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Original Article

Chloroquine attenuates LPS-mediated macrophage activation through miR-669n-regulated SENP6 protein translation

Yupeng Long[1], Xin Liu[1], Ning Wang[1], Hong Zhou[2], Jiang Zheng[1]

[1]Medical Research Center, Southwestern Hospital, The Third Military Medical University, Chongqing 400038, China; [2]Department of Pharmacology, College of Pharmacy, The Third Military Medical University, Chongqing 400038, China

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Abstract: Chloroquine (CQ) has been shown to inhibit Toll-like receptor 4 (TLR4)-mediated monocyte and macrophage activation induced by lipopolysaccharide (LPS). However, the underlying mechanisms have not been completely elucidated. Recently, SUMO-specific protease 6 (SENP6) has been reported to suppress LPS-induced activation of macrophages through deSUMOlation of NF-κB essential modifier (NEMO). Here, we studied whether this molecular pathway may also be involved in CQ/LPS model. We found that CQ dose-dependently increased SENP6 protein, but not mRNA, in mouse macrophages, RAW264.7 cells. Overexpression of SENP6 in RAW264.7 cells significantly decreased the LPS-induced release of pro-inflammatory proteins, TNF-α, IL-6 and IFN-γ, while depletion of SENP6 in RAW264.7 cells significantly increased these proteins. Moreover, in LPS-treated RAW264.7 cells, CQ dose-dependently decreased the levels of microRNA-669n (miR-669n), which bound to 3'-UTR of SENP6 mRNA to inhibit its translation. Overexpression of miR-669n decreased SENP6, resulting in increased production of TNF-α, IL-6 and IFN-γ in RAW264.7 cells, while depletion of miR-669n increased SENP6, resulting in decreased production of TNF-α, IL-6 and IFN-γ in RAW264.7 cells. In vivo, delivery of miR-669n plasmids augmented the effects of LPS, while delivery of antisense of miR-669n (as-miR-669n) plasmids abolished the effects of LPS. Taken together, our data demonstrate a previously unappreciated molecular control of LPS-induced macrophage activation by CQ, through miR-669n-regulated SENP6 protein translation.

Keywords: Chloroquine (CQ), LPS, SENP6, miR-669n, bioinformatics analyses, miRNAs

Introduction

The mammalian Toll-like receptors (TLRs) are germline-encoded receptors expressed by the immune cells, when they are stimulated by structural motifs from bacteria, viruses and fungi known as pathogen-associated molecular patterns (PAMPs) [1-3]. Among all TLRs, TLR4 is an important factor, activation of which triggers the production of pro-inflammatory cytokines as well as the functional maturation of antigen presenting cells of the innate immune system, to mediate the immune response to PAMPs [1-3]. If the immune responses are under control, the effects will be beneficial, while excessive signals may induce systemic inflammation and sepsis to be lethal to patients [4-6].

Lipopolysaccharide (LPS) is an important structural component of the outer membrane of Gram-negative bacteria [4]. Upon LPS recognition, TLR4 undergoes oligomerization and recruits its downstream adaptors through interactions with the TIR (Toll-interleukin-1 receptor) domains [4]. TIR domains contain 3 highly conserved regions, which mediate protein-protein interactions between the TLRs and signal transduction adaptor proteins. There are five TIR domain-containing adaptor proteins in TIR for TLR4: MyD88 (myeloid differentiation primary response gene 88), TIRAP (TIR domain-containing adaptor protein, also known as Mal, MyD88-adapter-like), TRIF (TIR domain-containing adaptor inducing IFN-b), TRAM (TRIF-related adaptor molecule), and SARM (sterile a and HEAT-Armadillo motifs-containing protein) [7-11]. Different combinations of adaptor proteins determine the signaling downstream TLR activation [12-14].

MicroRNA (miRNA) is a class of 18-23-nucleotide non-coding small RNAs that regulate gene expression at translational level, through their base-pairing to the 3'-untranslated region (3'-UTR) of target mRNAs [15, 16]. It has been acknowledged that miRNAs regulate many biological events. Moreover, miRNAs have been shown to play a role in the regulation of TLR signaling [17-21]. Among all miRNAs, miR-669n is newly defined and has been rarely studied. However, whether miR-669n may play a role in the TLR4-mediated macrophage activation during infection is unknown.

We have previously shown that LPS is internalized into human peripheral blood mononuclear cells to promote release of TNF-α, rather than directly activates TLR4 on the cell surface to secrete TNF-α [22]. Moreover, Chloroquine (CQ) attenuates the LPS-induced immune response [22], with ill-defined mechanisms.

Recently, SUMO-specific protease 6 (SENP6) has been reported to suppress LPS-induced activation of macrophages through deSUMOlation of NF-κB essential modifier (NEMO) [23]. This study prompted us to examine whether this molecular pathway may also be involved in CQ-induced attenuation of the effects of LPS on macrophage activation.

In the current study, we found that Here, we studied whether this molecular pathway may also be involved in CQ/LPS model. We found that CQ dose-dependently increased SENP6 protein, but not mRNA, in mouse macrophages, RAW264.7 cells. Overexpression of SENP6 in RAW264.7 cells significantly decreased the LPS-induced release of pro-inflammatory proteins, TNF-α, IL-6 and IFN-γ, while depletion of SENP6 in RAW264.7 cells significantly increased them. Moreover, in LPS-treated RAW-264.7 cells, CQ dose-dependently decreased the levels of miR-669n, which bound to 3'-UTR of SENP6 mRNA to inhibit its translation. Overexpression of miR-669n decreased SENP6, resulting in increased production of TNF-α, IL-6 and IFN-γ in RAW264.7 cells, while depletion of miR-669n increased SENP6, resulting in decreased production of TNF-α, IL-6 and IFN-γ in RAW264.7 cells. In vivo, delivery of miR-669n plasmids augmented the effects of LPS, while delivery of antisense of miR-669n (as-miR-669n) plasmids abolished the effects of LPS. Taken together, our data demonstrate a previously unappreciated molecular control of LPS-induced macrophage activation by CQ, through miR-669n-regulated SENP6 protein translation.

Materials and methods

Mouse ethic issues

Female C57BL/6 mice of 10 weeks of age were purchased from the Shanghai SLAC Laboratory Animal Company (Shanghai, China). The mice were maintained under specific pathogen-free (SPF) conditions at the Southwestern Hospital of the Third Military Medical University. Animal experiments were carried out in strict accordance with the regulations in the Guide for the Care and Use of Laboratory Animals issued by the Ministry of Science and Technology of the People's Republic of China. The protocol was approved by the Institutional Animal Care and Use Committee of the Southwestern Hospital of the Third Military Medical University.

Cell culture

RAW264.7 cells are mouse macrophages, as have been previously described [23]. RAW264.7 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 1% L-glutamine and 1% penicillin-streptomycin (Invitrogen) in a humidified chamber with 5% CO_2 at 37°C. TNF-α, IL-6 and IFN-γ levels in the conditioned media were measured 24 hours after LPS/CQ treatment, using ELISA kits (R&D Systems, Los Angeles, CA, USA), according to manufacturer's instructions.

Reagents

CQ and LPS were both purchased from Sigma-Aldrich (St Louis, MO, USA). TNF-α, IL-6 and IFN-γ ELISA kits were purchased from Biosource International (Camarillo, CA, USA).

Plasmid and transfection

SENP6- and miR-669n-modified plasmids were prepared using routine methods. Briefly, the constructs for SENP6, short hairpin small interfering RNA for SENP6 (shSENP6), control scrambled sequence (scr), miR-669n, anti-

sense of miR-669n (as-miR-669n) or control null were cloned into pcDNA3.1-EGFP to generate the corresponding plasmids. The sequences were: miR-669n: 5'-AUUUGUGUGUGGAUGUGUGU-3', as-miR-669n: 5'-ACACACAUCCACACACAAAU-3', shSENP6: 5'-GGGUGAUAAAGCCUGUAAATT-3'. These plasmids of 2 μg were transfected into cultured RAW264.7 cells using Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen). The plasmids also contained a GFP reporter to allow determination of transfection efficiency, which was nearly 100% in the current study.

Quantitative PCR (RT-qPCR)

MiRNA and total RNA were extracted from the cultured or primary cells with miRNeasy mini kit or RNeasy kit (Qiagen, Hilden, Germany), respectively. For cDNA synthesis, complementary DNA (cDNA) was randomly primed from 2 μg of total RNA using the Omniscript reverse transcription kit (Qiagen). RT-qPCR was subsequently performed in triplicate with a 1:4 dilution of cDNA using the Quantitect SyBr green PCR system (Qiagen) on a Rotorgene 6000 series PCR machine. All primers were purchased from Qiagen. Data were collected and analyzed using 2-ΔΔCt method. Values of genes were first normalized against α-tubulin, and then compared to the experimental controls.

Western blot

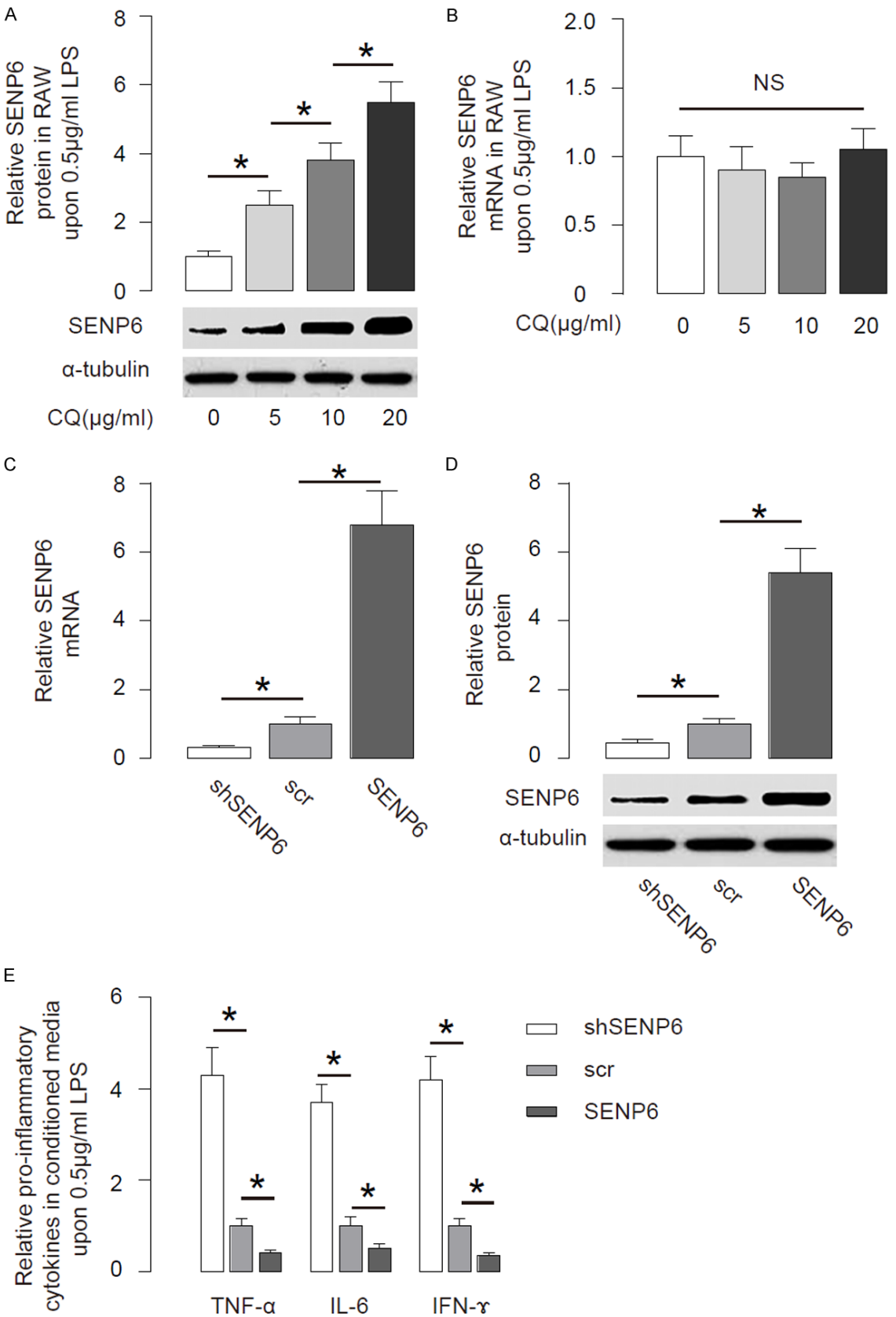
The protein was extracted from the cultured or isolated primary cells, and homogenized in RIPA lysis buffer (1% NP40, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, in PBS) on ice. The supernatants were collected after centrifugation at 12000× g at 4°C for 20 min. Protein concentration was determined using a BCA protein assay kit (Biorad, China), and whole lysates were mixed with 4×SDS loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/l DTT, and 0.2% bromophenol blue) at a ratio of 1:3. Protein samples were heated at 100°C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies for Western Blot are anti-SENP6 and α-tubulin (all from Cell Signaling, San Jose, CA, USA). α-tubulin was used as protein loading controls. Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). Images shown in the figures were representative from 5 individuals. Densitometry of Western blots was quantified with NIH ImageJ software (Bethesda, MA, USA). The protein levels were first normalized to α-tubulin, and then normalized to experimental controls.

MicroRNA target prediction and Luciferase-reporter activity assay

MiRNAs targets were predicted as has been described before, using the algorithms TargetScan (https://www.targetscan.org) [24]. Luciferase-reporters were successfully constructed using molecular cloning technology. Target sequence was inserted into pGL3-Basic vector (Promega) to obtain pGL3-SENP6-3'-UTR containing the miR-669n binding sequence (SENP6-3'-UTR sequence). MiR-669n-modified RAW264.7 cells were seeded in 24-well plates for 24 hours, after which they were transfected with 1 μg of Luciferase-reporter plasmids per well using PEI Transfection Reagent. Then luciferase activities were measured using the dual-luciferase reporter gene assay kit (Promega), according to the manufacturer's instructions.

In vivo plasmid transfection and in vivo endotoxic shock model

The plasmids carrying miR-669n, as-miR-669n or control null were delivered into C57BL/6 mice with JetPEI transfection reagent (PolyPlus Transfection, San Marcos, CA) according to the manufacturer's instructions. The plasmids and JetPEI were diluted into 100 ml of 5% glucose, then mixed and incubated for 15 minutes at room temperature, after which the mixture (200 ml) was injected into a mouse via tail vein. For the LPS-induced endotoxic shock study, 48 hours after plasmid delivery in vivo, the mice were challenged intraperitoneally with LPS at a dose of 20 mg/kg. Serum TNF-α, IL-6 and IFN-γ were measured 3 hours after intraperitoneal injection of LPS, using ELISA kits (R&D Systems, Los Angeles, CA, USA), according to manufacturer's instructions. For analysis of in vivo transfection efficiency, the liver kupffer cell extracts


A
Relative SENP6 protein in RAW upon 0.5μg/ml LPS
8
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SENP6
α-tubulin
CQ(μg/ml) 0 5 10 20
B
Relative SENP6 mRNA in RAW upon 0.5μg/ml LPS
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Relative pro-inflammatory cytokines in conditioned media upon 0.5μg/ml LPS
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TNF-α
IL-6
IFN-γ
shSENP6
scr
SENP6

Figure 1. CQ dose-dependently increases SENP6, which suppresses LPS-induced production of pro-inflammatory cytokines in macrophages. Mouse macrophages RAW264.7 cells were challenged with 500 ng/ml LPS for 2 hours, and then treated with different doses of CQ for 24 hours, after which the cellular levels of SENP6 were measured. (A) CQ dose-dependently increases SENP6 protein levels. (B) The mRNA levels of SENP6 were not altered by CQ treatment. (C, D) We either overexpressed (SENP6) or depleted SENP6 (shSENP6) in RAW264.7 cells. Scr: control by transfection with scrambled sequence. The modification of SENP6 levels in these cells were confirmed by RT-qPCR (C), and by Western blot (D). (E) The TNF-α, IL-6 and IFN-γ levels in the conditioned media from CQ-treated, LPS-challenged RAW264.7 cells. *p<0.05. NS: non-significant. N=5.

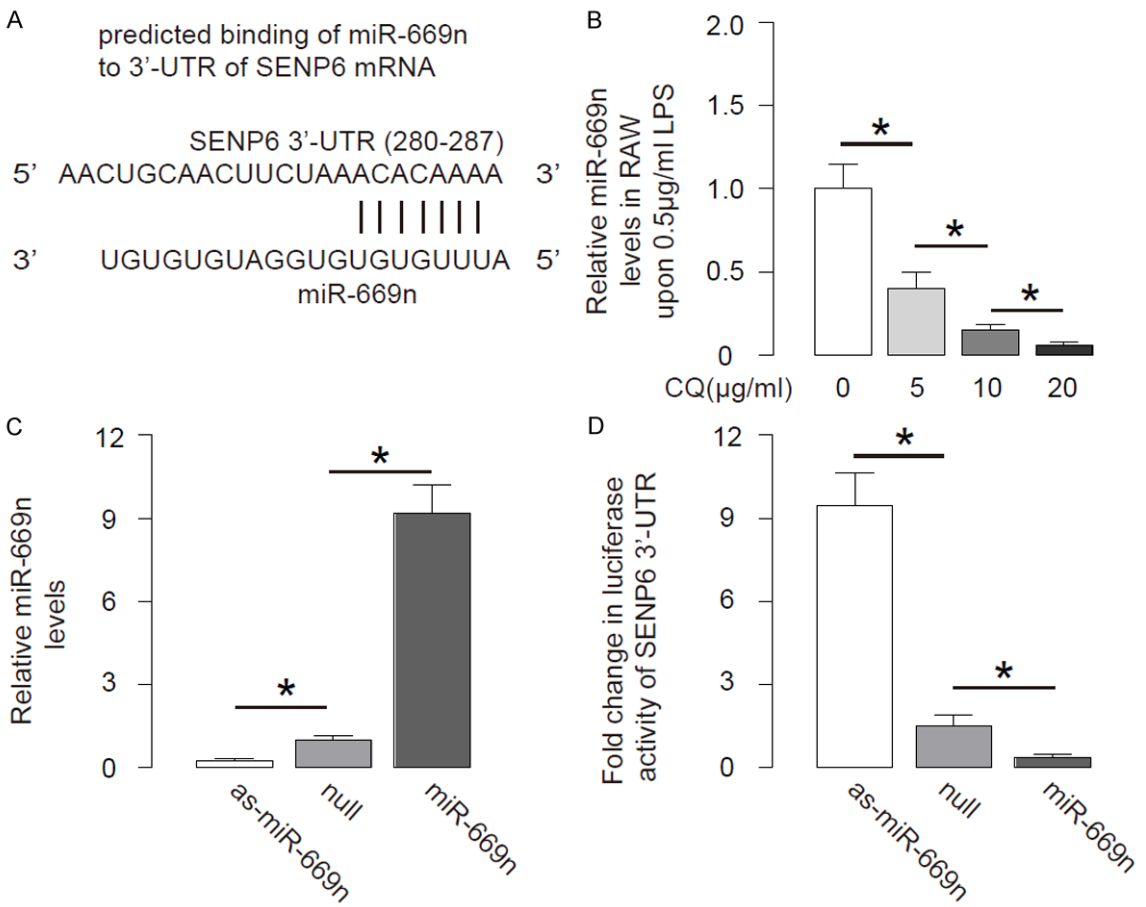

Figure 2. The effects of CQ on SENP6 are mediated through miR-669n. A. Bioinformatics analyses, showing that miR-669n binds to 3'-UTR of SENP6 mRNA. B. CQ dose-dependently decreased the levels of miR-669n in LPS-challenged RAW264.7 cells. C. RAW264.7 cells were transfected with plasmids carrying either miR-669n, or as-miR-669n, or null as a control. The modification of miR-669n levels in RAW264.7 cells was confirmed by RT-qPCR. D. The miR-669n-modified RAW264.7 cells were transfected with 1 μg of SENP6-3'-UTR Luciferase-reporter plasmid, and the luciferase activities in miR-669n-modified RAW264.7 cells were examined. *p<0.05. N=5.

were prepared for RT-qPCR and Western blot. The mice were monitored for lethality for 40 hours.

Isolation of kupffer cells from mouse liver

Kupffer cells were harvested as has been previously reported [23]. Briefly, the mice were anesthetized by intraperitoneal injection of 6 mg/ml sodium pentobarbital in saline, after which the liver was cannulated via the portal vein, and perfused with calcium- and magnesium-free HBSS (Invitrogen). Afterwards, the liver was perfused with HBSS containing 0.1% type IV collagenase (Invitrogen). The liver was then excised and the cells dispersed in RPMI1640 media (Invitrogen). The homogenate was filtered through a 70 mm cell strainer (Becton-Dickinson Biosciences, San Jose, CA, USA) and centrifuged at 50 g for 5 min to pellet the liver

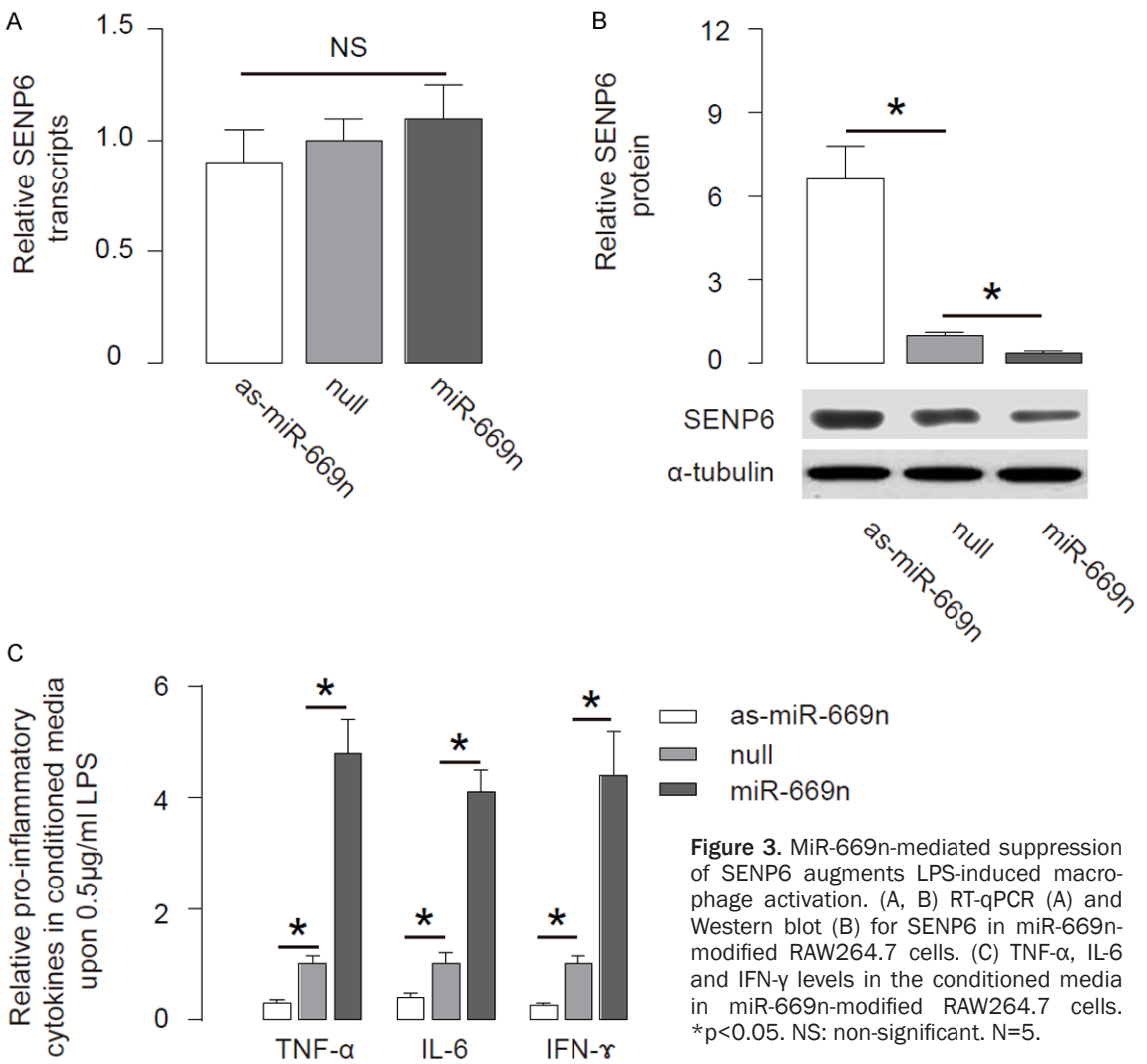

Figure 3. MiR-669n-mediated suppression of SENP6 augments LPS-induced macrophage activation. (A, B) RT-qPCR (A) and Western blot (B) for SENP6 in miR-669n-modified RAW264.7 cells. (C) TNF-α, IL-6 and IFN-γ levels in the conditioned media in miR-669n-modified RAW264.7 cells. *p<0.05. NS: non-significant. N=5.

cells. Subsequently, the supernatant containing non-parenchymal liver cells was re-centrifuged at 300 g for 10 min. The cell pellet was then re-suspended in 10 ml RMPI1640 media, loaded onto 25% and 50% Percoll gradients (Sigma-Aldrich), and centrifuged at 1400 g for 30 min. The cells at the interface were washed and re-suspended in RMPI1640 media. Kupffer cells were enriched by selective adherence to tissue culture plates.

Statistics

All statistical analyses were carried out using the GraphPad Prism 6.0 statistical software package (GraphPad Software, Inc. La Jolla, CA, USA). All values in cell and animal studies are depicted as mean ± standard deviation and are considered significant if $p < 0.05$. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher' Exact Test for comparison of two groups.

Results

CQ dose-dependently increases SENP6, which suppresses LPS-induced production of pro-inflammatory cytokines in macrophages

Mouse macrophages RAW264.7 cells were challenged with 500 ng/ml LPS for 2 hours, and then treated with different doses of CQ for 24 hours, after which the cellular levels of SENP6 were measured. We found that CQ dose-dependently increases SENP6 protein levels (**Figure 1A**). However, the mRNA levels of SENP6 were not altered by CQ treatment (**Figure 1B**), suggesting that CQ may regulate

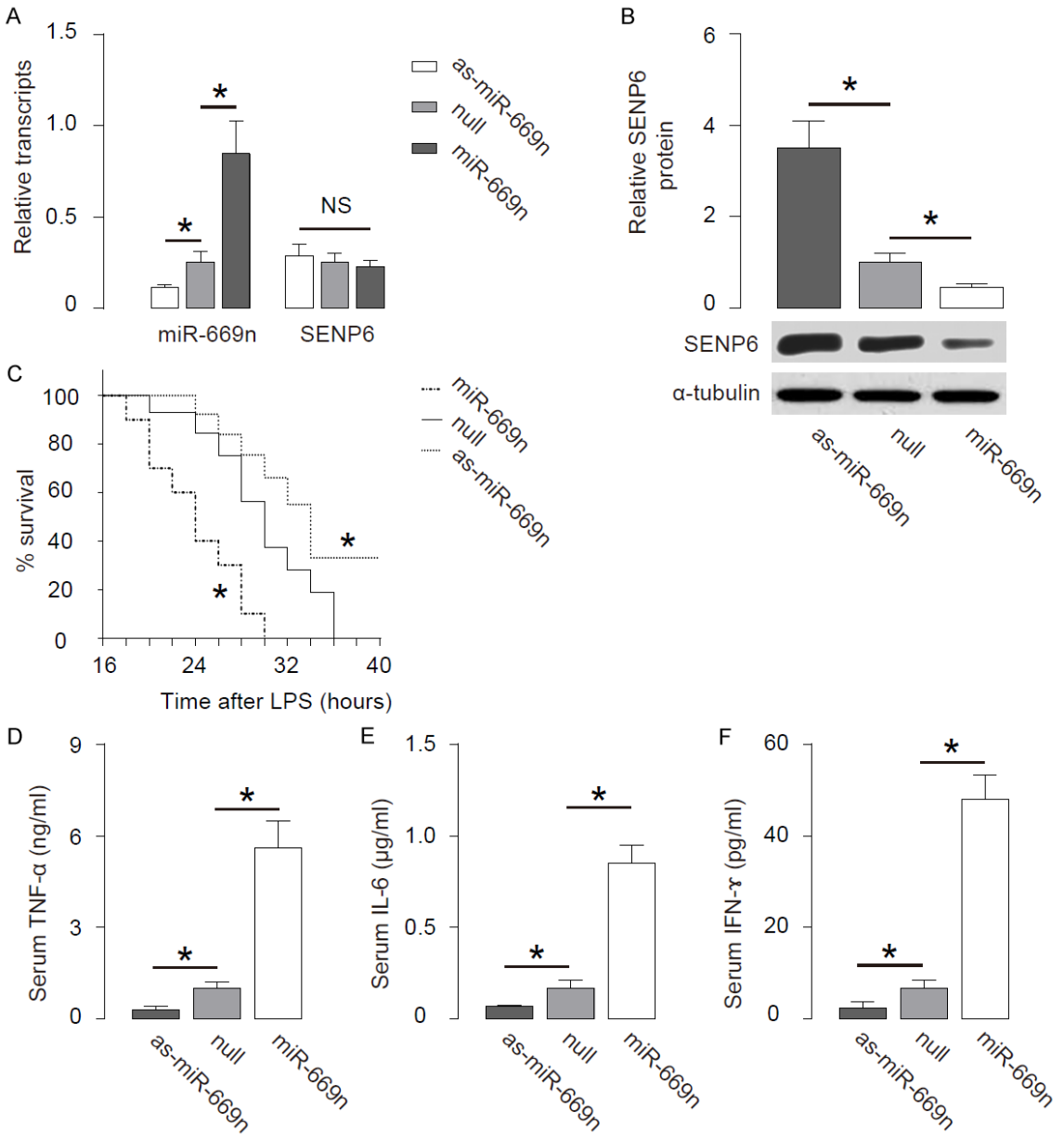

Figure 4. Suppression of miR-669n partially mimics the inhibitory effects of CQ on macrophage activation. (A, B) In a mouse endotoxin shock model, the plasmids of miR-669n, as-miR-669n or control null were delivered into mice via tail vein injection. After 48 hours, the mice were injected intraperitoneally with a sub-lethal dose of LPS. Liver kupffer cells were then isolated and examined their expression of miR-669n and SENP6 by RT-qPCR (A), and by Western blot (B). (C) The survival rates of the mice (10 per group) were monitored for 40 hours. (D-F) TNF-α (D), IL-6 (E) and IFN-γ (F) levels in the mouse serum after LPS/plasmids treatment. *$p<0.05$. NS: non-significant. N=5.

SENP6 at post-transcriptional level. In order to confirm that modulation of SENP6 levels may alter the LPS-induced macrophage activation, we either overexpressed or depleted SENP6 in RAW264.7 cells. First, we confirmed the modification of SENP6 levels in these cells, by RT-qPCR (**Figure 1C**), and by Western blot (**Figure 1D**). Since the activation of macrophages could be reflected by their release of pro-inflammatory cytokines like TNF-α, IL-6 and IFN-γ, we examined the TNF-α, IL-6 and IFN-γ levels in the conditioned media from CQ-treated, LPS-challenged RAW264.7 cells. We found that overexpression of SENP6 significantly decreased the levels of these cytokines, while SENP6 depletion significantly increased the

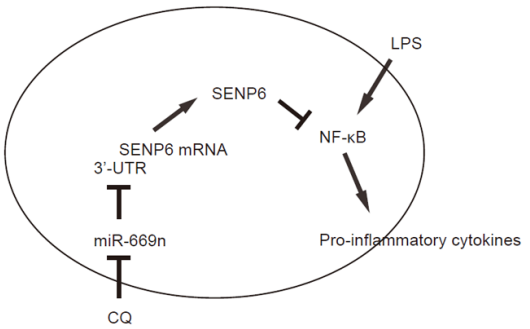

Figure 5. Schematic of the model. CQ may attenuates LPS-mediated macrophage activation partially through miR-669n-regulated SENP6 protein translation.

levels of these cytokines (**Figure 1E**). These data suggest that CQ dose-dependently increases SENP6, which suppresses LPS-induced pro-inflammatory cytokine release in macrophages.

The effects of CQ on SENP6 are mediated through miR-669n

Since CQ altered protein, but not mRNA levels of SENP6, we hypothesized that the post-transcriptional control of SENP6 might be regulated by CQ. Since miRNAs are the most important protein translational regulators, we screened all SENP6-targeting miRNAs, the levels of which were altered by CQ. Specifically, we found that miR-669n bound to 3'-UTR of SENP6 mRNA (**Figure 2A**), and CQ dose-dependently decreased the levels of miR-669n in LPS-challenged RAW264.7 cells (**Figure 2B**). In order to understand the biological relevance of the binding between miR-669n and 3'-UTR of SENP6 mRNA, we transfected RAW264.7 cells with plasmids carrying either miR-669n, or as-miR-669n, or null as a control. The modification of miR-669n levels in RAW264.7 cells was confirmed by RT-qPCR (**Figure 2C**). Afterwards, these miR-669n-modified RAW264.7 cells were transfected with 1 μg of SENP6-3'-UTR Luciferase-reporter plasmid. We found that the luciferase activities in miR-669n-depleted RAW264.7 cells were significantly higher than the control, while the luciferase activities in miR-669n-overexpressing RAW264.7 cells were significantly lower than the control (**Figure 2D**). Together, these data suggest that miR-669n may targets 3'-UTR of SENP6 mRNA to inhibit its translation in RAW264.7 cells. Moreover, CQ may suppress miR-669n in RAW264.7 cells, resulting in increases in SENP6, which subsequently attenuates the LPS-induced macrophage activation.

MiR-669n-mediated suppression of SENP6 augments LPS-induced macrophage activation

We found that modulation of miR-669n in RAW264.7 cells did not alter mRNA levels of SENP6 (**Figure 3A**). However, overexpression of miR-669n decreased SENP6 protein (**Figure 3B**), and increased TNF-α, IL-6 and IFN-γ release in RAW264.7 cells (**Figure 3C**). On the other hand, depletion of miR-669n increased SENP6 protein (**Figure 3B**), and decreased TNF-α, IL-6 and IFN-γ release in RAW264.7 cells (**Figure 3C**). These data suggest that miR-669n-mediated suppression of SENP6 augments LPS-induced macrophage activation.

Suppression of miR-669n partially mimics the inhibitory effects of CQ on macrophage activation

In order to figure out whether the regulatory axis of SENP6/miR-669n may play a substantial role in CQ-treated, LPS-challenged macrophage activation, we used the mouse endotoxin shock model. First, the plasmids of miR-669n, as-miR-669n or control null were delivered into mice via tail vein injection. After 48 hours, the mice were injected intraperitoneally with a sub-lethal dose of LPS. Liver kupffer cells were then isolated to confirm the in vivo modulation of miR-669n and SENP6 through plasmid transfection (**Figure 4A**, **4B**). The survival rates of the mice (10 per group) were monitored for 40 hours. We found that as-miR-669n-induced increases in SENP6 resulted in a significant improvement in mouse survival, compared to control mice, while miR-669n-induced decreases in SENP6 resulted in a significant regression in mouse survival, compared to control mice (**Figure 4C**). Moreover, the serum levels of pro-inflammatory cytokines TNF-α, IL-6 and IFN-γ in as-miR-669n-treated mice upon LPS stimulation were significantly lower than control mice, while the serum levels of pro-inflammatory cytokines TNF-α, IL-6 and IFN-γ in miR-669n-treated mice upon LPS stimulation were significantly higher than control mice (**Figure 4D-F**). Together, these data partially mimicked the inhibitory effects of CQ on macrophage activation, suggesting that CQ

may attenuates LPS-mediated macrophage activation partially through miR-669n-regulated SENP6 protein translation (**Figure 5**).

Discussion

TLR4 signaling has been divided into MyD88-dependent and MyD88-independent (TRIF-dependent) pathways [4-6]. According to the studies using MyD88-deficient macrophages, the MyD88-dependent pathway was shown to be mainly responsible for pro-inflammatory cytokine expression, while the MyD88-independent pathway is supposed to mediate the induction of type I interferons and interferon-inducible genes [4-6].

However, a recent study has shown that NEMO, a critical protein of the TLR signaling pathways, is covalently modified by SUMO-2/3 [23]. Moreover, this modification is reversed by the de-SUMOylation activity of SENP6. Thus, SENP6 facilitates CYLD to bind NEMO and to remove the polyubiquitin chains on NEMO, which eventually dampens the IKK activation [23]. This study has revealed another important regulatory pathway for TLR4 activation. The duration of the signaling is modulated via protein post-translational modifications, which control the invading microbes to be quickly eliminated and the damages to the host to be reduced to the minimum.

Generally, there are two ways to control protein regulation. First, like in the abovementioned study [23], protein levels are regulated by protein degradation via various protein modifications, e.g. phosphorylation, SUMOylation, acetylation and ubiquitination [25-27]. Second, protein levels are regulated at the translation control point through miRNAs. In our study, we further showed that CQ may attenuates LPS-induced macrophage activation partially through miR-669n-regulated SENP6 protein translation.

First, we found that CQ dose-dependently increased SENP6 protein, but not mRNA, in mouse macrophages, RAW264.7, upon LPS challenge. These data suggest the presence of the post-transcriptional control of SENP6, which is regulated by CQ. Our next approaches confirmed the finding in the previous report [23], by showing that overexpression of SENP6 in RAW264.7 cells significantly decreased the LPS-induced release of pro-inflammatory proteins, TNF-α, IL-6 and IFN-γ, while depletion of SENP6 in RAW264.7 cells significantly increased them. Since production and secretion of these cytokines are signatures of macrophage activation into a classical phenotype, or M1 macrophages, our data suggest that SENP6 suppresses macrophage activation. Of note, macrophages have another activation pathway towards an alternative phenotype, or M2 macrophages, which do not express these pro-inflammatory cytokines, but other cytokines related to vascularization, epithelial-mesenchymal transition and tissue remodeling, e.g. transforming growth factor β1 [28, 29]. Future studies may be applied to examine whether CQ may induce this alternative way of activation in the LPS-challenged macrophages.

Moreover, we found that in LPS-treated RAW-264.7 cells, CQ dose-dependently decreased miR-669n, which bound to 3'-UTR of SENP6 mRNA to inhibit its translation. Overexpression of miR-669n decreased SENP6, resulting in increased TNF-α, IL-6 and IFN-γ release in RAW264.7 cells, while depletion of miR-669n increased SENP6, resulting in decreased TNF-α, IL-6 and IFN-γ release in RAW264.7 cells. In vivo, delivery of miR-669n plasmids augmented the effects of LPS, while delivery of as-miR-669n plasmids abolished the effects of LPS. These data demonstrate that the SENP6 is upregulated by CQ through miRNA-mediated protein translational control. Future approaches may be performed to improve our understanding of the regulation of TLR4 activity in macrophages by SENP6 and miR-669n. First, post-translational modification of TLR4-signaling-related factors other than NEMO should be examined. Second, it is possible that miR-669n may have targets other than SENP6 that are also involved in this model. Elucidation of these targets of miR-669n other than SENP6 may improve the completeness of our understanding of the role of miR-669n in LPS/TLR4 signaling-controlled immune responsiveness.

In summary, our data demonstrate a previously unappreciated role of miR-669n in the regulation of SENP6-mediated LPS-induced macrophage activation by CQ, and this regulation may be at least partially conducted through modulation of SENP6 protein translation.

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Disclosure of conflict of interest

None.

Address correspondence to: Jiang Zheng, Medical Research Center, Southwestern Hospital, The Third Military Medical University, 29 Gaotanyan Street, Shapingba District, Chongqing 400038, China. Tel: +86-23-68754435; Fax: +86-23-68765468; E-mail: zhengj99219@gmail.com; zhengjian99219@163.com; Hong Zhou, Department of Pharmacology, College of Pharmacy, The Third Military Medical University, 30 Gaotanyan Street, Shapingba District, Chongqing 400038, China. Tel: +86-23-68752266; Fax: +86-23-68752266; E-mail: zhouh64@163.com

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