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ORIGINAL ARTICLE

miR-132 inhibits lipopolysaccharide-induced inflammation in alveolar macrophages by the cholinergic anti-inflammatory pathway

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ABSTRACT

Objective: Although microRNA-132 (miR-132) has been shown to be involved in the inflammatory regulation, its role in sepsis-induced lung injury is unknown. We hypothesized that miR-132 attenuated lipopolysaccharide (LPS)-induced inflammation of alveolar macrophages by targeting acetylcholinesterase (AChE) and enhancing the acetylcholine (ACh)-mediated cholinergic anti-inflammatory response. *Methods*: The LPS-treated rat alveolar macrophage cell line NR8383 was used as the inflammatory model. To assess the effect of miR-132, alveolar macrophages were transfected with miR-132 mimic or inhibitor. *Results*: We found that miR-132 was upregulated in LPS-stimulated alveolar macrophages. Induction of AChE mRNA showed an inverse pattern with respect to AChE protein and activity, suggesting posttranscriptional regulation of AChE. Utilizing miR-132 mimic transfection, we found that overexpression of miR-132 enhanced the ACh-mediated cholinergic anti-inflammatory reaction by targeting AChE mRNA in LPS-treated alveolar macrophages. Blockage of miR-132 using miR-132 inhibitor reversed the Ach action upon LPS-induced release of inflammatory mediators and reduction in AchE protein/activity. Moreover, in the presence of ACh, upregulation of miR-132 suppressed LPS-induced nuclear translocation of NF-κB and production of STAT3 and phosphorylated STAT3, while downregulation of miR-132 enhanced the nuclear translocation of NF-κB. *Conclusion*: We propose that miR-132 functions as a negative regulator of the inflammatory response in alveolar macrophages by potentiating the cholinergic anti-inflammatory pathway, and represents a potential therapeutic leverage point in modulating inflammatory responses.

KEYWORDS: alveolar macrophage, cholinergic anti-inflammatory pathway, microRNA-132, NF-κB, STAT3

INTRODUCTION

Sepsis refers to the systemic inflammatory response to infection caused by various pathogens. Severe sepsis induces dysfunction of certain organs, including initially the lung, where it leads to acute respiratory distress syndrome (ARDS), a leading cause of death in critically ill patients [1, 2]. The production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 by activated alveolar macrophages plays a pivotal role in triggering sepsis-induced lung injury [3, 4], suggesting that their neutralization represents a potential therapeutic approach in this condition. However, the failure of the monoclonal antibody against proinflammatory cytokine therapy to reduce mortality [5, 6] has led to increased interest in suppression of alveolar macrophages activation as a therapeutic intervention in sepsis-induced lung injury.

The cholinergic anti-inflammation pathway comprises efferent vagus nerve fibers, the neurotransmitter ACh and the α7 nicotinic receptor (α7nAChR). ACh released by efferent vagus nerve fibers in response to an inflammation reaction binds to macrophage α7nAChR, resulting in inhibition of NF-κB and JAK2/STAT3 signaling and repression

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of proinflammatory cytokine levels [7–10]. Given its role in the inhibition of systemic and local inflammation, this pathway represents a potential leverage point in modulating the inflammatory response in sepsis-induced lung injury. In this vein, recent evidence has documented the part played by the immunomodulatory microRNA miR-132 in regulating this pathway. For example, miR-132 has been shown to attenuate inflammation by targeting AChE mRNA, which leads to enhanced ACh-mediated cholinergic signaling [11]. Moreover, elevated miR-132 levels and lower AChE activity in intestinal biopsies from patients with inflammatory bowel disease (IBD) imply that miR-132 plays an inflammation-dependent homeostatic role in IBD patients through regulation of AChE [12]. It remains not known whether miR-132 functions as a protective regulator in sepsis-induced lung injury via the cholinergic anti-inflammatory pathway. Here, we set out to determine the therapeutic potential of miR-132 in sepsis-induced lung injury by evaluating its suppression of AchE and enhancement of the cholinergic anti-inflammatory pathway in LPS-stimulated rat alveolar macrophages.

MATERIALS AND METHODS

Cell Culture

The rat alveolar macrophage cell line NR8383 was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Rat NR8383 alveolar macrophages were cultured in Ham's F-12K complete medium (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 15% pyrogen-free fetal bovine serum (Gibco, USA) in a humidified incubator (Thermo Scientific, Waltham, MA, USA) with 5% CO_2 at 37°C. The media was renewed every 2–3 days, and macrophages were subcultured at 80% confluence. All materials used for the experimental procedure underwent depyrogenation.

Enzyme-Linked Immunosorbent Assay

TNF-α, IL-1β, and IL-6 levels in the cell culture supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols (Senxiong Technology Industrial Co., Ltd., Shanghai, China). Absorbance was read at 450 nm and all absorbance results were normalized via standard curves.

TABLE 1. Sequences of primers for reverse transcription and real-time quantitative PCR

Gene	Primer sequences
miR-132	RT primer: 5′-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC GAC CA-3′
	PCR primer F: 5′-GCC GCT AAC AGT CTA CAG CCA T-3′
	PCR primer R: 5′-GTG CAG GGT CCG AGG T-3′
U6	RT primer: 5′-AAC GCT TCA CGA ATT TGC GT-3′
	PCR primer F: 5′-CTC GCT TCG GCA GCA CA-3′
	PCR primer R: 5′-AAC GCT TCA CGA ATT TGC GT-3′
AChE	PCR primer F: 5′-AAA CAT GCA GAA GAT GAG GAT-3′
	PCR primer R: 5′-GAC CAC TAT AGC AAG CAG GAA C-3′
β-actin	PCR primer F: 5′-TAC TGC CCT GGC TCC TAG CA-3′
	PCR primer R: 5′-TGG ACA GTG AGG CCA GGA TAG-3′

F: forward, R: reverse.

Real-Time Quantitative PCR

The relative expression levels of miR-132 and AChE mRNA were detected by real-time quantitative PCR (RT-qPCR). Total RNA was extracted from alveolar macrophages with TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. Agarose gel electrophoresis was used to verify the integrity of RNA. The concentration and purity of RNA were determined by optical density (OD) values at 260 and 280 nm using an ultraviolet spectrophotometer (Hitachi, Japan). Reverse transcription reactions were performed using a PrimeScript RT reagent Kit (TaKaRa Biotechnology Co. Ltd., Dalian, China) following the manufacturer's protocols, with 10 μL total RNA per reaction. RT-qPCR was performed using a SYBR Premix Ex Taq II kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocols, with 20 μL total RNA per reaction. PCR amplification was performed using a StepOne quantitative PCR system (Applied Biosystems, Foster City, CA, USA). The relative expression levels of miR-132 and AChE mRNA were normalized to those of the endogenous references U6 snRNA and β-actin following the $2^{-\Delta\Delta Ct}$ method. Primers for miR-132, AChE, snRNA, and β-actin are shown in Table 1.

Cell Transfection

Alveolar macrophages in exponential growth phase were seeded at 0.5×10^6 cells per well in a

6-well plate 24 hours before transfection. According to the instructions of the PowerFect siRNA Transfection Reagent (SignaGen Co., USA), mimic FAM negative control (determining the optimal concentration for transfection), mimic negative control (mimic NC), miR-132 mimic, inhibitor FAM negative control (inhibitor FAM NC), inhibitor negative control (inhibitor NC), and miR-132 inhibitor (Invitrogen, USA) were transfected into alveolar macrophages. The transfection efficiency was detected using a fluorescence microscope (Olympus, Japan) 5 hours after transfection or to examine the relative expression level of miR-132 by RT-qPCR 24 hours after transfection.

Western Blot Analysis

For detection of AChE, STAT3, p-STAT3, and internal control β-actin proteins, total proteins were extracted from alveolar macrophages using RIPA buffer (Beyotime Co., China). For detection of NF-κB p65 and internal controls including β-actin and Lamin B, cytoplasmic and nuclear proteins were extracted using a Nuclear Extract Kit (Active Motif, USA). A bicinchoninic acid assay was used to determine protein concentrations. 20 μg protein was loaded per lane and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), prior to transfer onto nitrocellulose membranes and blocking with 5% skimmed milk for 1 hour. Membranes were incubated overnight with the following primary antibodies: anti-AChE at 1:200 dilution (Abcam, USA), anti-STAT3 at 1:500 dilution, anti-p-STAT3 at 1:500 dilution (Cell Signaling Technology Co., USA), anti-β-actin at 1:3000 dilution (anbo, USA), anti-NF-κB p65 at 1:500 dilution, and anti-Lamin B at 1:100 dilution (Santa Cruz Biotechnology Co., USA). Membranes were then washed and exposed to a horseradish peroxidase–conjugated secondary antibody (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). Protein bands were visualized using a chemiluminescent substrate (Thermo scientific, Waltham, MA, USA) and quantification of the bands was performed by scanning densitometry.

AChE Activity Assay

Culture supernatants were collected for AChE activity assay using an AchE assay kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols, and OD values were read at 412 nm. AChE activity was calculated using the following equation: (U/mL) = (OD of assay – OD of sample blank)/(OD of standard – OD of blank) $\times$ Standard concentration (1 μmol/mL) $\times$ Dilution coefficient of the sample.

Immunofluorescence

Alveolar macrophages were collected and seeded on sterile coverslips. The cells were fixed with 4% paraformaldehyde for 20 minutes followed by PBS washing, permeabilized with 0.2% Triton X-100 for 5 minutes followed by PBS washing and exposed to PBS containing 5% bovine serum albumin for 1 hour. Cells were then incubated with anti-NF-κB p65 primary antibody (1:50) (Santa Cruz Biotechnology Co., USA) at 4°C overnight followed by PBS washing prior to incubation with Alexa Flour 488-labeled secondary antibody (Life technologies Co., USA) from light at room temperature for 1 hour. After PBS washing, the cells were counterstained with DAPI (Beyotime Co., China) for 2 minutes to visualize the cell nuclei and then mounted with glycerol. Total cells and nuclear NF-κB p65 positive cells were counted at 400$\times$ magnification by fluorescence microscopy, followed by Image-Pro Plus software analysis.

Statistical Analysis

Statistical analysis was carried out using SPSS software, version 17.0. Data are expressed as mean $\pm$ SD. Statistical significance was determined using a Student's t test and one-way ANOVA, and $P < .05$ was considered statistically significant.

RESULTS

Upregulation of miR-132 Expression in LPS-Stimulated Alveolar Macrophages

To determine the role of miR-132 in the inflammatory response in alveolar macrophages, we first validated the rat alveolar macrophage NR8383 inflammatory reaction model. Treatment of NR8383 cells with 1 μg/mL LPS induced TNF-α, IL-1β, and IL-6 levels in the cell culture supernatant, reaching a peak at 12 hours posttreatment, then decreasing at 24 hours, higher than the levels in the control group (Figure 1A–1C).

Compared with the control group, expression of miR-132 was higher at 6 hours (5.98- $\pm$ 0.65-fold induction) and 12 hours (7.64- $\pm$ 0.53-fold induction) after LPS treatment (Figure 1D). In contrast to LPS-induced changes in the levels of proinflammatory cytokines, induction of miR-132 (8.92 $\pm$ 0.83)

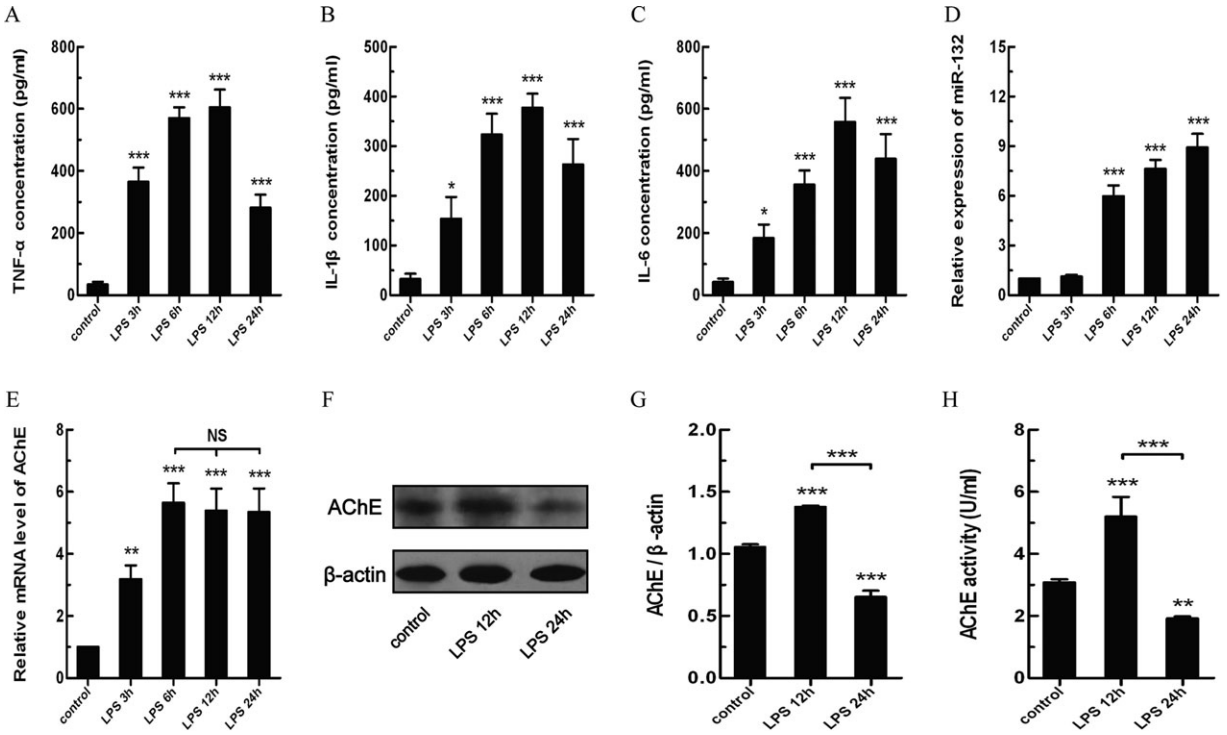

FIGURE 1. Upregulation of miR-132 and posttranscriptional regulation of AChE in LPS (1 μg/mL)-stimulated alveolar macrophages. A–C. ELISA for TNF-α (A), IL-1β (B), and IL-6 (C) in the culture supernatants after LPS treatment. D and E. RT-qPCR for miR-132 (D) and AChE mRNA (E) in alveolar macrophages after LPS treatment. F. Western blotting for AChE protein in alveolar macrophages after LPS treatment, normalized to β-actin. G. Analysis of the densitometry of the bands for (F). H. AChE activity in the culture supernatants after LPS treatment. Data are expressed as mean ± SD from three independent experiments. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, NS: not significant ($P > .05$).

was maintained up to 24 hours after LPS treatment. Collectively, these data validated the alveolar macrophage model and indicated that miR-132 might be involved in inflammatory regulation of these cells.

The expression of ACh mRNA increased at 3 hours after LPS stimulation, reaching a plateau at a higher level at 6 hours and sustaining until 24 hours (Figure 1E). The intracellular level of AChE protein (Figure 1F, 1G) and AChE activity in supernatants (Figure 1H) increased at 12 hours after LPS stimulation, but reduced at 24 hours showing an inverse pattern to AChE mRNA. These results suggested that AChE might be involved in the inflammatory regulation of alveolar macrophages and be regulated at posttranscriptional level.

ACh-Mediated Anti-Inflammatory Reaction in LPS-Treated Alveolar Macrophages

To determine the minimum effective dose of ACh required for an anti-inflammatory effect in LPS-treated alveolar macrophages, we evaluated the dose-dependent effect of ACh on supernatant levels of proinflammatory cytokines. Compared with the macrophages stimulated with LPS for 12 hours in the absence of ACh, a minimum concentration of 10 μM ACh was required for significant repression of LPS-induced supernatant levels of TNF-α, IL-1β, and IL-6 (Figure 2A-2C), and was, therefore, chosen for the experiments. To corroborate the involvement of ACh in modulating the inflammatory reaction of alveolar macrophages, we found that the acetylcholinesterase inhibitor physostigmine (Phy, 1 mM) substantially enhanced the ACh-mediated anti-inflammatory reaction (Figure 2D).

miR-132 Enhances ACh-Mediated Anti-Inflammatory Reaction in LPS-Stimulated Alveolar Macrophages

We further characterized the role of miR-132 in LPS-induced inflammatory reaction in NR8383 alveolar macrophages using a miR-132 mimic. An initial concentration optimization experiment in which alveolar macrophages were transfected with fluorescein amidite (FAM)-labeled mimic negative control (20 and 50 nM). (Figure 3A) established

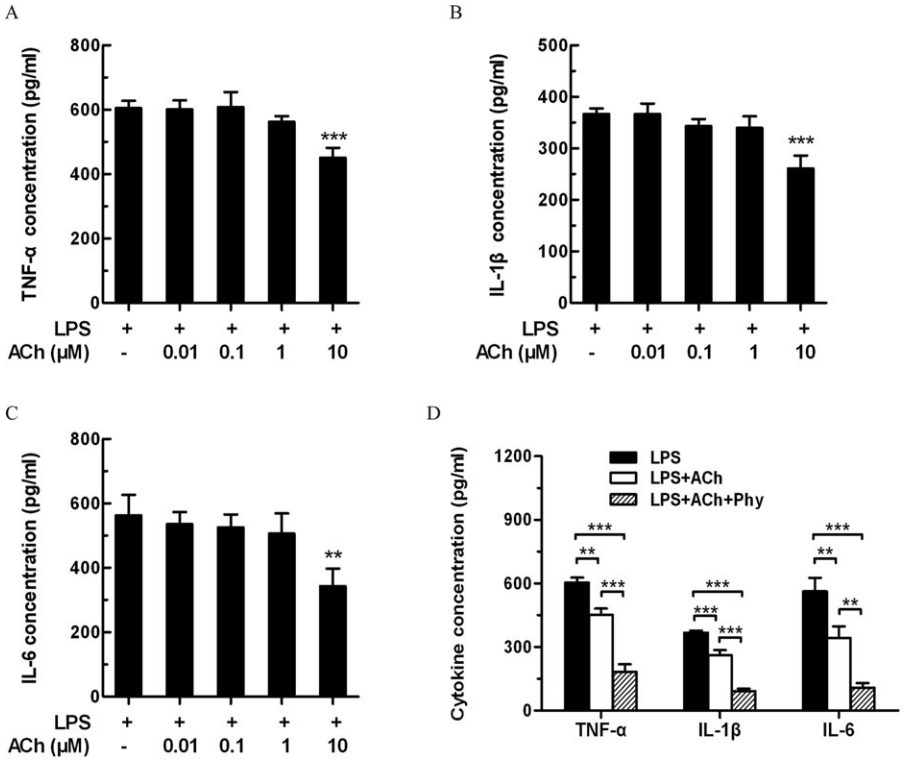

FIGURE 2. ACh-mediated anti-inflammatory effect on LPS (1 μg/mL)-stimulated alveolar macrophages. A–C. ELISA for TNF-α (A), IL-1β (B), and IL-6 (C) in the culture supernatants 12 hours after LPS exposure to ACh-pretreated alveolar macrophages compared to the group with LPS treatment alone. D. ELISA for TNF-α, IL-1β, and IL-6 in the supernatants of alveolar macrophages treated for 12 hours with LPS and with ACh (10 μM, 5 minutes) pretreatment or with ACh (10 μM, 5 minutes) pretreatment plus Phy (1 mM, 5 minutes). Data are expressed as mean $\pm$ SD from three independent experiments. $^{**}P < .01$, $^{***}P < .001$.

50 nM as the optimal concentration for transfection. Next, RT-qPCR analysis of alveolar macrophages transfected with 50 nM miR-132 mimic for 24 hours indicated induction (21.54 $\pm$ 2.14) of miR-132 relative to the mimic negative control (Figure 3B). Although no significant difference was observed in the levels of TNF-α, IL-1β, and IL-6 in the culture supernatant between miR-132 mimic-transfected and mimic-NC-transfected macrophages challenged by LPS for 12 hours. With pretreatment of the same cells with 10 μM ACh prior to LPS treatment, overexpression of miR-132 resulted in significant repression of the levels of TNF-α, IL-1β, and IL-6 relative to mimic NC-treated cells (Figure 3C–3E). Through a concentration optimization experiment in which alveolar macrophages were transfected with inhibitor FAM NC (20, 50, and 100 nM), 100 nM was confirmed as the optimal concentration for the following transfection (Figure 3F). When blocking miR-132 with miR-132 inhibitor, Ach-induced decreased levels of TNF-α, IL-1β, and IL-6 were reversed compared with inhibitor NC transfected cells (Figure 3G–3I). Together these results indicated that miR-132 modulated the ACh-mediated anti-inflammatory response of LPS-treated alveolar macr-ophages.

miR-132 Regulates AChE Expression Posttranscriptionally and Suppresses Activation of NF-κB and STAT3

Next, we evaluated the effect of transfected miR-132 mimic or inhibitor on AChE mRNA, protein, and activity in alveolar macrophages. While miR-132 overexpression or blocking had no effect on AChE mRNA (Figure 4A), AChE protein level and activity were significantly reduced by miR-132 mimic transfection compared with mimic NC-treated cells or increased by miR-132 inhibitor transfection compared with inhibitor NC-treated cells. (Figure 4B–4D).

Based on the current consensus that ACh modulates production of proinflammatory cytokines in immunocytes via the NF-κB and JAK2/STAT3

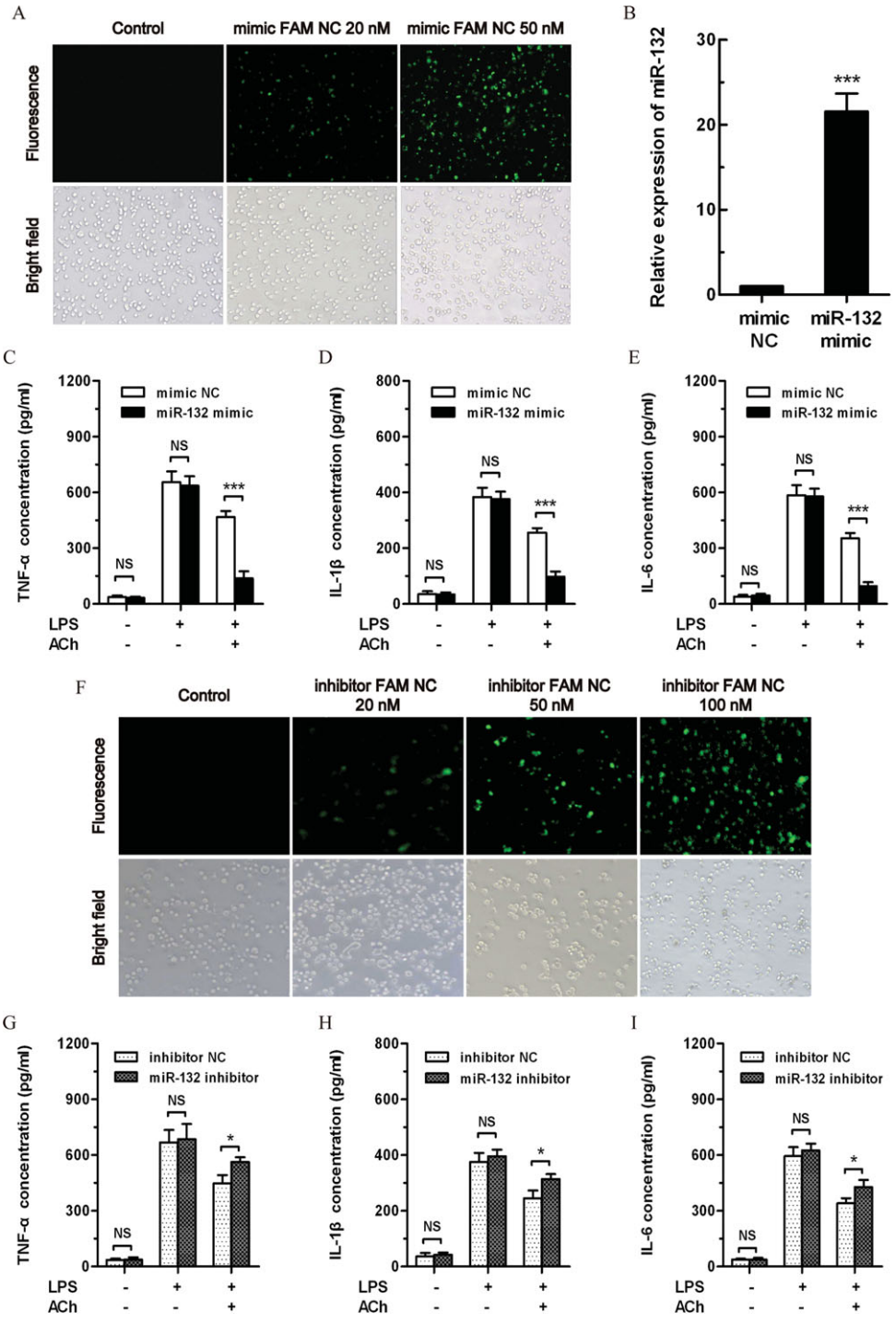

FIGURE 3. miR-132 enhances the ACh-mediated anti-inflammatory response in LPS (1 μg/mL)-stimulated alveolar macrophages. Fluorescence microscopy analysis of transfection efficiencies 5 hours after transfection with green FAM-labeled minic negative control (mimic FAM NC) (20 and 50 nM) compared with the control group (A) or FAM-labeled inhibitor negative control (inhibitor FAM NC) (20, 50, and 100 nM) compared with the control group (F). B. RT-qPCR analysis of miR-132 24 hours levels in miR-132 mimic-transfected and mimic negative control (mimic NC)-transfected alveolar macrophages. ELISA detection of TNF-α (C, G), IL-1β (D, H), and IL-6 (E, I) in the supernatants 12 hours after LPS exposure to alveolar macrophages transfected with miR-132 mimic (C-E) or inhibitor (G-H) for 24 hours in the presence or absence of ACh (10 μM, 5 minutes) pretreatment. Data are expressed as mean $\pm$ SD from three independent experiments. $^{*}P < .05$, $^{***}P < .001$, NS: not significant ($P > .05$).

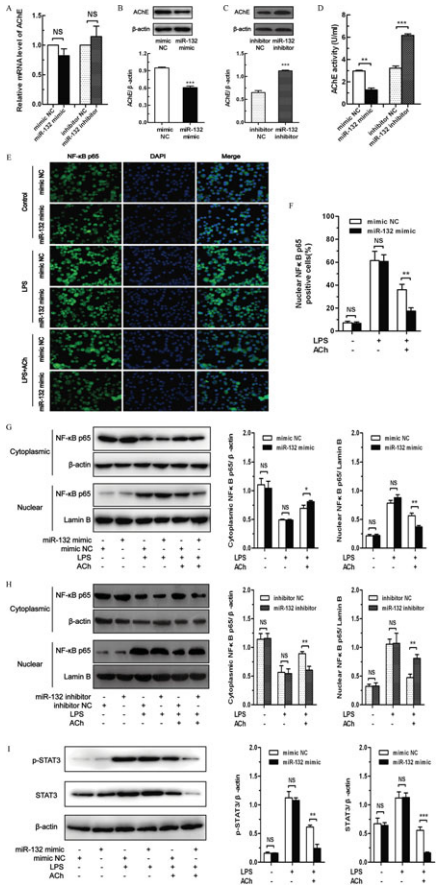

FIGURE 4. Mechanistic basis of miR-132 regulation of LPS-induced inflammatory reaction in alveolar macrophages. A. RT-qPCR detection of the relative expression of AChE mRNA 24 hours after transfection of alveolar macrophages with miR-132 mimic or inhibitor. B and C. Western blot for AChE protein 24 hours after transfection of alveolar macrophages with miR-132 mimic (B) and miR-132 inhibitor (C), normalized to β-actin. D. AChE activity in the supernatants 24 hours after transfection of alveolar macrophages with miR-132 mimic or inhibitor. E. Immunofluorescence detection of NF-κB p65 (green) nuclear translocation 12 hours after LPS exposure to alveolar macrophages transfected with miR-132 mimic for 24 hours in the presence or absence of ACh (10 μM, 5 minutes) pretreatment ($\times$400). Cell nuclei were stained by DAPI (blue). F. Quantitative analysis for (E). NF-κB p65 (green) nuclear translocation was expressed as the percentage of nuclear NF-κB p65 positive relative to total cells. G and H. Western blot detection of nuclear and cytoplasmic NF-κB p65 levels. I. Western blot detection of STAT3 and p-STAT3 proteins. Data are expressed as mean $\pm$ SD from three independent experiments. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, NS: not significant ($P > .05$).

pathways, as well as our observed regulation by miR-132 of this process in alveolar macrophages, we next asked whether miR-132 exerted its effect via these pathways. Immunofluorescence and Western analysis showed that miR-132 mimic suppressed LPS-induced NF-κB nuclear translocation (Figure 4E–4G) and STAT3 and p-STAT3 production (Figure 4I) in ACh-treated alveolar macrophages, but not untreated cells, while miR-132 inhibitor enhanced LPS-induced NF-κB nuclear translocation in ACh-treated alveolar macrophages. These results suggested that miR-132 targeted AChE mRNA to suppress the NF-κB and STAT3-mediated inflammatory reaction in alveolar macrophages.

DISCUSSION

In the present study, we have demonstrated miR-132 enhances the ACh-mediated anti-inflammatory response in LPS-stimulated alveolar macrophages, and have shown that this process involves inhibition of the NF-κB and STAT3 pathways.

Consistent with a previous report [11], we found that that levels of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 increased in tandem with miR-132 in short-term LPS-induced rat alveolar NR8383 macrophages. However, whereas levels of proinflammatory cytokines decreased in response to prolonged LPS stimulation, those of miR-132 continued to increase. These data indicated to us that miR-132 was involved in regulation of the inflammatory reaction in alveolar macrophages. In support of this notion, a variety of previous studies have implicated microRNAs in regulation of the inflammatory response. For example, miR-132 has been shown to be induced in LPS-stimulated human acute monocytic leukemia THP-1 cells [13], peptidoglycan/Toll-like receptor 2-stimulated THP-1 monocytes, human peripheral blood mononuclear cells, primary macrophages [14], and IBD [12], suggestive of a close relationship between miR-132 and the inflammation response.

The absence of a significant effect on the LPS-induced inflammatory reaction in miR-132 mimic-transfected NR8383 alveolar macrophages may be ascribed to the absence of certain *in vivo* substrates in the *in vitro* model. It was only in ACh-pretreated cells that an anti-inflammatory effect of miR-132 was observed, suggesting that AChE is involved in the inflammatory regulation of alveolar macrophages, and is regulated by miR-132 at the posttranscriptional level. Consistent with our findings, miR-132 has been shown to downregulate

AChE levels by targeting the 3'UTR of AChE mRNA, and AChE is highly expressed in neuron, immunocytes and macrophages [15]. Moreover, ACh has been shown to suppress production of proinflammatory cytokines via the macrophage-expressed α7 nAChR, establishing a mechanistic basis for the cholinergic anti-inflammatory pathway [16–18].

Since ACh is released by the vagus nerve *in vivo* and was likely deficient in our *in vitro* model, we pretreated NR8383 cells with ACh prior to LPS challenge. We found that ACh suppression of LPS-induced proinflammatory cytokine levels was attenuated by the acetylcholinesterase inhibitor Phy and by miR-132 overexpression. We propose therefore that miR-132 exerts its cholinergic anti-inflammatory effects by targeting AChE to inhibit hydrolysis of ACh, a model that will be verified by future *in vivo* experiments.

Activation of NF-κB has been shown to boost production of proinflammatory cytokines such as TNF-α [19]. We found that miR-132 enhancement of the suppressive effects of ACh involved inhibition of LPS-induced nuclear translocation of NF-κB and production of STAT3 and p-STAT3. Consistent with our own results, previous studies have shown that the cholinergic agonist nicotine suppresses activation of NF-κB, resulting in repression of proinflammatory cytokines in macrophages via activation of α7nAChR [9, 20]. Studies regarding the JAK2/STAT3 signaling pathway, however, are controversial. Some studies, aligned with our own data, have shown that LPS-induced STAT3 phosphorylation in RAW 264.7 cells resulted in production of interleukin 1 beta (IL-1beta) and interleukin-6 (IL-6); whereas nicotine suppressed production of proinflammatory cytokines by downregulation of STAT3 and p-STAT3 levels [21, 22]. On the contrary, de Jonge *et. al.* demonstrated that nicotine upregulated the expression of p-STAT3 and suppressor of cytokine signaling 3 (SOCS3) in LPS-stimulated primary peritoneal macrophages in a dose-dependent manner [23]. We presume that this discrepancy may arise from the interaction of multiple proinflammatory cytokines in these different studies.

In conclusion, we have shown that induction of miR-132 in LPS-stimulated alveolar macrophages enhances the ACh-mediated cholinergic anti-inflammatory reaction, and involves inhibition of NF-κB and STAT3 activation. We propose that miR-132 is a negative regulator of the inflammatory response in alveolar macrophages and, as such, represents a potential therapeutic leverage point in inflammatory diseases.

Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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REFERENCES

[1] Guirgis FW, Khadpe JD, Kuntz GM, Wears RL, Kalynych CJ, Jones AE: Persistent organ dysfunction after severe sepsis: A systematic review. J Crit Care. 2014;29:320–326.

[2] Matthay MA, Ware LB, Zimmerman GA: The acute respiratory distress syndrome. J Clin Invest. 2012;122:2731–2740.

[3] Crimi E, Slutsky AS: Inflammation and the acute respiratory distress syndrome. Best Pract Res Clin Anaesthesiol. 2004;18:477–492.

[4] Maus UA, Koay MA, Delbeck T, Mack M, Ermert M, Ermert L, Blackwell TS, Christman JW, Schlöndorff D, Seeger W, Lohmeyer J: Role of resident alveolar macrophages in leukocyte traffic into the alveolar air space of intact mice. Am J Physiol Lung Cell Mol Physiol. 2002;282:L1245-L1252.

[5] Morris PE, Zeno B, Bernard AC, Huang X, Das S, Edeki T, Simonson SG, Bernard GR: A placebo-controlled, double-blind, dose-escalation study to assess the safety, tolerability and pharmacokinetics/pharmacodynamics of single and multiple intravenous infusions of AZD9773 in patients with severe sepsis and septic shock. Crit Care. 2012;16:R31.

[6] Fisher CJ Jr, Agosti JM, Opal SM, Lowry SF, Balk RA, Sadoff JC, Abraham E, Schein RM, Benjamin E: Treatment of septic shock with the tumor necrosis factor receptor:Fc fusion protein. The Soluble TNF Receptor Sepsis Study Group. N Engl J Med. 1996;334:1697–1702.

[7] Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, Li JH, Yang H, Ulloa L, Al-Abed Y, Czura CJ, Tracey KJ: Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. Nature. 2003;421:384–388.

[8] Tracey KJ: Physiology and immunology of the cholinergic anti-inflammatory pathway. J Clin Invest. 2007;117:289–296.

[9] Yoshikawa H, Kurokawa M, Ozaki N, Nara K, Atou K, Takada E, Kamochi H, Suzuki N: Nicotine inhibits the production of proinflammatory mediators in human monocytes by suppression of I-kappaB phosphorylation and nuclear factor-kappaB transcriptional activity through nicotinic acetylcholine receptor alpha7. Clin Exp Immunol. 2006;146:116–123.

[10] Metz CN, Tracey KJ: It takes nerve to dampen inflammation. Nat Immunol. 2005;6:756–757.

[11] Shaked I, Meerson A, Wolf Y, Avni R, Greenberg D, Gilboa-Geffen A, Soreq H: MicroRNA-132 potentiates cholinergic anti-inflammatory signaling by targeting acetylcholinesterase. Immunity. 2009;31:965–973.

[12] Maharshak N, Shenhar-Tsarfaty S, Aroyo N, Orpaz N, Guberman I, Canaani J, Halpern Z, Dotan I, Berliner S, Soreq H: MicroRNA-132 modulates cholinergic signaling and inflammation in human inflammatory bowel disease. Inflamm Bowel Dis. 2013;19:1346–1353.

[13] Taganov KD, Boldin MP, Chang KJ, Baltimore D: NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A. 2006;103:12481–12486.

[14] Nahid MA, Yao B, Dominguez-Gutierrez PR, Kesavalu L, Satoh M, Chan EK: Regulation of TLR2-mediated tolerance

and cross-tolerance through IRAK4 modulation by miR-132 and miR-212. J Immunol. 2013;190:1250–1263.
[15] Meshorer E, Soreq H: Virtues and woes of AChE alternative splicing in stress-related neuropathologies. Trends Neurosci. 2006;29:216–224.
[16] Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, Wang H, Abumrad N, Eaton JW, Tracey KJ: Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature. 2000;405:458–462.
[17] Su X, Matthay MA, Malik AB: Requisite role of the cholinergic alpha7 nicotinic acetylcholine receptor pathway in suppressing Gram-negative sepsis-induced acute lung inflammatory injury. J Immunol. 2010;184:401–410.
[18] Lv Y, Hu S, Lu J, Dong N, Liu Q, Du M, Zhang H: Upregulating nonneuronal cholinergic activity decreases TNF release from lipopolysaccharide-stimulated RAW264.7 cells. Mediators Inflamm. 2014;2014:Article ID 873728, 10 pages.
[19] Zingarelli B: Nuclear factor-kappaB. Crit Care Med. 2005;33:S414-S416.
[20] Saeed RW, Varma S, Peng-Nemeroff T, Sherry B, Balakhaneh D, Huston J, Tracey KJ, Al-Abed Y, Metz CN: Cholinergic stimulation blocks endothelial cell activation and leukocyte recruitment during inflammation. J Exp Med. 2005;201:1113–1123.
[21] Sun Y, Li Q, Gui H, Xu DP, Yang YL, Su DF, Liu X: MicroRNA-124 mediates the cholinergic anti-inflammatory action through inhibiting the production of pro-inflammatory cytokines. Cell Res. 2013;23:1270–1283.
[22] Samavati L, Rastogi R, Du W, Huttemann M, Fite A, Franchi L: STAT3 tyrosine phosphorylation is critical for interleukin 1 beta and interleukin-6 production in response to lipopolysaccharide and live bacteria. Mol Immunol. 2009;46:1867–1877.
[23] de Jonge WJ, van der Zanden EP, The FO, Bijlsma MF, van Westerloo DJ, Bennink RJ, Berthoud HR, Eumatsu S, Akira S, van den Wijngaard RM, Boeckxstaens GE: Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. Nat Immunol. 2005;6:844–851.