Original paper

miR-21 regulates LPS-induced apoptosis and inflammatory injury in rat cardiomyocytes by targeting PLD1 and STAT3

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This study aims to elucidate the role and molecular mechanism of microRNA-21 (miR-21) in LPS-induced inflammatory injury in H9c2 cardiomyocytes.
H9c2 cardiomyocytes were treated with lipopolysaccharide (LPS) to establish an *in vitro* model. The expression of miR-21 was quantified using RT-qPCR, while protein levels were assessed via Western blot analysis. The impact of miR-21 on inflammatory response, cell proliferation, and apoptosis in LPS-treated H9c2 cells was evaluated using ELISA, CCK-8/EdU assays, and flow cytometry. TargetScan predictions and dual-luciferase reporter assays were employed to identify potential miR-21 targets. The regulatory effects of miR-21 on inflammation, proliferation, and apoptosis in cells were further examined following transfection with phospholipase D1 (PLD1) overexpression constructs or signal transducer and activator of transcription 3 (STAT3) activation. The expression levels of miR-21, PLD1, and p-STAT3 were significantly elevated in LPS-treated H9c2 cells. Knockdown of miR-21 markedly inhibited the LPS-induced inflammatory response, enhanced cell proliferation, and reduced apoptosis in H9c2 cells. PLD1 and STAT3 were confirmed as direct targets of miR-21. Overexpression of PLD1 or activation of STAT3 significantly reversed the protective effects of miR-21 downregulation in LPS-treated H9c2 cells. Downregulation of miR-21 protects cardiomyocytes against LPS-induced inflammatory injury and apoptosis by inhibiting PLD1 expression and STAT3 phosphorylation.

Key words: HmiR-21, PLD1, STAT3, sepsis, myocardial injury.

Introduction

Sepsis is an infection-induced condition characterised by a systemic inflammatory response syndrome (SIRS), which is characterised by host immune damage mediated by inflammatory mediators and cytokines [1]. Septic cardiomyopathy (SCM) is myocardial dysfunction induced by sepsis [2]. Lipopolysaccharide (LPS) plays a crucial role in the pathogenesis of sepsis, stimulating the release of large amounts of pro-inflammatory cytokines and chemokines from host cells, inducing an inflammatory response and leading to multi-organ failure [3]. Currently, there are no specific and effective drugs available for LPS-induced sepsis. Therefore, identifying new therapeutic targets and developing more effective treatments are of critical importance [4]. MicroRNAs (miRNAs) can regulate the expression of target genes at the transcriptional level by being partially complementary to the target mRNA molecule [5]. Recent studies have increasingly shown that miRNAs are promising molecular targets for the treatment of sepsis [6]. Signal transducer and

activator of transcription 3 (STAT3) can systemic inflammation by regulating the expression of various inflammatory mediators. Targeting and inhibiting STAT3 activation can suppress LPS-induced cellular inflammation and oxidative stress [7]. Phospholipase D1 (PLD1) is an enzyme involved in multiple cellular processes, including inflammation. Recent studies suggest that PLD1 may be a novel target for regulating sepsis [8].

In this study, we used LPS to establish an in vitro model of septic cardiomyopathy, and we verified that STAT3 and PLD1 are direct targets of miR-21. Our findings suggest that miR-21 may serve as an effective target for treating LPS-induced sepsis, providing a new strategy for miR-21-targeted therapy in sepsis treatment.

Material and methods

Reagents

Colivelin was purchased from MCE (MedChemExpress, New Jersey, USA). Protein marker, SDS-PAGE protein loading buffer, and BeyoECL Plus were purchased from Beyotime Biotechnology (Shanghai, China). ELISA kits were purchased from Beijing Solebao Technology Co., Ltd. PBS buffer, CCK-8 kit, penicillin-streptomycin solution, universal cell tissue fixative, trypsin 0.25%, annexin V-FITC/PI Double Staining Cell Apoptosis Detection Kit, and RIPA lysis buffer were purchased from Seven biotech (Beijing, China). Rabbit monoclonal antibodies GAPDH, STAT3, Phospho-STAT3, Bax, and Bcl-2 were purchased from ZENBIO (Chengdu, China). PLD1 was purchased from Cell Signaling Technology.

Cell culture and transfection

Rat myocardial H9c2 cells, obtained from Suzhou Haixing Biotechnology Co., Ltd., were cultured in DMEM/F12 medium supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin in a 37°C incubator with 5% CO_2.

For cell transfection, miR-21 inhibitor and plasmids were purchased from Seven biotech (Beijing, China). Transfection was started when the H9c2 cells grew to 70–80%. Lipofectamine™ 2000 was used according to the instructions for experimental operation. After transfecting the cells for 6 h, the transfection was terminated by changing the complete medium, and the culture was continued for 48 h.

CCK-8 assay

Cells were seeded in 96-well plates at a density of 10^4 cells per well. Cells were then treated with various concentrations of LPS (0, 0.1, 0.5, 1, 5, and 10 μg/ml) for 24 h, with 6 replicates per concentration. After treatment, CCK-8 solution was added to each well, and the plate was gently shaken to ensure even distribution of the reagent. The 96-well plate was then incubated at 37°C in a 5% CO_2 incubator for 1–2 h. Absorbance (OD value) was measured at 450 nm using a microplate reader.

For the cell proliferation assay, cells were seeded in 96-well plates at a density of 10^4 cells per well. Following this, the cells were treated with 5 μg/ml LPS for 24 h. Proliferation activity was assessed at 0 hours and 24 h, with subsequent steps performed as described above.

Real-time fluorescence quantitative PCR

Total RNA was extracted using an RNA extraction kit, and cDNA was synthesised from the extracted RNA using a reverse transcription kit. RT-qPCR was then performed to measure the expression levels of miR-21 or the target mRNA. The PCR reaction mixture was prepared and added to an 8-tube strip, with 20 μl of sample per well. PCR amplification was then performed. Using GAPDH as an internal reference for target genes. The specific primer sequences are listed in Suppl. Table 1.

Western blot

Cell samples were lysed to extract proteins. Protein concentration was determined using the BCA method and adjusted to be consistent across all samples. Protein samples are then mixed with SDS-PAGE Sample Buffer and heated at 95°C for 3 minutes. Electrophoresis was performed in polyacrylamide gels to separate proteins based on their molecular weight. The proteins were transferred from the gel to a PVDF membrane using the electroblotting method. Membranes were incubated at room temperature with 5% skimmed milk, target protein-specific primary antibody, and HRP-conjugated secondary antibody. Protein signals were detected using chemiluminescence (ECL) and visualised on a chemiluminescence imaging system.

Enzyme-linked immunosorbent assay

Inflammatory factor levels in H9c2 cell culture supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits. 100 μl of coated antibody was added to each well of a 96-well plate and incubated overnight at 4°C to ensure that the antibody is fully adsorbed to the plate surface. The plate was then washed 3 times with PBS buffer, shaking thoroughly after each wash. Next, 200 μl of blocking solution was added to each well, and the plate was incubated at 37°C for one hour. Afterward, the plate was washed 3 times with PBS buffer. Cell supernatant was then added to the wells and incubated at room tempera-

ture for 2 h. The plate was washed again 3 times with PBS buffer. Subsequently, 100 μl of HRP-conjugated secondary antibody was added to each well, and incubation continued for one hour. After washing 3 times with PBS buffer, 100 μl of substrate solution was added to each well and incubated at room temperature for 10–30 minutes, or until a colour change was observed. Absorbance (OD value) at 450 nm was measured using a microplate reader. A standard curve was generated based on the OD values of known concentration standards (X-axis representing standard concentration and Y-axis representing absorbance). The concentration of the target molecule in the sample was calculated using the OD value and the standard curve.

5-Ethynyl-2'-deoxyuridine

After culturing cells to an appropriate density, they were treated with LPS for 24 h. 5-Ethynyl-2'-deoxyuridine (EdU) solution was then added to the cell culture medium, and cells were incubated for an additional 2–4 h to allow EdU incorporation into newly synthesised DNA during DNA replication. After incubation, cells were washed with PBS to remove the culture medium. Cells were fixed at room temperature for 15–20 minutes using 4% paraformaldehyde. After fixation, cells were washed with PBS to remove the fixative. To enhance the binding of EdU to the dye, cells were permeabilised with 0.1% Triton X-100 at room temperature for 15 minutes. The click reaction solution was prepared according to the instructions provided in the Click-iT assay kit. 50 μl of the click reaction solution was added to the cell samples and incubated at room temperature for 30 minutes to 1 h, allowing EdU to react with the fluorescently labelled probe. To stain the cell nuclei, DAPI was added and incubated for 5 minutes. Cells were observed under a fluorescence microscope. Fluorescent images were quantitatively analysed using ImageJ, and the proportion of EdU-positive cells was determined.

Flow cytometry

After culturing the cells to the appropriate density, they were treated with LPS for 24 hours. Cells were then harvested, gently pipetted to ensure a single-cell suspension, and washed twice with PBS. Cell counts were performed using a haemocytometer to ensure each sample contained at least 1×10^6 cells. Following centrifugation to remove the supernatant, the cell pellet was resuspended in sample buffer. Subsequently, 5 μl of annexin V-FITC and 10 μl of PI solution were added, and the cells were incubated at room temperature for 10–15 minutes, protected from light. After staining, cells were analysed by flow cytometry. Fluorescence signals were analysed using FlowJo software, and apoptotic cell distribution was determined based on the Annexin V and PI dual staining pattern.

Target gene prediction and dual-luciferase reporter assay

The TargetScan database (https://www.targetscan.org/) was utilised to predict potential target genes of miR-21. In the GeneCards (https://www.genecards.org/) and Disgenet (https://www.disgenet.org/) databases, the keyword "sepsis" was entered to retrieve sepsis-related targets. The predicted miR-21 target genes were then intersected with the identified disease targets, and a Venn diagram was generated. From this intersection, PLD1 and STAT3, both associated with sepsis, were selected for further investigation. Wild-type STAT3 (STAT3-WT) and mutant STAT3 (STAT3-Mut) sequences were inserted into the pmirGLO reporter vector (Promega, USA). Similarly, wild-type PLD1 (PLD1-WT) and mutant PLD1 (PLD1-Mut) sequences were inserted into the pmirGLO reporter vector. H9c2 cells were co-transfected with miR-21 mimic or negative control (NC) using Lipofectamine 2000. Forty-eight hours after transfection, luciferase activity was measured using the dual-luciferase reporter assay system (Promega).

Statistical analysis

GraphPad Prism 9 was used to perform t-test or one-way analysis of variance for each group of data, and all data are presented as the mean ±standard deviation (SD) of 3 independent experiments. *P*-value < 0.05 was considered statistically significant.

Bioethical Committee

Due to the nature of the study, the consent of the bioethics committee was not required.

Results

miR-21, PLD1, and STAT3 are highly expressed in LPS-treated H9c2 cells

The proliferative activity of H9c2 cells was evaluated following 24-hour LPS exposure. Results revealed an IC50 of 4.902 μg/ml, prompting selection of 5 μg/ml LPS for subsequent experiments (Fig. 1A). Also, 24-h LPS (5 μg/ml) treatment upregulated miR-21 (Fig. 1B) and elevated PLD1/p-STAT3 protein levels (Fig. 1C).

Downregulation of miR-21 inhibits inflammatory injury in septic H9c2 cells

To further investigate the impact of miR-21 on LPS-treated H9c2 cells, we transfected H9c2 cells with a miR-21 inhibitor. The results of RT-qPCR indicated a significant increase in miR-21 expression following LPS treatment, whereas transfection with

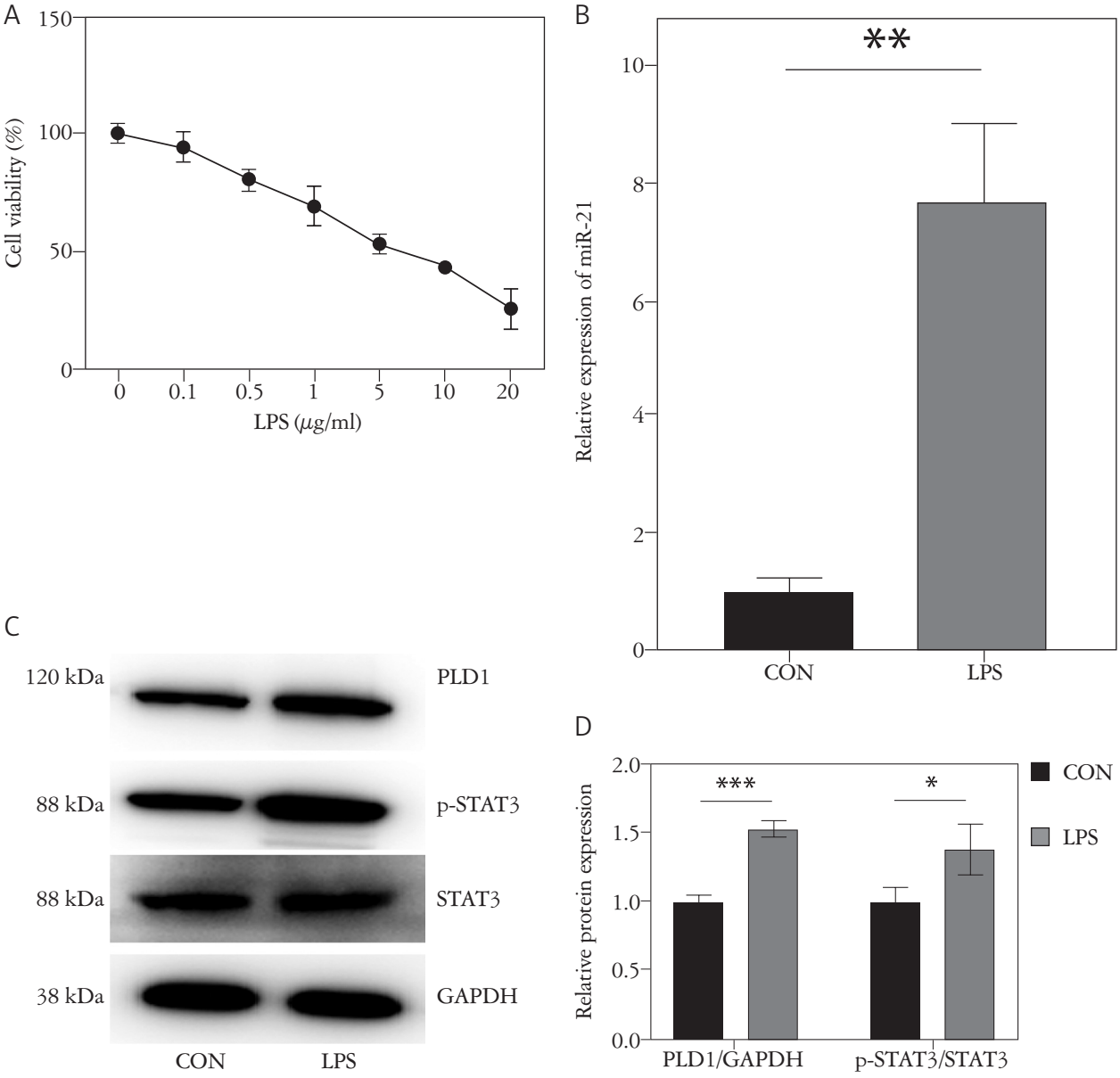

Fig. 1. miR-21, PLD1, and STAT3 are highly expressed in LPS-treated H9c2 cells: **A)** CCK-8 assay to detect the proliferation activity of cells treated with LPS at a series of concentrations; **B)** RT-qPCR assay to detect the expression of miR-21 at the transcriptional level; **C)** Western blot assay to detect the expression of phospholipase D1 (PLD1), p-STAT3, and signal transducer and activator of transcription 3 (STAT3) proteins. The data are presented as the mean ±SD of 3 independent experiments. Compared with the CON group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

the miR-21 inhibitor led to a reduction in miR-21 levels (Fig. 2A). ELISA showed that compared to the control group (CON), LPS treatment resulted in a marked increase in the levels of inflammatory factor. However, in the LPS + miR-21 inhibitor group, these cytokine levels were significantly lower compared to the LPS + inhibitor NC group (Fig. 2B–E).

CCK-8 and EdU assays revealed that the proliferation capacity of H9c2 cells was significantly lower in the LPS group, while reducing miR-21 expression restored cell proliferation (Fig. 2F, G). Flow cytometry analysis of apoptosis revealed that the apoptosis rate in the LPS-treated group was significantly higher than that in the control group. Inhibition of miR-21 expression was found to reduce LPS-induced apoptosis (Fig. 2H). Western blot analysis supported these findings, showing that LPS treatment increased Bax expression and decreased Bcl-2 expression. Transfection with the miR-21 inhibitor reversed this effect, leading to a reduction in Bax expression and an increase in Bcl-2 expression (Fig. 2I). These results suggest that downregulation of miR-21 can mitigate LPS-induced inflammatory injury in H9c2 cells.

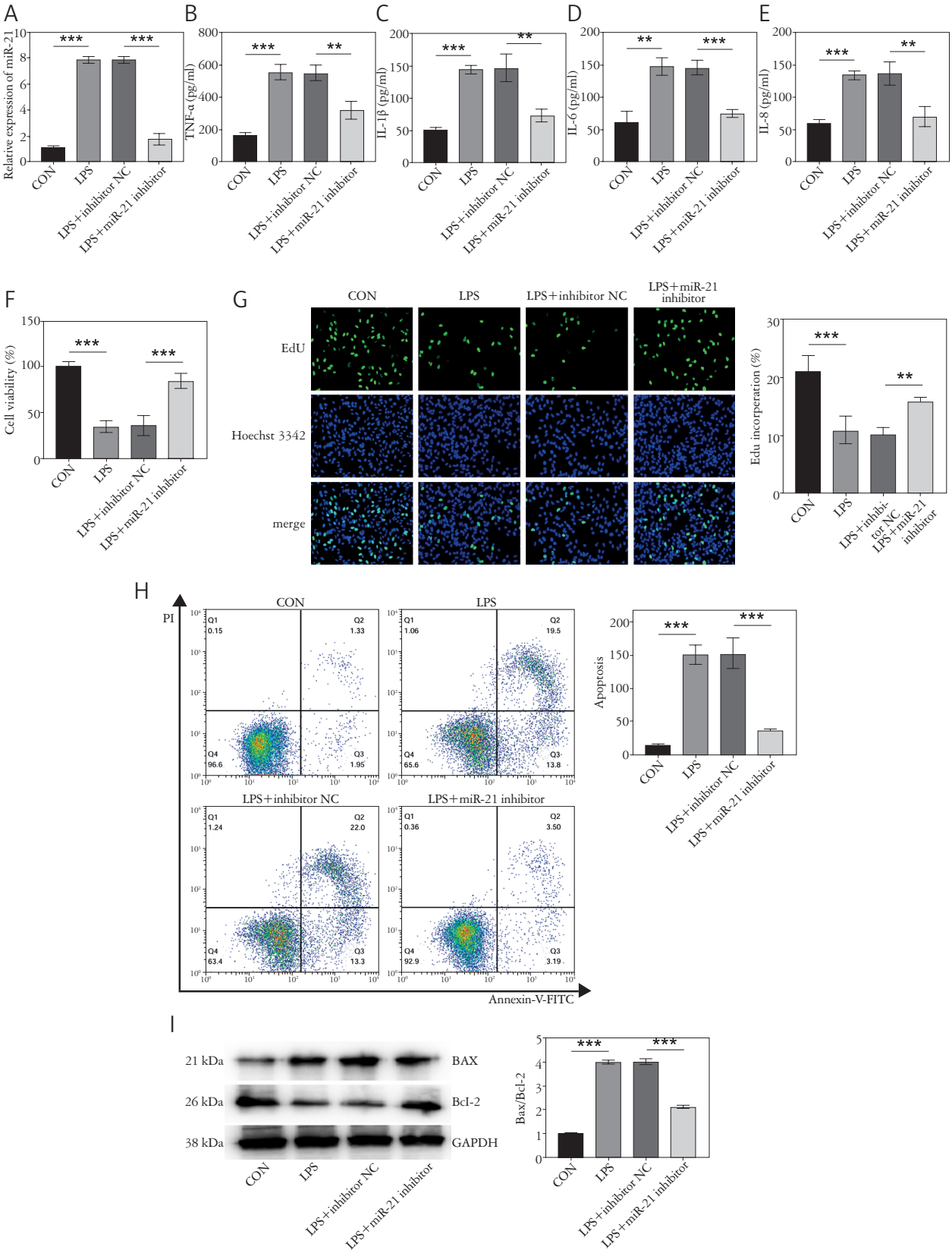

Fig. 2. Downregulation of miR-21 inhibits inflammatory injury in septic H9c2 cells: **A)** RT-qPCR was used to detect the expression of miR-21; **B–E)** ELISA was used to detect the levels of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-8 (IL-8) in each group of cells; **F)** CCK-8 was used to detect the proliferation activity of each group of cells; **G)** EdU was used to detect the proliferation ability of each group of cells; **H)** flow cytometry was used to detect the apoptosis rate of each group of cells; **I)** Western blot was used to detect the expression of BAX and Bcl-2 proteins in each group of cells. The data are presented as the mean ±SD of 3 independent experiments. ** $p < 0.01$, *** $p < 0.001$

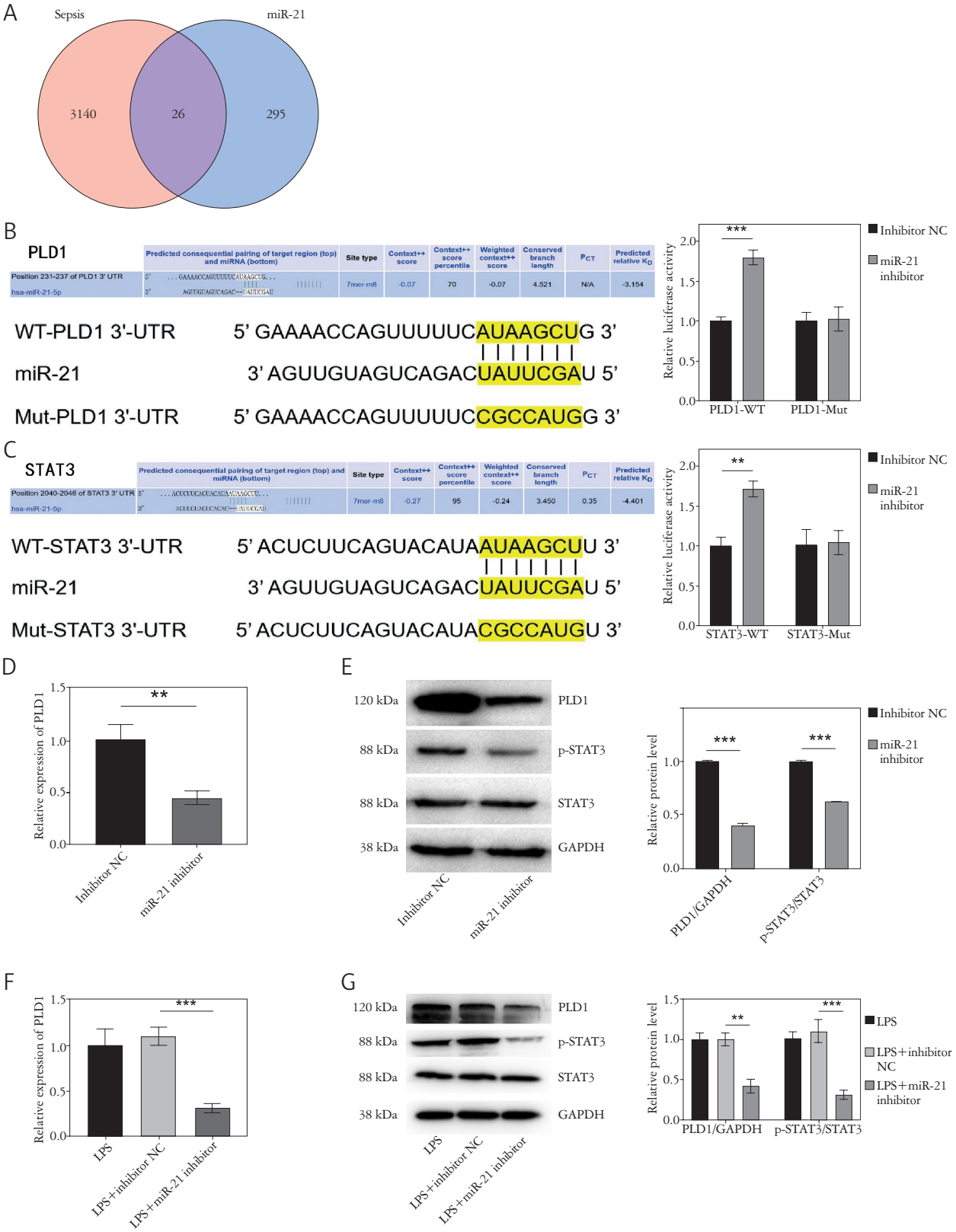

Fig. 3. miR-21 targets PLD1 and STAT3: **A)** Venn diagram of miR-21 potential targets and disease targets; **B)** TargetScan and dual luciferase reporter gene predicted and verified the binding sites of miR-21 and PLD1; **C)** TargetScan and dual luciferase reporter gene predicted and verified the binding sites of miR-21 and STAT3; **D)** RT-qPCR experiment detected the expression of PLD1 mRNA in each group of cells; **E)** Western blot experiment detected the expression of PLD1, p-STAT3, and STAT3 protein in each group of cells; **F)** RT-qPCR experiment detected the expression of PLD1 mRNA in each group of cells; **G)** Western blot experiment detected the expression of PLD1, p-STAT3, and STAT3 protein in each group of cells. The data are presented as the mean ±SD of 3 independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$

miR-21 targets PLD1 and STAT3

A total of 3140 sepsis-related targets were identified from the GeneCards and Disgenet databases, and 295 potential target genes of miR-21 were predicted using the TargetScan database. The intersection of these miR-21 targets with the sepsis-related targets resulted in 26 overlapping genes, including STAT3, PLD1, BCL-2, PDCD4, and CCL20. Among these, the genes most likely to be targeted by miR-21 and previously unreported in the literature were prioritised based on their scores. Consequently, PLD1 and STAT3 were selected for further investigation (Fig. 3A).

Next, this study further identified the downstream target genes of miR-21. TargetScan predicted the binding sites between miR-21 and PLD1, as well as STAT3. Dual-luciferase reporter assays were employed to validate the interactions between miR-21 and these targets. As expected, the miR-21 inhibitor significantly enhanced the luciferase activity of PLD1-3'-UTR-WT and STAT3-3'-UTR-WT, but it did not affect the luciferase activity of PLD1-3'-UTR-MUT and STAT3-3'-UTR-MUT (Fig. 3B–C).

RT-qPCR results indicated that, compared to the NC group, transfection with the miR-21 inhibitor significantly reduced the mRNA expression level of PLD1 in H9c2 cells (Fig. 3D). Western blot analysis further revealed that PLD1 and p-STAT3 protein levels were decreased following miR-21 inhibitor transfection (Fig. 3E).

Finally, RT-qPCR and Western blot results showed that the miR-21 inhibitor treatment significantly decreased both the mRNA and protein levels of PLD1, as well as the protein level of p-STAT3, in LPS-treated H9c2 cells (Fig. 3F, G).

Downregulation of miR-21 inhibits inflammatory injury in septic H9c2 cells by regulating PLD1 and STAT3

To further validate whether miR-21 modulates LPS-induced apoptosis and inflammation in H9c2 cells by regulating PLD1 and STAT3, rescue experiments were conducted. For LPS-treated H9c2 cells, PLD1 overexpression plasmid was transfected or STAT3 activator (Colivelin) was used to detect protein expression by Western blot. The results showed that PLD1 and p-STAT3 protein levels were significantly increased compared with the control group (Fig. 4A, B). ELISA analysis further showed that in LPS-treated H9c2 cells, transfection with the miR-21 inhibitor led to a marked decrease in inflammatory factor levels. However, PLD1 overexpression or STAT3 activation reversed this effect (Fig. 4C, D). CCK-8 assay results indicated that PLD1 overexpression or STAT3 activation significantly inhibited the proliferation-promoting effect of the miR-21 inhibitor on LPS-treated H9c2 cells (Fig. 4E, F). Flow cytometry results demonstrated that the reduction of miR-21 expression decreased LPS-induced cell apoptosis, while PLD1 overexpression or STAT3 activation significantly increased apoptosis (Fig. 4G, H). Western blot analysis supported these findings, showing that miR-21 inhibition reduced Bax expression and increased Bcl-2 expression. Overexpression of PLD1 or activation of STAT3 reversed these effects (Fig. 4I). These results indicate that downregulation of miR-21 improves LPS-induced inflammatory injury in septic H9c2 cells by regulating PLD1 and STAT3.

Discussion

Sepsis frequently leads to multiple organ dysfunction, with myocardial injury being a prevalent and severe complication. This condition significantly contributes to high mortality rates and poor prognosis in septic patients [9]. Key mechanisms underlying myocardial injury in sepsis include the excessive release of inflammatory cytokines, oxidative stress, metabolic dysregulation, and microcirculatory disturbances. Lipopolysaccharide (LPS), a key component of the outer membrane in Gram-negative bacteria, is a powerful immunostimulant that triggers strong inflammatory responses, playing a critical role as a pathogenic factor in sepsis [10].

Despite various clinical interventions for septic cardiomyopathy, therapeutic outcomes remain suboptimal, underscoring the urgent need for more precise therapeutic targets. Recently, microRNAs (miRNAs) have emerged as critical regulators of immunity, inflammation, and apoptosis, with significant implications for the pathogenesis, diagnosis, and treatment of septic cardiomyopathy [11]. Multiple studies have identified various miRNAs, such as miR-193b-5p, miR-107, miR-155, miR-452, and miR-106a-5p, as closely linked to the onset and progression of sepsis [12–15]. Among these, miR-21 has been extensively studied for its role in various physiological and pathological processes, particularly in cancer, inflammation, and cardiovascular diseases. It is highly expressed in multiple cancers, promoting malignancy in osteosarcoma, colorectal adenocarcinoma, and gastrointestinal cancers by inhibiting tumour suppressors such as PDCD4 and PTEN [16–18]. Additionally, miR-21 influences inflammation by regulating genes and signalling pathways related to inflammation. For instance, miR-21 knockout reduces lung function decline and inflammatory responses in acute lung injury [19]. miR-21 modulates the NF-κB signalling pathway by inhibiting A20 and activating the NLRP3 inflammasome, thereby promoting LPS-induced septic shock [20]. Moreover, miR-21 is implicated in several myocardial diseases, including heart failure, dilated cardio-

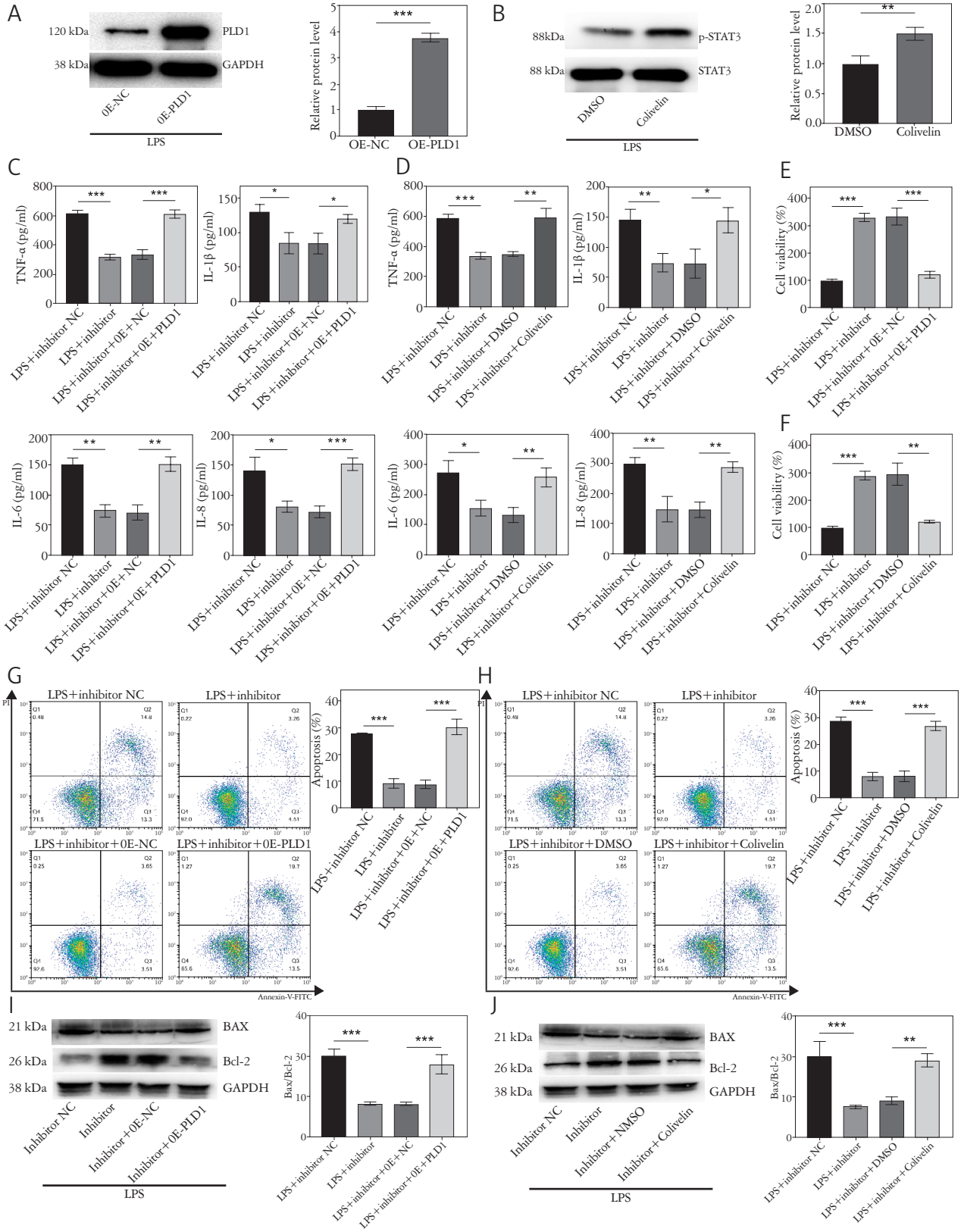

Fig. 4. Downregulation of miR-21 inhibits inflammatory injury in septic H9c2 cells by regulating PLD1 and STAT3. **A, B)** Western blot assay to detect protein expression of PLD1 **(A)** and p-STAT3 **(B)**; **C, D)** ELISA assay to detect TNF-α, IL-1β, IL-6, and IL-8 levels in cells after overexpression of PLD1 **(C)** or activation of STAT3 **(D)**; **E, F)** CCK-8 assay to detect cell proliferation activity after overexpression of PLD1 **(E)** or activation of STAT3 **(F)**; **G, H)** Flow cytometry to detect cell apoptosis rate after overexpression of PLD1 **(G)** or activation of STAT3 **(H)**; **I, J)** Western blot assay to detect BAX and Bcl-2 protein expression in cells after overexpression of PLD1 (I) or activation of STAT3 **(J)**. The data are presented as the mean ±SD of 3 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

myopathy, and myocardial infarction [21]. The precise mechanisms of miR-21 in LPS-induced septic myocardial injury are still not fully understood. In this study, we developed an in vitro model of septic myocardial injury by exposing H9c2 cells to LPS. RT-qPCR analysis demonstrated a marked upregulation of miR-21 expression in LPS-treated H9c2 cells. LPS exposure induced inflammation and apoptosis in H9c2 cells while suppressing cell proliferation. Downregulation of miR-21 reversed these effects, inhibiting LPS-induced inflammation and apoptosis while promoting cell proliferation. These findings indicate that miR-21 is pivotal in LPS-induced sepsis.

MiRNAs can bind to the mRNAs of target genes, regulating protein expression and modulating various cellular physiological processes, such as inflammatory responses. In this study, we used the TargetScan tool to predict that PLD1 and STAT3 are downstream targets of miR-21, and we validated these interactions using dual-luciferase reporter assays. Additionally, RT-qPCR and Western blot results showed that downregulation of miR-21 inhibited PLD1 expression at both the transcriptional and protein levels and reduced STAT3 protein phosphorylation, regardless of LPS treatment. We also found that both PLD1 and p-STAT3 were upregulated in LPS-treated cardiomyocytes.

PLD1 is critically involved in various inflammatory diseases, and its knockdown can attenuate inflammatory responses by regulating the activity of the NF-κB and Wnt/β-catenin pathways [22]. Studies suggest that selective PLD1 inhibitors notably enhance survival rates in a mouse model of sepsis induced by caecal ligation and puncture (CLP) by alleviating lung inflammation, reducing leukocyte apoptosis, and decreasing the production of pro-inflammatory factors. The STAT protein family, consisting of 7 members, is crucial in cell signal transduction and gene expression regulation, with STAT3 playing a pivotal role in inflammatory responses and immune regulation. High expression of STAT3 can promote LPS-induced inflammation and apoptosis. Our rescue experiments further confirmed that overexpression of PLD1 or activation of STAT3 reversed the inhibitory effects of miR-21 downregulation on LPS-induced inflammation and apoptosis. These results indicate that knocking down miR-21 reduces LPS-induced inflammatory damage in H9c2 cells by downregulating PLD1 and inhibiting STAT3 phosphorylation.

To date, there is no effective treatment for septic cardiomyopathy. Targeting miR-21 offers a promising new approach for therapy. The future development of miR-21-targeted drugs and their clinical translation could have significant implications for treating septic cardiomyopathy. However, this study has some limitations, particularly the lack of *in vivo* animal model experiments. Moving forward, we plan to validate the targeting effects of miR-21 on PLD1 and STAT3 in animal models and to further elucidate the roles of miR-21, PLD1, and STAT3 in the pathogenesis of sepsis.

Conclusions

Our study demonstrates that miR-21 is significantly upregulated in LPS-induced H9c2 cells. Knockdown of miR-21 targets and inhibits the phosphorylation of PLD1 and STAT3, thereby suppressing LPS-induced inflammatory responses and apoptosis in H9c2 cells while promoting cell proliferation. These results indicate that miR-21 could be a promising therapeutic target for the treatment of LPS-induced septic cardiomyopathy.

Disclosures

1. Institutional review board statement: Not applicable.
2. Assistance with the article: None.
3. Financial support and sponsorship: None.
4. Conflicts of interest: None.

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