImageLab software version 4.1 (Bio-Rad).

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of inflammatory cytokines TNF-α, IL-6 and Interleukin-8 (IL-8) were examined using Human ELISA Kits (Invitrogen), as per the operating procedures provided by the producer.

Dual-luciferase reporter assay

Circ_VMA21 and KLF4 3'UTR sequences containing the miR-409-3p binding sites were respectively cloned into the pmirGLO vector (Promega, Madison, WI, USA) to construct the circ_VMA21-wt and KLF4-wt plasmids. The miR-409-3p binding sites in circ_VMA21 and KLF4 3'UTR were mutated, then circ_VMA21-mut and KLF4-mut plasmids were obtained by molecular cloning. Cells were plated into the 24-well plates overnight, and co-transfected with the above circ_VMA21 or KLF4 plasmid and miR-409-3p or miR-NC for 48 h. Finally, cell lysate was harvested and the relative luciferase activity was detected using the dual-luciferase reporter assay system (Promega).

Statistical analysis

Data were shown as the mean \pm standard deviation (SD) and SPSS 24.0 was applied for the statistical analysis. The linear correlation was analyzed using Pearson's correlation coefficient. Student's *t*-test and one-way analysis of variance (ANOVA) followed by Tukey's test were respectively performed to compare the difference between two groups and among multiple groups. $P < 0.05$ represented the significant statistical difference.

Results

LPS inhibited cell cycle progression and proliferation but induced apoptosis in WI-38 cells

After the treatment of LPS, cellular behaviors were analyzed to assess the effects of LPS on WI-38 cells. Cell cycle was blocked from G0/G1 phase to S phase in 10 $\mu\text{g/mL}$ group compared to control and 5 $\mu\text{g/mL}$ groups (Fig. 1A), showing that LPS impeded the progression of cell cycle in WI-38 cells. Colony formation assay (Fig. 1B) and MTT (Fig. 1C) demonstrated that cell proliferation was repressed by LPS. In addition, cell apoptotic rate was elevated in 5 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$ LPS groups in comparison to control group (Fig. 1D). These results indicated that cell injury was successfully induced by LPS *in vitro*.

Circ_VMA21 was downregulated in pneumonia patients and LPS-treated WI-38 cells

The expression of circ_VMA21 in serum samples and cells was examined using qRT-PCR. As shown in Fig. 2A-B, circ_VMA21 expression was significantly declined in IP samples and LPS-treated WI-38 cells contrasted with the normal samples and control cells. Circ_VMA21 was found to be resistant to RNase R digestion (Fig. 2C) and Actinomycin D treatment (Fig. 2D) while

the VMA21 expression was evidently inhibited, which suggested that circ_VMA21 was more stable than the linear VMA21 and it was a circRNA. Circ_VMA21 downregulation might be associated with the progression of pneumonia.

Circ_VMA21 relieved the LPS-induced apoptosis and inflammation in WI-38 cells

Circ_VMA21 vector was constructed to investigate the function of circ_VMA21 in pneumonia. The qRT-PCR manifested that circ_VMA21 transfection upregulated the expression of circ_VMA21 by 9-fold changes while it did not affect the VMA21 level (Fig. 3A). Circ_VMA21 vector and control vector were respectively transfected into WI-38 cells under the treatment of 10 µg/mL LPS. It has been observed that the introduction of circ_VMA21 reversed the LPS-induced cell cycle arrest (Fig. 3B), cell proliferation inhibition (Fig. 3C-D) and Ki67 (a proliferation marker) protein downregulation (Fig. 3E). Cell apoptotic rate was lower in LPS+circ_VMA21 group than that in LPS+Vector group (Fig. 3F). Also, the downregulation of anti-apoptotic Bcl-2 but upregulation of pro-apoptotic Bax and Cleaved PARP caused by LPS were mitigated following the transfection of circ_VMA21 (Fig. 3G). Data of ELISA suggested that LPS treatment promoted the concentrations of inflammatory cytokines (TNF- α , IL-6 and IL-8), while the overexpression of circ_VMA21 ameliorated this inflammatory response (Fig. 3H). These findings showed that circ_VMA21 could abate the LPS-induced apoptotic and inflammatory injury in WI-38 cells.

Circ_VMA21 targeted miR-409-3p

CircBank software was employed to predict the binding sites between circ_VMA21 and miR-409-3p (Fig. 4A). Dual-luciferase reporter assay manifested that miR-409-3p overexpression suppressed the relative luciferase activity of circ_VMA21-wt plasmid while no distinct difference was found in circ_VMA21-mut group (Fig. 4B), affirming that miR-409-3p could bind to circ_VMA21. The qRT-PCR analysis exhibited that miR-409-3p expression was downregulated by the upregulation of circ_VMA21 in WI-38 cells (Fig. 4C). In addition, the level of miR-409-3p was notably upregulated in LPS-treated WI-38 cells (Fig. 4D) and IP serum samples (Fig. 4E) compared to the control groups. Interestingly, a negative relationship ($r=-0.6382$, $P<0.0001$) between the expression of circ_VMA21 and miR-409-3p was found in IP serum samples (Fig. 4F). Together, circ_VMA21 could directly target miR-409-3p.

Circ_VMA21 lightened the LPS-induced cell injury via sponging miR-409-3p

The qRT-PCR data displayed that miR-409-3p expression was increased in

circ_VMA21+miR-409-3p group in contrast with the circ_VMA21+miR-NC group (Fig. 5A), implying that miR-409-3p was greatly overexpressed by miR-409-3p mimic. Subsequently, LPS-treated WI-38 cells were transfected with circ_VMA21, circ_VMA21+miR-409-3p and the corresponding control groups. Functional experiments indicated that the effects of circ_VMA21 on cell cycle (Fig. 5B), cell proliferation (Fig. 5C-E) and cell apoptosis (Fig. 5F-G) were abolished after the overexpression of miR-409-3p. Meanwhile, ELISA and western blot indicated that the concentration inhibition (Fig. 5H) and protein expression downregulation (Fig. S1A) of inflammatory cytokines were also relieved by miR-409-3p mimic. Therefore, the protective function of circ_VMA21 against the LPS-induced cell injury was achieved by sponging miR-409-3p.

KLF4 acted as a target gene for miR-409-3p

The miRDB analysis presented the complementary sites for miR-409-3p in the 3'UTR sequence of KLF4 (Fig. 6A). The interaction between miR-409-3p and KLF4 was further validated by the dual-luciferase reporter assay (Fig. 6B). The miR-409-3p mimic and anti-miR-409-3p were both efficient on the expression of miR-409-3p by comparison with the miR-NC and anti-miR-NC groups (Fig. 6C). KLF4 mRNA and protein levels were found to be inhibited by miR-409-3p overexpression but increased by miR-409-3p inhibition (Fig. 6D-E). The expression analysis for KLF4 by qRT-PCR and western blot revealed that it was downregulated in LPS-treated WI-38 cells (Fig. 6F-G) and IP serum samples (Fig. 6H-I). Pearson's correlation coefficient exhibited that there was a negative relation ($r=-0.5496$, $P=0.0011$) between miR-409-3p and KLF4 (Fig. 6J) but a positive correlation ($r=0.5392$, $P=0.0015$) between circ_VMA21 and KLF4 (Fig. 6K) in IP samples. Altogether, KLF4 was a target of miR-409-3p which related to circ_VMA21.

Downregulation of miR-409-3p weakened cell injury caused by LPS via upregulating the KLF4 expression

Si-KLF4 was used for the knockdown of KLF4 expression. As illustrated in Fig. 7A-B, si-KLF4 transfection restored the anti-miR-409-3p-mediated upregulation of KLF4 mRNA and protein expression. Cell cycle progression was enhanced by miR-409-3p inhibitor in LPS-treated WI-38 cells, which was eliminated by the knockdown of KLF4 (Fig. 7C). Cell proliferation by colony formation assay (Fig. 7D), MTT (Fig. 7E) and Ki67 protein detection (Fig. 7F) was also shown to be facilitated by anti-miR-409-3p transfection, whereas this promotion was reverted following the silence of KLF4 expression. Moreover, flow cytometry and western blot showed that KLF4

downregulation abrogated the anti-miR-409-3p-induced apoptosis inhibition (Fig. 7G-H). Data from ELISA suggested that the downregulation of TNF- α , IL-6 and IL-8 caused by anti-miR-409-3p was counteracted after KLF4 knockdown in LPS-treated WI-38 cells (Fig. 7I). Consistently, the protein levels of TNF- α and IL-6 were also upregulated by the introduction of si-KLF4 relative to alone anti-miR-409-3p transfection in LPS-treated cells (Fig. S1B). Taken together, miR-409-3p inhibitor could recede the LPS-induced cell injury via targeting KLF4.

Circ_VMA21 upregulated the expression of KLF4 as a sponge of miR-409-3p

To reveal whether circ_VMA21 could regulate the KLF4 expression via targeting miR-409-3p, reverted transfection (Vector, circ_VMA21, circ_VMA21+miR-NC and circ_VMA21+miR-409-3p) was performed in WI-38 cells. The qRT-PCR and western blot demonstrated that circ_VMA21 transfection upregulated the mRNA (Fig. 8A) and protein (Fig. 8B) expression of KLF4, which was subsequently returned by miR-409-3p overexpression. Thus, KLF4 could be regulated by the circ_VMA21/miR-409-3p axis.

Discussion

Pneumonia is a typical inflammatory disease of human respiratory system. The functional analysis and mechanism exploration in this study confirmed that circ_VMA21 acted as an effective biomarker to inhibit the apoptotic and inflammatory injury in pneumonia by the miR-409-3p/KLF4 axis.

A handful of circRNAs have been revealed to participate in the regulation of pneumonia (Zhao et al. 2019; Zhong et al. 2019). For instance, Jia *et al.* stated that circ_406961 inhibited the particulate matter (PM)-induced inflammatory response in human bronchial epithelial 16HBE cells by interacting with ILF2 and activating the STAT3/JNK pathway (Jia et al. 2020). Guo *et al.* reported that the silence of circANKRD36 lightened the LPS-aroused cell injury in human embryonic lung fibroblast cells (Guo et al. 2020). Knockdown of circ_0038467 remitted the LPS-induced inflammatory damage in 16HBE cells by regulating the expression of miR-338-3p (Liu et al. 2020), and circZNF652 retarded the progression of pneumonia in WI-38 cells by sponging miR-181 (Liu et al. 2019). The functional experiments for circ_VMA21 have shown that its overexpression weakened the LPS-triggered cell cycle retardation, proliferation inhibition, apoptotic phenomenon and inflammatory response in WI-38 cells. Circ_VMA21 was identified as an anti-apoptotic and anti-inflammatory factor in the pathological injury of pneumonia.

CircRNAs are important regulatory molecules in human diseases via acting as the sponges of miRNAs. Hsa_circ_0000523 repressed cell proliferation and promoted cell apoptosis in colorectal cancer by serving as a miR-31 sponge (Jin et al. 2018). CircADAMTS13 could sponge miR-484 to inhibit the proliferation of hepatocellular carcinoma cells (Qiu et al. 2019). Circ-ZNF609 has been found to regulate the retinal neurodegeneration by the sponge effect on miR-615 (Wang et al. 2018). Our results manifested that circ_VMA21 combined with miR-409-3p and incurred the negative regulation of miR-409-3p expression. Additionally, the restoration of miR-409-3p for the circ_VMA21-mediated effect suggested that circ_VMA21 attenuated the LPS-induced cell injury by sponging miR-409-3p.

MiR-409-3p is a common tumor regulator by targeting different genes. Wu *et al.* declared that miR-409-3p repressed osteosarcoma cell proliferation and invasion by downregulating the ZEB1 expression (Wu et al. 2019). Zhang *et al.* found that miR-409-3p targeted Akt1 to regulate the development of breast cancer (Zhang et al. 2016). In this study, KLF4 was proved to be a downstream target of miR-409-3p and miR-409-3p could regulate the LPS-aroused WI-38 injury by the negative regulation of KLF4. More interestingly, circ_VMA21 was positively correlated with KLF4 expression in IP serum samples. The expression analysis indicated that circ_VMA21 could sponge miR-409-3p to induce the promoting effect on the KLF4 level. Wang *et al.* have shown that circ_0001023 facilitated cell growth and metastasis in gastric cancer by regulating the expression of PHF10 via sequestering miR-409-3p (Wang et al. 2020). Herein, circ_VMA21 was considered to alleviate the LPS-induced injury in WI-38 cells by targeting the miR-409-3p/KLF4 axis.

Conclusion

In conclusion, all these results clarified that circ_VMA21 protected WI-38 cells from the LPS-mediated injury by regulating the miR-409-3p/KLF4 axis. The circ_VMA21/miR-409-3p/KLF4 molecular network was discovered as a novel pathological mechanism in the progression of pneumonia.

Disclosure of interest. The authors declare that they have no financial conflicts of interest.

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Figure Legends

Figure 1. LPS inhibited cell cycle progression and proliferation but induced apoptosis in WI-38 cells. WI-38 cells were treated with 5 µg/mL and 10 µg/mL LPS with the untreated cells as the control group. (A) Cell cycle was detected by flow cytometry. (B-C) Cell proliferation was determined using colony formation assay (B) and MTT (C). (D) Cell apoptosis was assessed by flow cytometry. Each experiment was independently performed three times with three technical repetition (n=3). * $P < 0.05$.

Figure 2. Circ_VMA21 was downregulated in pneumonia patients and LPS-treated WI-38 cells. (A-B) The expression of circ_VMA21 was examined by qRT-PCR in IP/Normal serum samples (A) and control/LPS treatment groups (B). (C-D) The qRT-PCR was used for the analysis of circ_VMA21 and VMA21 levels after RNase R (C) or Actinomycin D (D) treatment (0 h, 8 h, 16 h and 24 h). Each experiment was independently performed three times with three technical repetition (n=3). * $P < 0.05$.

Figure 3. Circ_VMA21 relieved the LPS-induced apoptosis and inflammation in WI-38 cells. (A) Circ_VMA21 and VMA21 detection was performed by qRT-PCR after transfection of circ_VMA21 or Vector. (B) The measurement of cell cycle was conducted by flow cytometry in Control, LPS, LPS+Vector and LPS+circ_VMA21 groups. (C-E) Colony formation assay (C), MTT assay (D) and Ki67 detection by western blot (E) were applied for cell proliferation analysis. (F-G) Flow cytometry (F) and apoptotic protein detection by western blot (G) were carried out to evaluate cell apoptosis. (H) ELISA was used to assay the levels of inflammatory cytokines. Each experiment was independently performed three times with three technical repetition (n=3). * $P < 0.05$.

Figure 4. Circ_VMA21 targeted miR-409-3p. (A-B) The binding between circ_VMA21 and miR-409-3p was analyzed by circBank prediction (A) and dual-luciferase reporter assay (B). (C) The miR-409-3p expression was determined in Vector and circ_VMA21 transfection groups. (D-E) The qRT-PCR was used for the expression analysis of miR-409-3p in LPS-treated WI-38 cells (D)

and IP samples (E). (F) The linear relation between circ_VMA21 and miR-409-3p was analyzed by Pearson's correlation coefficient. Each experiment was independently performed three times with three technical repetition (n=3). * $P < 0.05$.

Figure 5. Circ_VMA21 lightened the LPS-induced cell injury via sponging miR-409-3p. (A) MiR-409-3p level was assayed using qRT-PCR following transfection of Vector, circ_VMA21, circ_VMA21+miR-NC, circ_VMA21+miR-409-3p. (B) Cell cycle detection was performed by flow cytometry in the Control, LPS, LPS+Vector, LPS+circ_VMA21, LPS+circ_VMA21+miR-NC and LPS+circ_VMA21+miR-409-3p groups. (C-E) Cell proliferation was evaluated by colony formation assay (C), MTT assay (D) and proliferation protein analysis by western blot (E) in the above six groups. (F-G) The assessment of cell apoptosis was performed by flow cytometry (F) and the apoptotic marker detection by western blot (G). (H) The examination of inflammatory cytokines was performed by ELISA. Each experiment was independently performed three times with three technical repetition (n=3). * $P < 0.05$.

Figure 6. KLF4 acted as a target gene for miR-409-3p. (A-B) The miRDB software (A) and dual-luciferase reporter assay (B) were used for the prediction and affirmation of miR-409-3p and KLF4 binding. (C) The promoting and inhibitory effects of miR-409-3p mimic and miR-409-3p inhibitor were respectively analyzed by qRT-PCR. (D-E) The mRNA and protein expression levels of KLF4 were assayed via qRT-PCR and western blot in miR-409-3p, anti-miR-409-3p and the relative control groups. (F-I) The qRT-PCR and western blot were implemented for KLF4 detection in LPS-treated WI-38 cells (F-G) and IP serum samples (H-I). (J-K) Pearson's correlation coefficient was used for the analysis of linear relationship between KLF4 and miR-409-3p (J) or circ_VMA21 (K). Each experiment was independently performed three times with three technical repetition (n=3). * $P < 0.05$.

Figure 7. Downregulation of miR-409-3p weakened cell injury caused by LPS via upregulating the KLF4 expression. (A-B) KLF4 mRNA and protein levels were measured by qRT-PCR and western blot after transfection of anti-miR-NC, anti-miR-409-3p, anti-miR-409-3p+si-NC or anti-miR-409-3p+si-KLF4. (C) Flow cytometry was conducted to determine cell cycle in different groups of Control, LPS, LPS+anti-miR-NC, LPS+anti-miR-409-3p, LPS+anti-miR-409-3p+si-NC or LPS+anti-miR-409-3p+si-KLF4. (D-F) The analysis of cell proliferation was performed using colony formation assay (D), MTT assay (E) and western blot for Ki67 expression detection (F).

(G-H) Cell apoptosis was assessed by flow cytometry for the apoptotic rate (G) and western blot for the apoptotic protein(H). (I) The levels of inflammatory cytokines were detected via ELISA. Each experiment was independently performed three times with three technical repetition (n=3). * $P < 0.05$.

Figure 8. Circ_VMA21 upregulated the expression of KLF4 as a sponge of miR-409-3p. (A-B) The qRT-PCR and western blot were used to analyze the expression changes of KLF4 after WI-38 cells were transfected with Vector, circ_VMA21, circ_VMA21+miR-NC or circ_VMA21+miR-409-3p. Each experiment was independently performed three times with three technical repetition (n=3). * $P < 0.05$.

Figure S1. Circ_VMA21/miR-409-3p axis and miR-409-3p/KLF4 axis regulated the protein levels of TNF- α and IL-6. (A-B) The protein detection of TNF- α and IL-6 was performed by western blot for Fig. 5H (A) and Fig. 7I (B). Each experiment was independently performed three times with three technical repetition (n=3).







