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MiR-520c-3p alleviates LPS-induced A549 cell and mice lung injury via targeting NLRC5

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MicroRNAs (miRNAs) play important roles in the progression of acute lung injury (ALI). So far, little is known about the role of miR-520c-3p in ALI. In this study, the mechanism of the occurrence and development of ALI was explored. We firstly found that miR-520c-3p could inhibit the expression of NLRC5. A549 cells were treated with lipopolysaccharide (LPS) *in vitro* to simulate ALI cells and inducing ALI model mice. Moreover, miR-520c-3p overexpression enhanced the viability of damaged cells, inhibited LPS-induced apoptosis, and decreased LPS-induced IL-1β, IL-6 and TNF-α. In addition, the NLRC5 was a direct target of miR-520c-3p. And NLRC5 partially aggravated inflammation injury. In conclusion, miR-520c-3p alleviates LPS-induced inflammatory injury *via* regulating NLRC5.

1. Introduction

Acute lung injury (ALI) is a progressive syndrome with a high incidence and mortality rate, which is related to inflammation in pulmonary system (Cai et al. 2016). The pathological features of ALI are extremely complex (Mitchell et al. 2020). Previous studies have found that ALI is an acute inflammatory process involving simultaneous production of inflammatory cytokines and chemokines (Li et al. 2020; Liu et al. 2020a). Therefore, it is highly important to study molecular mechanisms that might reduce inflammatory reactions that lead to the development and progression of ALI. However, its specific pathogenesis is still unknown.

MicroRNAs (miRNAs), as non-coding small RNA molecules with endogenous gene expression regulation, can bind to target gene mRNAs to regulate the target gene expression at the post-transcriptional level and further participate in various disease processes including a variety of miRNAs such as miR-297 (Xi et al. 2020), miR-205-5p (Yu et al. 2019), and miR-216a (Kong et al. 2020), which have been reported to play key regulatory roles in the development of ALI. As an emerging miRNA in recent years, miR-520c-3p has been found to be involved in many cancers (Xu et al. 2014; Peng et al. 2019), acute myeloid leukemia (Dong et al. 2018), inflammasome activation and inflammatory cascade (Liu et al. 2019). Nevertheless, whether it participates in the pathogenesis and its possible mechanism of action has not been studied.

NOD-like receptor CARD domain containing 5 (NLRC5), expressed in a variety of cells and tissues, is a member of the NLR family proteins that contain a nucleotide-binding domain and leucine-rich repeats. Human NLRC5 is located in the 16q13 locus and consists of 1,866 amino acids (aa) while mouse NLRC5 is at chromosome 8 and contains 1,915 aa (Mótyán et al. 2013; Rodriguez et al. 2016). NLRC5 is involved in regulating inflammatory responses and cell death. Recently, novel studies suggest that NLRC5 may have a closely relationship with inflammation-associated diseases which overexpression of NLRC5 are highly crucial in inflammation-induced diseases (Wang et al. 2019; Wu et al. 2019). Nevertheless, the role of NLRC5 in ALI remains to be further explored.

Therefore, in this study, we investigated the expression of miR-520c-3p *in vivo* and *in vitro* in order to explore whether miRNA-520c-3p affected the progression of ALI through regulating the expression of NLRC5. This study would provide a novel candidate therapeutic target for blocking the development of ALI.

2. Investigations and results

2.1. miR-520c-3p and NLRC5 are decreased after LPS treatment and miR-520c-3p targets directly NLRC5

First, as shown in Fig. 1A, RT-qPCR was used to detect the transfection efficiency. The results showed that the expression of miR-520c-3p and NLRC5 was significantly decreased after

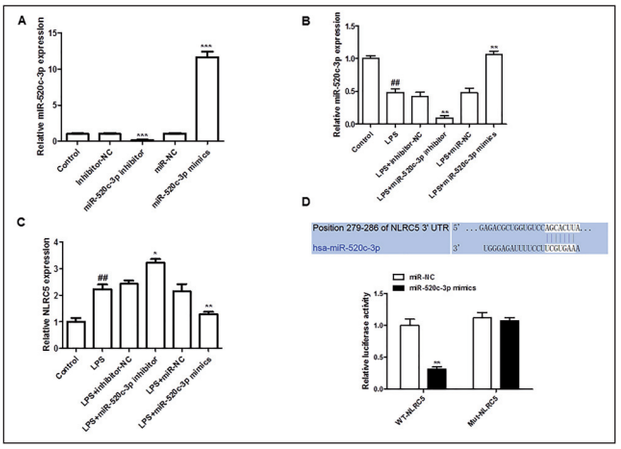

Fig. 1: Expression of miR-520c-3p and NLRC5 in LPS-treatment A549. (A) The transfection efficiency was measured by RT-qPCR. (B) Expression of miR-520c-3p was detected by RT-qPCR. (C) NLRC5 mRNA levels detected by RT-qPCR in each group cells. (D) The luciferase assay proved miR-520c-3p targets NLRC5 directly proved. ##P<0.01, ###P<0.001 *vs*. the Control group; **P<0.01, ***P<0.001 *vs*. the LPS group.

LPS treatment (Figs. 1B and 1C) and the overexpression of miR-520c-3p significantly inhibited the level of NLRC5. We further used the luciferase assay to determine that miR-520c-3p targets NLRC5 directly (Fig. 1D).

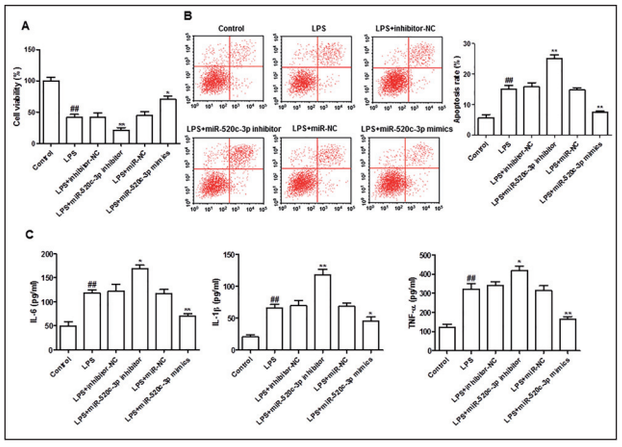

Fig. 2: Sliencing the miR-520c-3p reduces the cell viability, increases the cell apoptosis and promotes the inflammatory factors caused by LPS-treatment. (A) Cell viability was measured by CCK-8 assay. (B)Apoptosis was detected by Flow cytometry. **P<0.01 *vs*. the control group. (C) TNF-α, IL-1β, IL-6 ELISA kits were used to detect their levels. ##P<0.01, ##P<0.001 *vs*. the Control group; *P<0.05, **P<0.01 *vs*. the LPS group.

2.2. Silencing the miR-520c-3p reduces the cell viability, increases the cell apoptosis and promotes the inflammatory factors

As shown in Fig. 2A, blocking the expression of miR-520c-3p led to the decrease of cell viability accompany with the increased apoptosis (Fig. 2B). Moreover, ELISA assay showed that miR-520c-3p overexpression the decrease of TNF-α, IL-6 and IL-1β levels induced by LPS (Fig. 2C).

2.3. NLRC5 overexpression decreases cell viability, and increases apoptosis in vitro

As shown in Figs. 3A and 3B, we found a significant increase expression of NLRC5 after LPS treatment, while miR-520c-3p overexpression could alleviate its expression. Furthermore, we found that the high NLRC5 level significantly decrease cell viability, and increased cell apoptosis under LPS treatment, but this recovery effect was inhibited by the overexpression of miR-520c-3p (Figs. 3C and D).

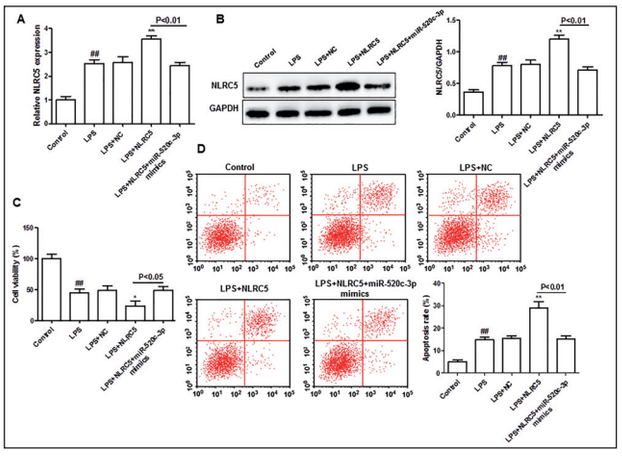

Fig. 3: NLRC5 overexpression decreases cell viability, and increases apoptosis in *vitro* caused by LPS-treatment. (A) The level of NLRC5 mRNA was detected by RT-qPCR. (B) NLRC5 protein level was measured by Western blot and was calculated by the band intensity of NLRC5 normalized by that of GAPDH. (C) Cell viability was measured by CCK-8 assay. (D) Cell apoptosis detected by Flow cytometry. ##P<0.01 *vs*. the Control group; *P<0.05, **P<0.01 *vs*. the LPS group.

2.4. NLRC5 overexpression promotes inflammatory response in vitro

As shown in Fig. 4, overexpression of NLRC5 promoted the levels of TNF-α, IL-6 and IL-1β compared with LPS group.

2.5. miR-520c-3p overexpression reduces NLRC5 expression and ameliorates to resistance to ALI in vivo

As shown in Fig. 5A, HE staining showed that miR-520c-3p overexpression could reduce the degree of lung lesions. And IHC showed that in the ALI lung tissues, the expression of NLRC5 was highly increased compared with the control mice. miR-520c-3p overexpression could reduce its expression (Fig. 5B).

Moreover, the mRNA and protein levels of NLRC5 increased significantly in the pathological tissues of ALI model mice, and this reduction was restored under the treatment of miR-520c-3p mimics (Figs. 5D and E).

Further, increased levels of TNF-α, IL-1β and IL-6 in lungs were observed in the ALI model mice (Fig. 6A). In addition, we found the decrease of SOD and GSH-Px content and the increase of MDA levels in the lung tissues of ALI, while overexpression of miR-520c-3p could reduce these changes and restore them to normal value (Fig. 6B). These results suggested that overexpression of miR-520c-3p could reduce inflammation response and oxidative stress. Overall, our results suggested that miR-520c-3p may inhibit apoptosis and decrease inflammation by inhibiting NLRC5 expression, and aggravate tissue lesions and ALI.

3. Discussion

To investigate the molecular mechanisms of ALI in humans, various experimental models of ALI have been used of which the most common is the endotoxin model using LPS (Cong et al. 2020; Yang et al. 2020). The present study used an *in vivo* LPS-induced ALI model as well as an *in vitro* model using A549 cells to investigate the regulatory mechanism of the inflammatory response. Our results showed that the expression of miR-520c-3p and NLRC5 were significantly decreased after LPS treatment. Moreover, blocking miR-520c-3p accelerated the increase of NLRC5 expression, consistent with this, overexpression of miR-520c-3p contributed to the further decrease of NLRC5 expression, which implied that miR-520c-3p regulated the expression of NLRC5. Luciferase reporter showed miR-520c-3p targets NLRC5 directly.

Although many studies have confirmed that miRNAs is a critical regulator in inflammatory bowel disease, ALI and other immunoreactive diseases (Lin et al. 2019; Liu et al. 2020b; Yan et al. 2020), it is not yet clear about the role of miRNAs in NLRC5 inflammasome in LPS induced ALI. Next, our data indicated that inhibiting the expression of miR-520c-3p significantly decreased the viability of damaged cells and increased the cell apoptosis, and promoted inflammation *in vivo* and *in vitro*. Overexpression of NLRC5 *in vitro* had similar effects. In further to detect the role of miR-520c-3p in ALI, the results from ALI mouse model showed that miR-520c-3p overexpression significantly reduce the degree of lung tissue damage, inflammation and oxidative stress response. This may be the mechanism by which miR-520c-3p participates in regulating the development of ALI. In conclusion, our results suggested that miR-520c-3p overexpression may be a potential and important clinical treatment for ALI.

Overall, this study demonstrated that miR-520c-3p inhibits inflammatory responses *via* targeting NLRC5. These findings highlights the miR-520c-3p might be a potential prognostic biomarker and part of therapeutic strategies for ALI.

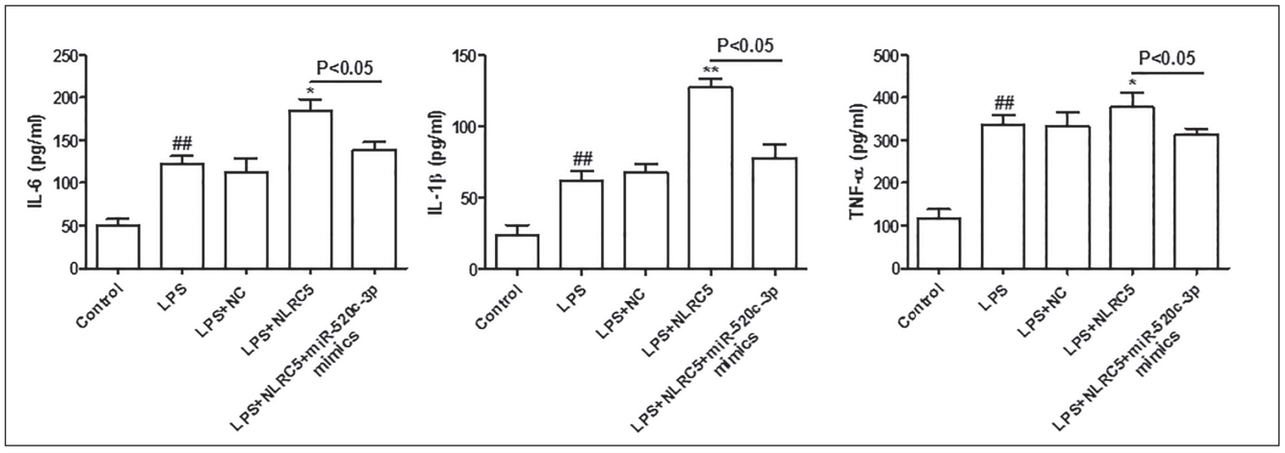

Fig. 4: NLRC5 overexpression promotes inflammatory response *in vitro.* Levels of TNF-α, IL-6 and IL-1β detected by ELISA essay in control group, LPS group, LPS + NLRC5 NC group, LPS + NLRC5 group and LPS + NLRC5 + miR-520c-3p mimics group. *P<0.05, ##P<0.01 *vs*. the Control group; *P<0.05, **P<0.01 *vs*. the LPS group.

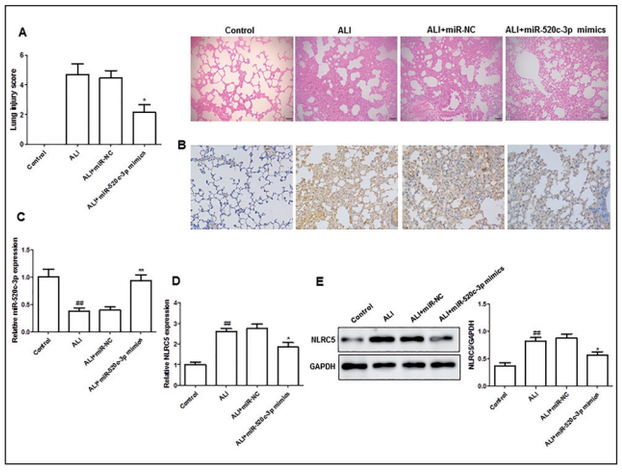

Fig. 5: miR-520c-3p overexpression reduces NLRC5 expression and ameliorates to resistance to ALI *in vivo.* (A) Representative photographs of lung in different treatment groups, stained with hematoxylin and eosin (HE). (B) IHC of NLRC5. RT-qPCR indicates a reduction in miR-520c-3p levels (C). RT-qPCR (D) and (E) western blot were detected the mRNA and protein expression of NLRC5. ##P<0.01 *vs*. the Control group; *P<0.05, **P<0.01 *vs*. the ALI group.

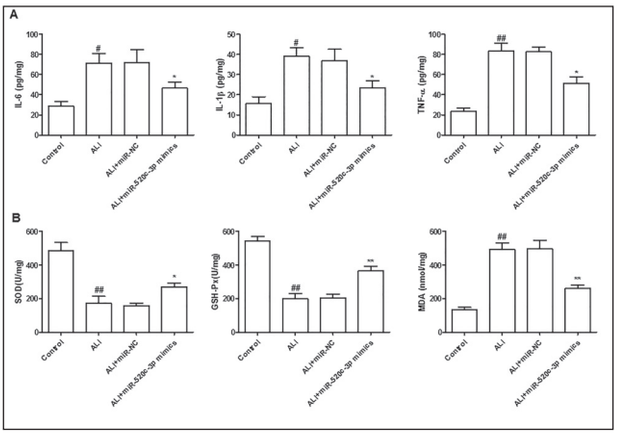

Fig. 6: miR-520c-3p overexpression reduces inflammatory response and oxidative response in ALI model mice. (A) Levels of TNF-α, IL-6 and IL-1β in lung tissues were measured by ELISA kit (B) Measurements of the levels of SOD, GSH-Px and MDA. #P<0.05, ##P<0.01 *vs*. the Control group; *P<0.05, **P<0.01 *vs*. the ALI group.

4. Experimental

4.1. Cell culture, treatment and transfection

The human lung cancer cell line, A549, was purchased from ATCC cell bank (Manassas, VA, USA) and cultured in the Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL. Co. Ltd., Grand Island, New York, USA) containing 10% fetal bovine serum (FBS, Beyotime Biotech. Shanghai, China) and supplementing with 1% penicillin-streptomycin (Beyotime Biotech, Shanghai, China) at 37 °C and 5% CO_2. Lipofectamine 3000 was used to perform the transfection. The miR-520c-3p mimic, mimic NC (miR-NC), inhibitor NC (inhibitor-NC), miR-520c-3p inhibitor, NLRC5, NLRC5-NC were synthesized by GenePharma. And A549 cells were treated with lipopolysaccharide (LPS) at concentrations of 1000 ng/ml.

4.2. CCK-8 analysis

Cell Counting Kit-8 (CCK-8) was used to detect the proliferation of A549 according to the manufacturer's instructions. The absorbance was measured at a wavelength of 450 nm using a microplate reader (BioTek ELx808, BioTek Instruments, Winooski, VT, USA).

4.3. Luciferase activity assay

According to TargetScan (http://www.targetscan.org) and MiRanda (http://www.microrna.org/microrna/home.do) databases, the wild-type NLRC5 3'UTR (NLRC5-WT) or the mutant NLRC5 3'UTR (NLRC5-Mut) was constructed into the pGL3 luciferase reporter vector. The above luciferase reporter plasmid was co-transfected with miR-520c-3p mimic or NC mimic into HEK293T cells, and the pRL-TK luciferase reporter vector was used as negative control. Luciferase assay was performed the firefly luciferase 48h post-transfection.

4.4. Animal and model establishment

Specific pathogen free (SPF) male C57BL/6 mice, weighing from 20 g to 22 g, were purchased from Charles River (Beijing, China). The mice were housed in the SPF conditions with water and food freely available. The ALI mice were intratracheally challenged with 50 µl of LPS (*Escherichia coli* 055:B5; Sigma-Aldrich, 100 µg/mouse). The control mice were received the same volumes of sterile water. After 3d treatment of LPS, the animals were sacrificed to collect tissues for analysis. In the treatment groups, mice were treated with LPS plus the miR-520c-3p inhibitor vector (1×10^9 pfu/ mouse).

4.5. Real-time quantitative PCR

Total RNA in tissues and cells were routinely acquired by TRIZOL reagent (Thermo Fisher Scientific, Waltham, MA, USA), and reverse-transcribed to corresponding cDNAs. Next, the concentration and purity of cDNAs were measured via Nanodrop nucleic acid quantitation, and the targeted gene expression was quantitatively detected in accordance with Power Up SYBR Green Kit instruction (Invitrogen). PCR reaction system (reaction mixture 20 µL) was set as follows: up to 40 cycles consisting of pre-denaturation at 95 °C for 5 min; amplification at 95 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 1 min. At the same time, a negative control was used to exclude PCR contamination and primer-dimer interference in the reaction system.

4.6. Western blot analysis

Total proteins were routinely acquired by using RIPA cell lysis buffer, and were quantified with Bradford protein assay kit (Beyotime, Shanghai, China). Proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to polyvinylidene fluoride (PVDF) membrane for 2 h blocking with 5% skim milk powder. Primary antibody was used to incubate the PVDF membrane overnight at 4 °C. The membrane was then washed with TBS buffer three times, and subsequently being incubated with horseradish peroxidase-labeled second antibody

for 1 h at room temperature. The membrane was washed with TBS buffer 3 times again. ECL kit was used to visualize blots and image was processed by gel imaging analysis system. The members were analyzed using the Image Lab for quantification.

4.7. Apoptosis assay

Cell apoptosis was assessed using an Annexin V-FITC/PI Apoptosis Kit (Solarbio Life Sciences, cat. no. CA1020). A549 cells were centrifuged after harvested. After washing, the cells were suspended in 1× Binding buffer, and 100 µL of cell suspension was mixed with 5 µL of FITC and 5 µL of PI. After incubation for 15 min in the dark, cell apoptosis was measured with a FACSCalibur flow cytometer (BD, FACSCalibur).

4.8. ELISA assay

The levels of IL-1β, IL-6 and TNF-α were detected using ELISA kits (Affymetrix, Santa Clara, CA, USA) according to instructions provided by the manufacturers.

4.9. Histological examination

The lung tissues were fixed in formaldehyde solution for 24 h at room temperature, embedded in paraffin, sliced into sections 4 µm thick, and stained with hematoxylin–eosin (HE).

4.10. Immunohistochemistry

The diagnosis and staining area for IHC was confirmed by a senior pathologist. The method of IHC was used according to previous studies. After deparaffinization of the specimen with xylene, the samples were rehydrated with graded ethanol. To block the endogenous peroxidase activity, 3% hydrogen peroxide was used to incubate slides, while EDTA buffer (pH = 9.0) was used for antigen retrieval. Phosphate buffered saline (PBS) with 5% bovine serum albumin was used to incubate the specimen for unspecific antigen binding, and primary of NLRC5 (1:200) was added to the slides at 4 °C overnight. After incubation in the biotin-labeled secondary antibody (Sangon, Shanghai, China), streptavidin-peroxidase and 3,3'-diaminobenzidine substrate were used for antigen visualization.

4.11. Measurements of the levels of SOD, GSH-Px and MDA

The levels of SOD, GSH-Px and MDA were analyzed using kits (Thermo Fisher Scientific) according to manufacturer's instructions.

4.12. Statistical analysis

Data were exhibited in the form of mean±standard deviation (SD). All data were analyzed with software SPSS 22.0 (IBM cooperation, USA). Significant differences between means were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparisons. P less than 0.05 was considered as statistically significant.

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Conflicts of interest: The authors declare that they have no competing interests

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