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Original Article

Simvastatin inhibits inflammatory response in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages through the microRNA-22/Cyr61 axis

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Abstract: Simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, has been shown to improve atherosclerosis (AS) via its anti-inflammatory activity. Recently, several studies have reported the involvement of macrophages in chronic inflammation associated with AS. However, it is unknown whether macrophages participate in the anti-inflammatory activity of simvastatin in AS. This study was designed to investigate the roles and underlying mechanisms of simvastatin in LPS-stimulated RAW264.7 macrophages. First, we examined the anti-inflammatory effects of simvastatin on LPS-treated macrophage RAW264.7 cells using an enzyme-linked immunosorbent assay (ELISA) and a quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Then, a microarray assay was used to analyze the microRNA (miRNA) expression profile in RAW264.7 cells incubated with or without simvastatin in the presence of LPS. MicroRNA-22 (miR-22) with the highest change was validated independently by qRT-PCR. Luciferase reporter assays were conducted to determine the association between miR-22 and the cysteine-rich protein 61 (Cyr61). Subsequently, we investigated the molecular mechanism by which miR-22 functions in the anti-inflammation of simvastatin in LPS-stimulated macrophages. We found that simvastatin treatment could significantly inhibit inflammation by modulating the expression of mediators, such as IL-1β, TNF-α and IL-6, whose expression were increased remarkably in the activated RAW264.7 cells. miR-22 was found to be one of the most significantly upregulated miRNAs in LPS-stimulated RAW264.7 macrophages after treatment with simvastatin. Pre-treatment of simvastatin in LPS-stimulated RAW264.7 macrophages enhanced miR-22 expression in a dose dependent manner. Interestingly, Cyr61, a novel pro-inflammatory factor involved in the pathogenesis of atherosclerosis (AS), was identified as a direct target of miR-22. Overexpression of miR-22 enhanced the anti-inflammatory effects of simvastatin, whereas inhibition of miR-22 had an opposite effect. More importantly, further study demonstrated that the knockdown of Cyr61 by siRNA could attenuate the inhibitory effects of miR-22 inhibition on anti-inflammatory activities of simvastatin. The results clearly show that simvastatin inhibits the inflammation response in LPS-stimulated RAW264.7 macrophages through the miR-22/Cyr61 axis and suggests that targeting the miR-22/Cyr61 axis may be a promising molecular target for AS therapy.

Keywords: Atherosclerosis, inflammation, RAW264.7 macrophages, simvastatin, miR-22/Cyr61 axis

Introduction

Atherosclerosis (AS) is a common chronic inflammatory cardiovascular disease, which is characterized by the formation of atherosclerotic plaques [1, 2]. Although the pathogenesis of AS is still not fully clear, studies have demonstrated the key role of macrophage activation-mediated inflammation in the development and progression of atherosclerotic plaques [3, 4]. At this standpoint, it is widely accepted that statins inhibit inflammation by decreasing pro-inflammatory cytokine production in activated macrophages. However, the targets for stain-reduced inflammation in AS remain unclear.

Cysteine-rich 61 (Cyr61/CCN1) is a member of the CCN protein family and its expression has been reported to act as an important regulator of macrophage function [5, 6]. A recent study conducted by Lin et al. showed that simvastatin diminished Cyr61 production in osteoblasts and attenuated macrophage infiltration and progression of induced rat periapical lesions [5].

Accordingly, this result strongly indicates that the anti-inflammatory properties of simvastatin are associated with the downregulation of Cyr61. Moreover, based on a previous report, lovastatin can completely inhibit arecoline-induced Cyr61 synthesis, and the inhibition is dose-dependent [7]. Thus, we can propose that the reduction of Cyr61 is a class effect of statins. However, the signaling pathways leading to Cyr61 suppression by statins have not been completely elucidated.

MicroRNA (miRNAs) is endogenous ~22 nucleotides, noncoding RNA which binds to partially complementary sites primarily found in the 3' untranslated region (3'UTR) of target mRNA and inhibits gene expression via the induction of mRNA degradation and translational repression [8]. Dysregulation of miRNAs has already been involved in a great variety of processes, which are linked to cardiovascular diseases and AS [9, 10]. MiR-342-5p and miR-155 have been reported to promote AS [11, 12], while miR-146a contributes to reduce pro-inflammatory cytokine secretion and lipid accumulation in macrophages during AS development [13]. Available data suggest that statins, such as simvastatin, exert their effects by inhibiting the synthesis of Cyr61 in osteoblasts and subsequently decreasing macrophage recruitment [5]. Therefore, it is interesting to establish whether simvastatin inhibits the synthesis of Cyr61 via the regulation of miRNAs in AS.

In the present study, we tried to assess the anti-inflammatory potential of simvastatin on LPS-treated RAW264.7 macrophages and the associated mechanisms. The results showed that simvastatin significantly suppressed the inflammation response of activated macrophages, possibly through miR-22/Cyr61 axis.

Materials and methods

Reagents

Simvastatin was purchased from Merck Sharp & Dohme (I.A.) Corp. and was dissolved in dimethyl sulfoxide (DMSO) to prepare a 30 mM stock solution followed by dilution as needed.

Cells and treatments

The mouse RAW264.7 macrophages were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, antibiotics (100 units/ml penicillin A and 100 units/ml streptomycin), and 10% heat-inactivated fetal bovine serum in a humidified 5% CO_2 atmosphere at 37°C. The cells were treated with simvastatin in the presence of LPS (1 μg/ml) for 24 h for further measurements.

Measurement of cytokines

To detect the effect of simvastatin on pro-inflammatory cytokine generation from LPS-stimulated RAW264.7 cells, the cells were cultured in 24-well plates and were pretreated with simvastatin (5, 10, or 30 μM) 1 h prior to LPS (1 μg/ml) treatment for 24 h. The concentrations of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) were measured using ELISA kits (Millipore, Temecula, CA, USA) according to the manufacturer's instructions.

microRNA expression profiling

MiRNA profiling of LPS-stimulated RAW264.7 macrophages after simvastatin treatment was performed with the 7th generation of miRCURYTM LNA Array (v.18.0, Exiqon) as previously described [14]. Expressed data were normalized using the median normalization. After normalization, significant differentially expressed miRNAs were identified through Fold Change filtering. Hierarchical clustering was performed using cluster3.0 and treeview 1.14. The miRNAs were considered to be significantly differentially expressed between the two groups (AD patient versus healthy control) if the fold change (FC) was > 2.0 and the P value was < 0.05.

Quantitative RT-PCR analysis

Total RNA from RAW264.7 macrophages treated as above-mentioned was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After reverse transcription, the cDNA was amplified using SYBR-Green Premix (Takara, Otsu, Japan). U6 and GAPDH functioned as a normalization control in the expression analyses of miR-22 and pro-inflammatory cytokines, respectively. qRT-PCR was performed using the Applied Biosys-

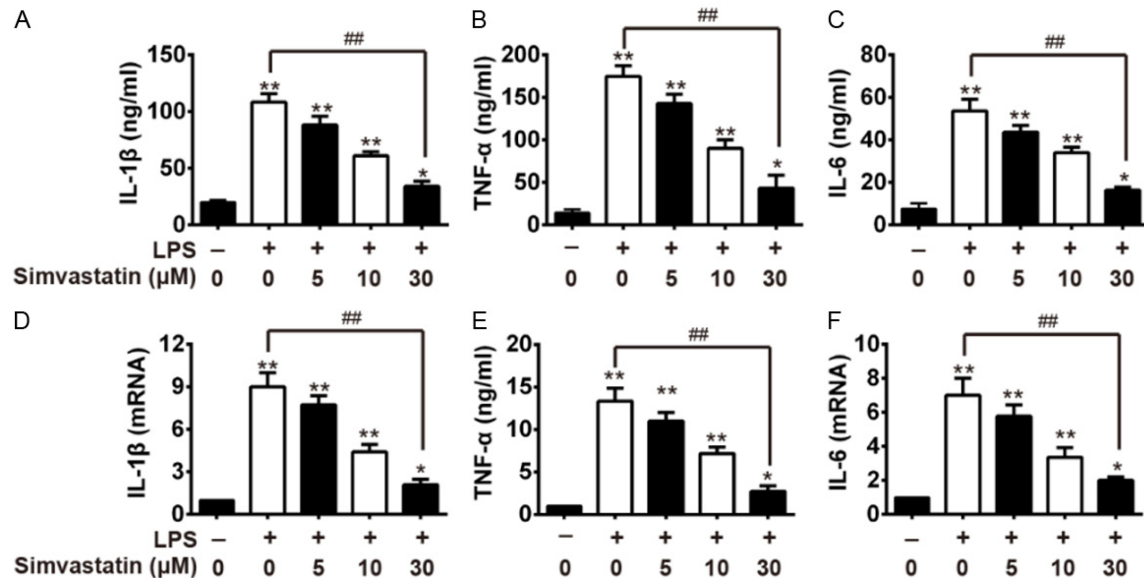

Figure 1. The effect of simvastatin on the inflammatory response in LPS-treated RAW264.7 macrophages. The cells were cultured in 24-well plates and were pretreated with simvastatin (5, 10, or 30 μM) 1 h prior to LPS (1 μg/ml) treatment for 24 h. A-C. The concentrations of IL-1β, TNF-α, and IL-6 were measured using ELISA kits. D-F. The mRNA expression of IL-1β, TNF-α, and IL-6 were measured using qRT-PCR. Data represent the mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$ vs. the control group, ##$P<0.01$ vs. L the PS-treated group.

tems 7900 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The data were analyzed by ΔCt method. The sequences of primers were purchased from Guangzhou RiboBio Co. Ltd: miR-22, forward, 5'-TGCGCAGTTCTTCAGTGGCAAG-3' and reverse, 5'-CCAGTGCAGGGTCCGAGGTATT-3'; and U6, forward, 5'-CGCTTCGGCAGCACATATAC-3' and reverse, 5'-AAATATGGAACGCTTCACGA-3'; TNF-α forward 5'-ACGGGCTTTACCTCATCTACTCR-3', reverse 5'-GGCTCTTGATGGCAGACAGG-3'; IL-1β forward 5'-FGGCAACCGTACCTGAACCCA-3', reverse 5'-CCACGATGACCGACACCACC-3'; IL-6 forward 5'-CCTTCACTCCATTCGCTGTCT-3', reverse 5'-TCCTGATTTCCCTCATACTCG-3' and GAPDH forward, 5'-GAAGATGGTGATGGGATTTC-3', and reverse, 5'-GAAGGTGAAGGTCGGAGT-3'.

Transfection

MiR-22 mimic, miR-22 inhibitor and the corresponding negative control (mimics NC and inhibitor NC) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The cyr-61 siRNA and NC siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Western blot

RAW264.7 cells after indicated treatment were harvested for Western blot analyses. The harvested cells were lysed, and their protein concentrations were determined using a BCA protein assay kit (Beyotime, Shanghai, China). Samples (30 μg of protein) were electrophoresed onto a 12% sodium dodecyl sulfate/polyacrylamide gel (SDS/PAGE), and transferred to PVDF membranes (Millipore, Mississauga, ON, Canada). The membranes were blocked in 5% nonfat milk for 1 h at room temperature (RT). The membranes were then incubated overnight at 4°C with anti-cyr-61 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin (1:5000) (Santa Cruz Biotechnology), followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000) for 1 h at RT. The proteins were visualized by enhanced chemiluminescence (ECL). Relative intensities were determined with Quantity One 4.6.2 software (Bio-Rad, CA, USA) and β-actin was used as the internal control. Data were given as mean ± SD of the percentage ratio of the control.

Luciferase activity assay

A cDNA fragment of the cyr-61 3'-UTR mRNA containing the seed sequence of the miR-

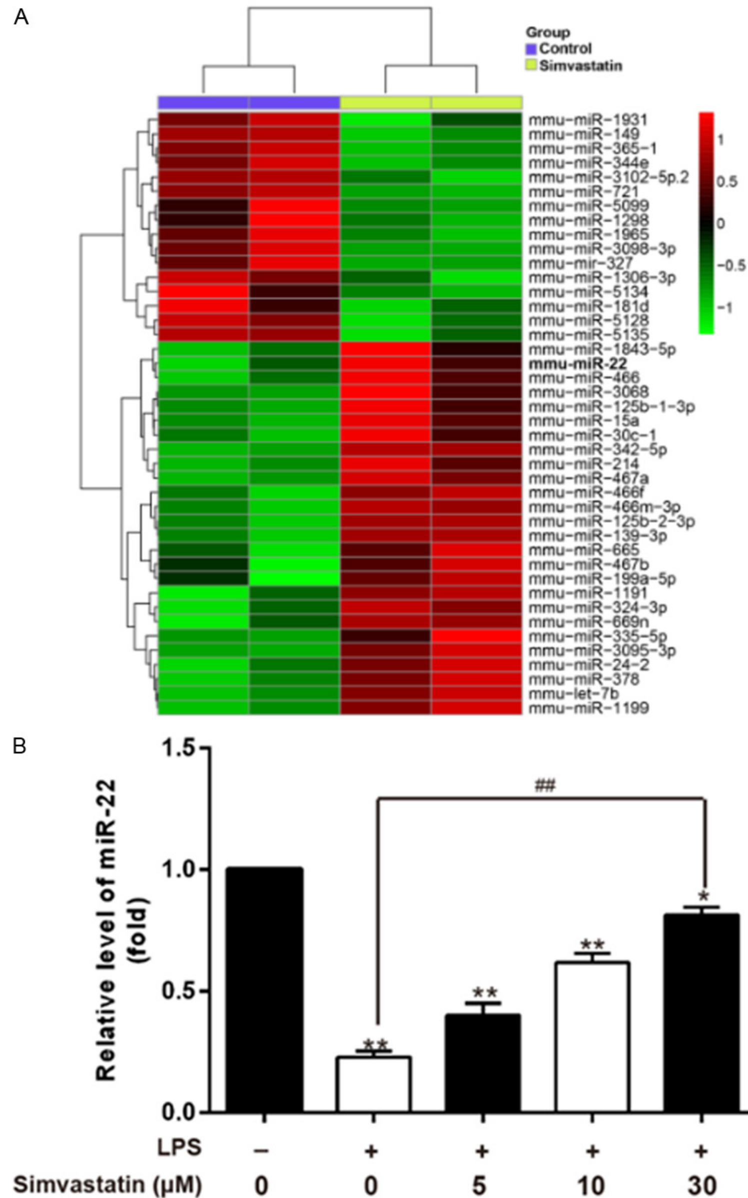

Figure 2. Differentially expressed miRNAs in LPS-treated RAW264.7 macrophages with or without simvastatin treatment. A. Comparison of observed miRNAs in LPS-treated RAW264.7 macrophages with or without simvastatin treatment. Expression levels are indicated by a color code, green = expression below, red = expression above mean levels of expression. B. RAW264.7 macrophages were pretreated with simvastatin (5, 10, or 30 μM) 1 h prior to LPS (1 μg/ml) treatment for 24 h. Then, the expression of miR-22 was evaluated by qRT-PCR. Data represent the mean ± SD of three independent experiments. **P* < 0.05, ***P* < 0.01 vs. Control group, ##*P* < 0.01 vs. LPS-treated group.

22-binding site or a mutated binding site was cloned into the pmirGLO dual-luciferase vector (Promega, Madison, WI, USA). Luciferase reporter plasmids plus miR-22 mimics, miR-22 inhibitor or miR-NC were co-transfected into HEK 293T cells using Lipofectamine 2000 (Invitrogen). The cells were harvested and lysed 24 h later, and the luciferase activity was measured by the Dual-Luciferase Assay System (Promega) in accordance to the manufacturer's instructions.

Statistical analysis

The data are expressed as the mean ± SD from the three independent experiments. The comparison of means between more than two groups was performed using one-way analysis of variance and Turkey's multiple comparison tests between groups using SPSS 13.0 software (SPSS, Chicago, IL, USA). A *p*-value of less than 0.05 was considered statistically significant.

Results

Effects of simvastatin on inflammation response in LPS-stimulated RAW264.7 macrophages

As is already known, the RAW-264.7 mouse macrophage cell line is applied to inflammation studies because of its highly reproducible response to lipopolysaccharide (LPS) [15]. In our experiment, RAW 264.7 cells were treated with 5, 10, or 30 μM of simvastatin for 1 h and then incubated with 1 μg/mL LPS for 24 h, then an ELISA assay and a qRT-PCR were performed to examine the effect of simvastatin on the levels of IL-1β, TNF-α, and IL-6. As shown in **Figure 1A-F**, the LPS challenge significantly increased the mRNA and protein levels of IL-1β, TNF-α, and IL-6 in RAW264.7 cells compared with the control group, and pretreatment with simvastatin significantly decreased the mRNA and protein levels of IL-1β, TNF-α,

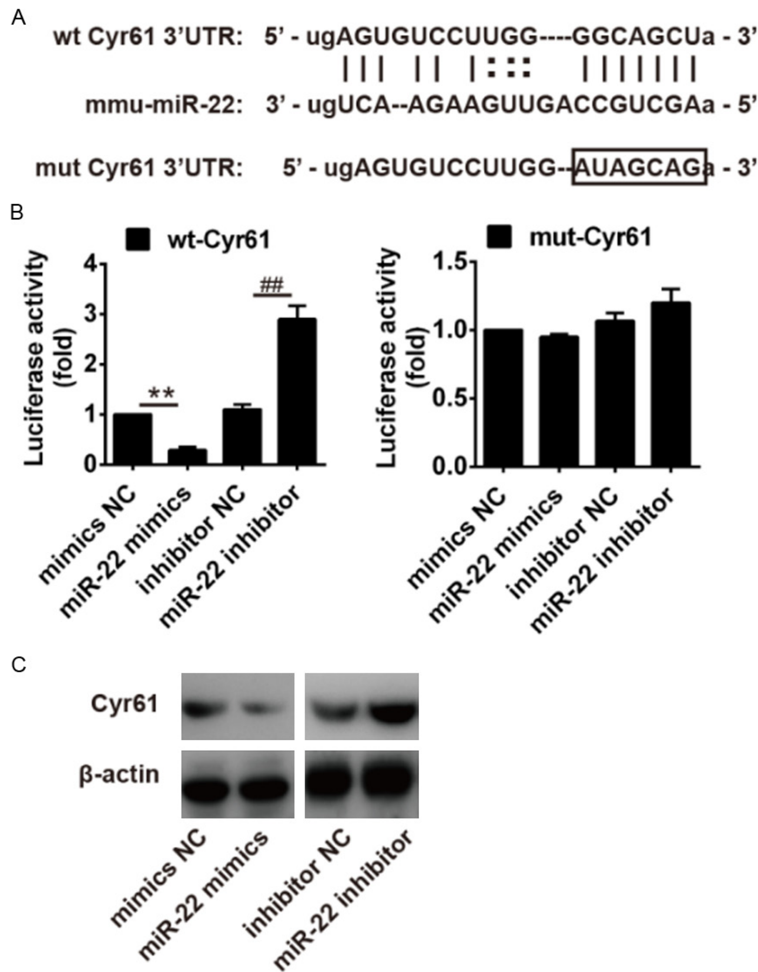

Figure 3. Cyr61 is a direct target of miR-22. A. The putative binding site of miR-22 and Cyr61 is shown. B. Luciferase assay of HEK293 cells co-transfected with firefly luciferase constructs containing the Cyr61 wild-type or mutated 3'-UTRs and miR-22 mimics, mimics NC, miR-22 inhibitor or inhibitor NC, as indicated (n = 3). Data represent the mean ± SD of three independent experiments. $^{**}P < 0.01$ vs. mimics NC, $^{\#\#}P < 0.01$ vs. inhibitor NC. C. The expressions of Cyr61 protein after treatment with miR-22 mimic or miR-22 inhibitor were measured by Western blot.

and IL-6 in a dose-dependent manner. These results suggested that simvastatin could inhibit the inflammation response induced by LPS in RAW264.7 macrophages.

miR-22 was upregulated by simvastatin in LPS-stimulated RAW264.7 macrophages

To further investigate the mechanism by which simvastatin inhibits the inflammation response, the miRNA expression profile in RAW264.7 macrophages was incubated with or without simvastatin in the presence of 10 μg/mL LPS for 12 hours using the miRNA microarray assay. As shown in **Figure 2A**, we identified 41 differentially expressed miRNAs in LPS-stimulated RAW264.7 macrophages treated with simvastatin, including 16 upregulated miRNAs and 25 downregulated miRNAs. miR-22 was identified as having one of the highest expression levels in the simvastatin group compared with the control. It has previously been reported that miR-22 is associated with an inflammation response in rheumatoid arthritis (RA) and in myocardial ischemia-reperfusion injuries [16, 17]. For this reason, miR-22 was chosen as the candidate for further study. To further validate the expression of miR-22, miR-22 levels in LPS-stimulated RAW 264.7 cells after treatment with various concentrations of simvastatin for 24 hours were determined. The results showed that miR-22 expression was dose-dependently upregulated in response to simvastatin treatment in LPS-treated RAW264.7 cells (**Figure 2B**). These data indicate that miR-22 may be involved in the anti-inflammatory effects of simvastatin against AS.

Cyr61 is a direct target of miR-22

A recent study identified Cyr61 as a target of miR-22 in the regulation of inflammation in rheumatoid arthritis (RA) [16]. Thus, we sought to determine whether miR-22 also modulates the Cyr61 in RAW264.7 macrophages. First, two publicly available databases, TargetScan and miRanda, were used to predict the potential downstream targets of miR-22. As suggested in **Figure 3A**, the complementary sequence of miR-22 was found in the 3'-UTR of Cyr61 mRNA. To test whether miR-22 could directly target 3'-UTR of Cyr61, a luciferase reporter assay was performed. The results showed that forced expression of miR-22 sig-

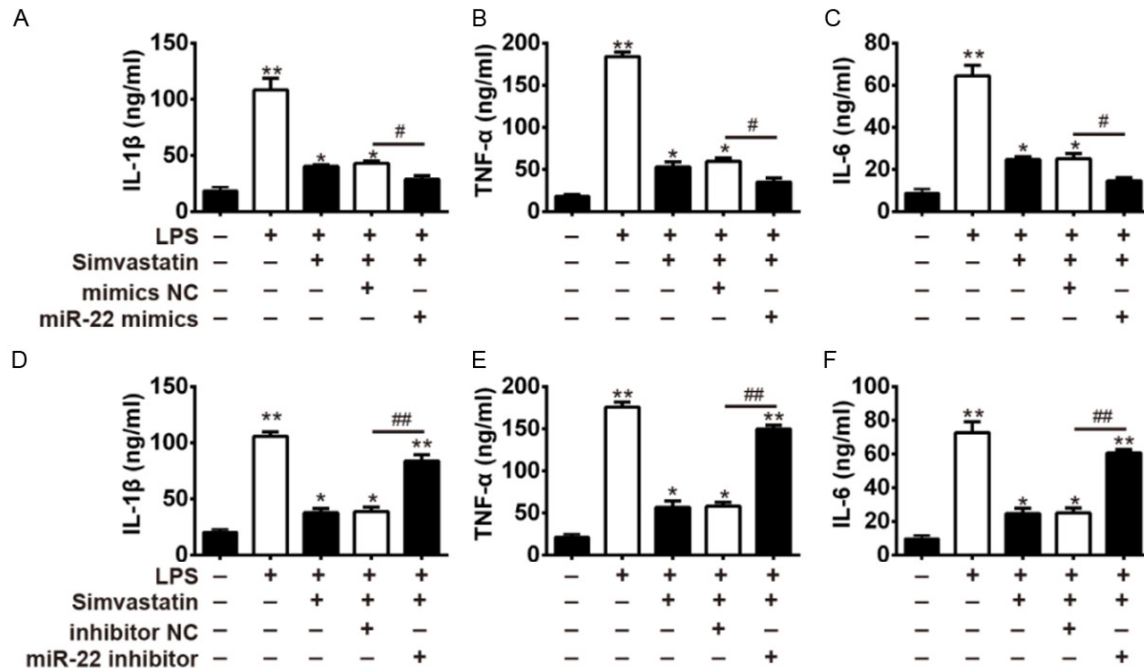

Figure 4. miR-22 is involved in the anti-inflammatory effect of simvastatin in LPS-treated RAW264.7 macrophages. miR-22 mimics or miR-22 inhibitor was transfected into LPS-stimulated RAW264.7 macrophages in the presence of simvastatin. The concentrations of IL-1β (A), TNF-α (B), and IL-6 (C) were measured after transfection with miR-22 mimics using ELISA kits, while the concentrations of IL-1β (D), TNF-α (E), and IL-6 (F) were measured after transfection with an miR-22 inhibitor using ELISA kits. Data represent the mean ± SD of three independent experiments. **P* < 0.05, ***P* < 0.01 vs. Control group, ##*P* < 0.01 vs. simvastatin + mimics NC + LPS-treated group or simvastatin + inhibitor NC+ LPS-treated group.

nificantly decreased the luciferase activity of Cyr61 with wt 3'-UTR, whereas knockdown of miR-22 increased the luciferase activity of Cyr61 with wt 3'-UTR (**Figure 3B**). Likewise, cells co-transfected with miR-22 mimics, miR-22 inhibitor, and Cyr61-mut-3'UTR showed no obvious change in their luciferase activity (**Figure 3B**). We further examined whether miR-22 could modulate the expression of Cyr61 in RAW264.7 cells. The results of a Western blot showed that the overexpression of miR-22 significantly reduced the expression of Cyr61, whereas the inhibition of miR-22 promoted the expression of Cyr61 (**Figure 3C**). Taken together, these data suggest that Cyr61 is also a direct downstream target of miR-22 in RAW 264.7 cells.

Inhibition of miR-22 abrogates simvastatin mediated anti-inflammatory effects in LPS-stimulated RAW264.7 macrophages

To determine the role of miR-22 on the anti-inflammatory effect of simvastatin, miR-22 mimics or an miR-22 inhibitor was transfected into LPS-stimulated RAW264.7 macrophages in the presence of simvastatin. As shown in **Figure 4A-C**, IL-1β, TNF-α, and IL-6, secretion in the supernatants was significantly enhanced after LPS stimulation for 24 h, and pretreatment with simvastatin prior to the LPS challenge notably attenuated the enhancement of these cytokines' secretion. Interestingly, miR-22 mimics enhanced the inhibitory effects of simvastatin on these cytokines' secretion, whereas the miR-22 inhibitor abolished the inhibitory effects of simvastatin on these cytokines' secretion (**Figure 4D-F**). These results suggest that simvastatin exerts an anti-inflammatory role in LPS-stimulated RAW264.7 macrophages by upregulating miR-22.

Simvastatin inhibits inflammation response through miR-22/Cyr61 axis

Following on the above findings, we sought to further explore whether the miR-22/Cyr61 axis was involved in the anti-inflammatory role of simvastatin in LPS-stimulated RAW264.7 macrophages. First, an miR-22 inhibitor and si-

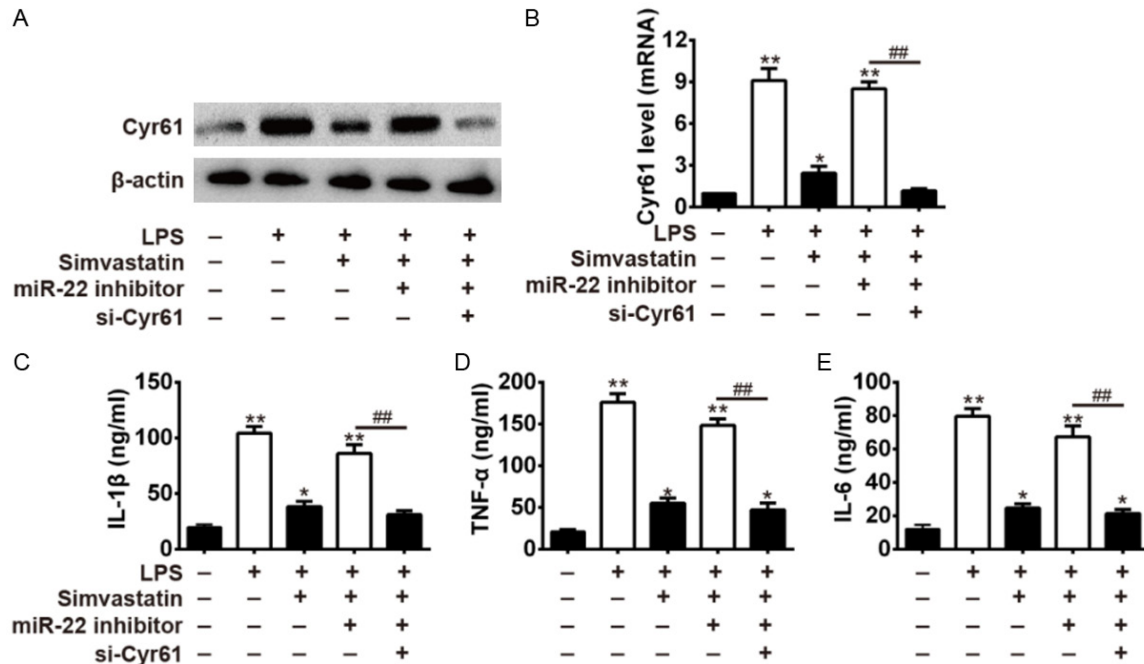

Figure 5. Simvastatin inhibits inflammation response through miR-22/Cyr61 axis. miR-22 inhibitor and si-Cyr61 were together transfected into LPS-stimulated RAW264.7 macrophages in the presence of simvastatin. A, B. The protein and mRNA expression of Cyr61 was measured by Western blot and qRT-PCR. C-E. The concentrations of IL-1β, TNF-α, and IL-6 were measured using ELISA kits. Data represent the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01 vs. Control group, ##P < 0.01 vs. miR-22 inhibitor + simvastatin + LPS-treated group.

Cyr61 were together transfected into LPS-stimulated RAW264.7 macrophages in the presence of simvastatin, and the expression of Cyr61 was measured at the protein and mRNA levels. Western blot and qRT-PCR analysis showed that the expression of Cyr61 was increased in LPS alone group compared with the control group, and that this promoting effect was removed by simvastatin treatment. Meanwhile, it was also observed that miR-22 inhibitor could reverse the reducing effects of simvastatin in LPS-stimulated RAW264.7 macrophages (**Figure 5A**, **5B**). In addition, Cyr61 was successfully knocked-down using si-Cyr61 in LPS-stimulated RAW264.7 macrophages. Moreover, we found that the miR-22 inhibitor attenuated the inhibitory effect of simvastatin on the secretion of IL-1β, TNF-α, and IL-6, but this inhibitory effect was recovered after the si-Cyr61 plus miR-22 inhibitor transfection (**Figure 5C-E**). These data indicated that simvastatin upregulates the expression of miR-22, resulting in the downregulation of Cyr61 and thus inhibits the activities of pro-inflammatory cytokine inflammation induced by LPS.

Discussion

In the present study, we found that simvastatin triggered and anti-inflammatory effect in LPS-stimulated RAW264.7 macrophages by inhibiting the expression of pro-inflammatory cytokines at the protein and mRNA levels. Our findings have also demonstrated that simvastatin exerted an anti-inflammatory effect by upregulating miR-22, thereby negatively regulating the expression of Cyr61 in LPS-stimulated RAW264.7 macrophages.

In addition to lowering lipids, statins have been reported to possess several beneficial properties, including an anti-inflammatory effect [18, 19]. Several observational studies and randomized clinical trials have documented that simvastatin exhibits potent anti-inflammatory effects mediated by reducing the production of pro-inflammatory cytokines in response to LPS [20], and the expression of cell adhesion molecules [21] and matrix metalloproteinase activity [22]. In the present study, we reinforce this anti-inflammatory activity of simvastatin by showing that the ability of simvastatin to inhibit

the expression of pro-inflammatory cytokines at the protein and mRNA levels in LPS-stimulated RAW264.7 macrophages, which was in accordance with previous reports [14]. However, the precise molecular mechanism remains to be shown.

In the present study, using a miRNA array, we successfully identified 41 miRNAs that altered in response to simvastatin treatment in LPS-stimulated RAW264.7 macrophages. Among them, we focused on the role of miR-22 because of its anti-inflammatory effects. Previous studies showed that miR-22 attenuated myocardial ischemia-reperfusion injury through anti-inflammation via the suppression of p38 MAPK, CBP and c-Jun-AP-1 in rats [17]. A recent study from Tu et al. demonstrated that the contribution of atorvastatin to cardiomyocyte hypertrophy may be associated with the alteration of miR-22 expression, which modulates the activity of PTEN in cardiomyocyte hypertrophy [23]. In this study, we found simvastatin increased miR-22 expression in a dose dependent manner. However, whether miR-22 participates in the anti-inflammatory effects of simvastatin in LPS-stimulated RAW264.7 macrophages and how it works is unknown.

Cysteine-rich 61 (Cyr61/CCN1) is a member of the CCN protein family and its expression has been reported to be associated with the pathogenesis of inflammation [24, 25]. A study from Bai et al. showed that Cyr61 induced the expression of multiple pro-inflammatory cytokines in macrophages, implicating Cyr61 as a novel regulator of macrophage function. Fromigue et al. identified for the first time that Cyr61 is a new target of statins using microarray analysis [26]. Lin et al. found that simvastatin targeted Cyr61 through modulating FoxO3a activity in osteoblasts, which subsequently diminished macrophage infiltration and progression of induced rat periapical lesions [5, 27]. In this study, we identified Cyr61 as a direct target of miR-22 that mediated the beneficial effects of simvastatin in LPS-stimulated RAW264.7 macrophages. A previous study demonstrated that the miR-22/Cyr61 axis played an important role in mediating joint inflammation and damage in RA [16]. Thus, we determined whether the regulation of Cyr61 expression by miR-22 is the potential mechanism underlying the anti-inflammatory effect of simvastatin. As expected, we found that miR-22 inhibitor attenuated the inhibitory effect of simvastatin on the secretion of IL-1β, TNF-α, and IL-6, but this inhibitory effect recovered after si-Cyr61 plus miR-22 inhibitor transfection. These data suggest that simvastatin inhibits the inflammation response in LPS-stimulated RAW264.7 macrophages through miR-22/Cyr61 axis.

In summary, we demonstrated that simvastatin inhibits the inflammation response by regulating the miR-22/Cyr61 axis in LPS-stimulated RAW264.7 macrophages. On this basis, it is proposed that targeting the miR-22/Cyr61 axis might be a novel therapeutic strategy for AS.

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Disclosure of conflict of interest

None.

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