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MiR-155 enhances phagocytosis of alveolar macrophages through the mTORC2/RhoA pathway

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Abstract

Alveolar macrophage phagocytosis is significantly reduced in Chronic obstructive pulmonary disease, and cigarette smoke extract is one of the chief reasons for this decrease. Nevertheless, the specific underlying mechanism remains elusive. In this study, the role and possible mechanism of miR-155-5p/mTORC2/RhoA in the phagocytosis of mouse alveolar macrophages (MH-S) were explored. Our results revealed that cigarette smoke extract intervention reduced MH-S cell phagocytosis and miR-155-5p expression. Meanwhile, the dual-luciferase reporter assay validated that Rictor is a target of miR-155-5p. On the one hand, transfecting miR-155-5p mimic, mimic NC, miR-155-5p inhibitor, or inhibitor NC in MH-S cells overexpressing miR-155-5p increased the Alveolar macrophage phagocytotic rate, up-regulated the expression level of RhoA and p-RhoA, and down-regulated that of mTOR and Rictor mRNA and protein. On the other hand, inhibiting the expression of miR-155-5p lowered the phagocytotic rate, up-regulated the expression of mTOR, Rictor mRNA, and protein, and down-regulated the expression of RhoA and p-RhoA, which taken together, authenticated that miR-155-5p participates in macrophage phagocytosis via the mTORC2/RhoA pathway. Finally, confocal microscopy demonstrated that cells overexpressing miR-155-5p underwent cytoskeletal rearrangement during phagocytosis, and the phagocytic function of cells was enhanced, signaling that miR-155-5p participated in macrophage skeletal rearrangement and enhanced alveolar macrophage phagocytosis by targeting the expression of Rictor in the mTORC2/RhoA pathway.

Abbreviations: 3′UTR = 3′untranslated region, AM = alveolar macrophage, COPD = chronic obstructive pulmonary disease, CSE = cigarette smoke extract, FITC = fluorescein isothiocyanate, miRNA = microRNA, mTOR = mammalian rapamycin target protein, qRT-PCR = quantitative real time polymerase chain reaction.

Keywords: alveolar macrophage, cytoskeleton, MICRORNA-155, mTORC2, phagocytosis

1. Introduction

Alveolar macrophages (AM) are the most abundant immune cells in the lung and play a decisive role in host defense, tissue repair, and pulmonary homeostasis.[1] AM dysfunction has been reported to be implicated in the pathogenesis of chronic obstructive pulmonary disease (COPD).[2–4] Cigarette smoke (CS) is the primary risk factor of COPD and can trigger lung inflammatory cascade reactions. Indeed, exposure to CS or cigarette smoke extract (CSE) changes the phenotype and function of AM, inhibits its exocytosis activity, and significantly reduces the phagocytotic rate.[2,5] So far, the exact mechanism underlying CSE leading to the decreased phagocytic function of macrophages remains poorly understood.

MicroRNA (miRNA) is an endogenous non-coding small RNA molecule (18–22 nucleotides) that combines with complementary sequences in mRNA and governs various cell functions through translation inhibition or mRNA degradation, eventually leading to target gene silencing. It modulates various aspects of macrophage biology, including macrophage development, polarization, plasticity, and phagocytic function.[6,7] Similarly, the activation of inflammatory pathways, oxidative stress, and phagocytic signal cascade can also mediate the miRNA spectrum of phagocytes such as macrophages. Regarding the role of macrophages in infection clearance, it has been established that Toll-like receptor signaling triggers different expressions of various miRNAs and different expressions of miRNA target genes.[8] However, reports on individual miRNAs and their effects on phagocytosis are currently limited. The overexpression of miR-155 elevates the phagocytosis of macrophages,[9,10] but the underlying mechanism is still unclear.

The phagocytosis process of AM is very complex, and AM dynamically alters its actin cytoskeleton to drive them to migrate and phagocyte.[11] Studies have confirmed that the

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The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

The experimental process did not involve animal, human and human samples, human embryonic stem cells; it was deemed negligible-risk research and was exempt from ethical review by the Ethics Committee of The First Hospital of Lanzhou University, thus, Ethical approval was not required.

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change in the phagocytotic rate of macrophages is related to cytoskeleton rearrangement.[12] Mammalian rapamycin target protein (mTOR) is an evolutionarily conserved serine/threonine kinase of the phosphatidylinositol-3 kinase family. There are 2 known complexes, mTORC1, and mTORC2, with unique structures and functions. The main function of the former is to regulate protein synthesis and cell cycle, while the latter is a rapamycin-insensitive complex that plays an instrumental role in regulating actin and cytoskeleton.[13,14] The basic components of mTORC2 include mTOR, mammalian lethal SEC13 protein 8 (mLST8), mTOR interacting protein containing DEP domain, and Tel2 interacting protein 1, and its specific core subunit is referred to as rapamycin-insensitive mTOR chaperone (Rictor).[15] mTORC2 inactivation can boost the efficiency and rate of phagocytosis.[16] Rictor is a main component of mTORC2, and thus its deletion leads to the selective destruction of mTORC2.[17] Besides, Rictor triggers the generation of mTORC2 and induces the activation of mTORC2 target molecules. Meanwhile, activated mTORC2 also regulates actin skeleton assembly and participates in the phagocytosis of macrophages through RhoA and Rac-1.[18] Consequently, mTORC2 deficiency destroys the polarization of the actin cytoskeleton and controls the actin cytoskeleton by activating Rho GTPase.[19] The activated RhoA can subsequently stimulate the formation of stress fibers and the extension of pseudopodia and promote cell deformation,[20] while a decrease in RhoA and Rac1 activities may impede the rearrangement of the actin cytoskeleton.[18] Macrophage phagocytosis dysfunction is speculated to be related to the regulation of the cytoskeleton by mTORC2/RhoA. Therefore, this study aimed to explore the role of miR-155-5p in macrophage phagocytosis. Considering that Rictor is the target gene of miR-155-5p, it can be inferred that miR-155-5p may promote the phagocytosis of MH-S intervened by CSE by targeting Rictor in mTORC2, thereby providing a new idea and insights for improving the phagocytosis of AM in patients with chronic obstructive pulmonary disease.

2. Methods

2.1. Cell culture and treatment

Mouse alveolar macrophage cells (MH-S) (BeNa Culture Collection, China) were cultured in RPMI1640 containing 10% fetal bovine serum and supplemented with 1% penicillin/streptomycin at 37°C in an incubator with 5% CO2.

2.2. CSE preparation

CSE was prepared following the methods of Li et al[21] One cigarette without filter (Lanzhou Brand; Gansu Tobacco Industry Co, Ltd, China) was aspirated using a vacuum pump. The smoke was drawn into 50 mL of PBS for 3 to 5 minutes. Then, the CSE solution pH was adjusted to 7.4 and sterilized through a 0.22 μm millipore needle filter and considered 100% CSE. CSE (100%) was diluted to the desired concentrations with a culture medium for the ensuing experiments and used within 30 minutes of preparation. The optical densities of CSE solutions prepared in this experiment were constant.

2.3. Cell viability assay

MH-S cells were seeded in 96-well plates at a density of 2×10^4 cells/well. The cells were exposed to 0, 2.5, 5, 7.5, and 10% CSE for 24 hours or 5% CSE for 0, 6, 12, 24, and 48 hours. Then, MTT reagent (Sigma-Aldrich, USA) was added, and the solution was incubated at 37°C for 4 hours in the dark. Next, the supernatant was discarded, and 150 μL DMSO was added to each well. A microplate reader (Synergy H1, Biotek, VT) was employed to measure the absorbance at 490 nm. Cell survival rate was calculated as follows: Cell survival rate = [(ODtreatment – ODblank)/(ODcontrol – ODblank)] × 100%.

2.4. Cell transfection and dual-luciferase reporter assay

According to the manufacturer's instructions, MH-S cells were seeded in 96-well plates at a density of 2×10^4 cells/well. 6-carboxyfluorescein (FAM)-labeled negative control (NC) (GenePharma, Shanghai, China) were transfected into MH-S cells using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) for 24 hours, and the transfection efficiency was subsequently observed under a fluorescence microscope (Olympus, Tokyo, Japan). Transfection efficiency was assessed by counting the number of cells transfected with FAM-labeled NC compared to the total number of cells (Number of cells transfected/Total cells in the sample × 100%). The binding sites of miR-155-5p and Rictor were predicted using TargetScan. The wild type (wt) or mutant (mut) 3′untranslated region (3′UTR) sequence of Rictor was inserted into the GP-miRGLO vector (GenePharma). Thereafter, the Rictor-3′UTR-wt vector or Rictor-3′UTR-mut vector was co-transfected with either miR-155-5p mimic or mimic NC into MH-S cells using the Lipofectamine 2000 reagent. After 24 hours, the transfected cells were lysed, and luciferase activity was measured using a dual-luciferase reporter assay system (Promega, Fitchburg, WI). Using the same method, miR-155-5p mimic/mimic NC/inhibitor/inhibitor NC (GenePharma) were transfected into MH-S cells. Further experiments were conducted 24 hours after transfection. The expression of miR-155-5p was determined by quantitative real-time polymerase chain reaction (qRT-PCR).

2.5. AM phagocytosis was detected by flow cytometry

The MH-S cells (1×10^5 cells/well) were incubated with 0.04 mg/mL fluorescein isothiocyanate (FITC)-labeled *E coli* (Invitrogen) in the dark for 6 hours. Each well was then supplemented with 4% trypan blue (Invitrogen) to quench the extracellular fluorescence of the FITC-labeled *E coli*. The mean fluorescence intensity (MFI) and percentage of phagocytic cells positive for FITC-labeled *E coli* (percent phagocytosis) were evaluated by flow cytometry (Becton Dickinson Co., USA). A higher MFI and percent phagocytosis indicated a greater phagocytic capacity.

2.6. qRT-PCR assay

Total RNA was extracted from MH-S cells using RNAiso Plus reagent (Takara, Dalian, China). The RNA was reverse transcribed into cDNA using PrimeScript™ RT reagent Kit or MirX™ miRNA First-Strand Synthesis Kit (Takara). Primers for mRNA and miRNA were acquired from Accurate Biotechnology (Hunan, China). The primers used are listed in Table 1. qRT-PCR was performed using the SYBR® Premix Ex Taq™ II kit (Takara) and an Mx3000P Real-Time Thermal Cycler (Agilent, Santa Clara, CA). The relative mRNA or miRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH or U6, respectively.

2.7. Western blot analysis

Total protein was extracted from MH-S cells using RIPA lysis buffer (Solarbio, Beijing, China) and centrifuged at 12,000 × g for 15 minutes at 4°C. Protein concentrations were determined by the Bicinchoninic acid method. Each protein sample (40 μg) was resolved by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked with 5% BSA for 1 hour and subsequently incubated overnight at 4°C with primary

antibodies against mTOR (BOSTER, Wuhan, China) at 1:1000 dilution, Rictor (Signalway Antibody, College Park, MD) at 1:1000 dilution, RhoA (Abcam, UK) at 1:5000 dilution, as well as Phospho-RhoA (p-RhoA) (Signalway Antibody) at 1:1000 dilution and β-actin (Signalway Antibody) at 1:2000 dilution. After overnight incubation, the membranes were washed with TBST buffer and incubated with goat anti-rabbit IgG (Signalway Antibody) for 2 hours at 1:5000 dilution at room temperature. Finally, blots were quantified using an enhanced chemiluminescence kit, and β-actin was used as an internal control.

2.8. Visualization of cytoskeletons by the confocal laser scanning microscope

The cells in different groups were inoculated onto coverslips in a 12-well plate (3×10^5 cells/well) and incubated in the dark for 6 hours with 400 μL/well of FITC-labeled *E coli* (final concentration: 0.04 mg/mL). Each well was then supplemented with 200 μL of 4% trypan blue (Invitrogen) to quench the extracellular fluorescence of FITC-labeled *E coli* for 1 minute. Following this, the cells were fixed with 4% paraformaldehyde (Solarbio) for 30 minutes and washed 3 times with PBS prior to being stained with 400 μL/well of Tetramethylrhodamine isothiocyanate-labeled phalloidin (final concentration: 100 nM) (Yeasen, Shanghai, China.) at room temperature for 1 hour. Lastly, the cells were washed thrice with PBS and mounted for subsequent visualization and imaging under the confocal laser scanning microscope (A1R + Ti2-E, Nikon, Japan) in a 2-line laser launch (488 nm and 561 nm laser lines).

Table 1

Primer sequences.

Gene	Primer sequence(5′–3′)
mTOR	Forward: ACCCTCCATCCACCTCAT
	Reverse: TGCCAAGACACAGTAGCG
Rictor	Forward: GGCTTGGTCGAGGTGATA
	Reverse: TGGCTATGCGGAAGAGG
RhoA	Forward: ATTTGGTTTCCCGCCTGAG
	Reverse: CCATAAGAACTGGTGGCTCCTC
GAPDH	Forward: TGTGTCCGTCGTGGATCTGA
	Reverse: TTGCTGTTGAAGTCGCAGGAG
miR-155-5p	TTAATGCTAATTGTGATAGGGGT
U6	Forward: GGAACGATACAGAGAAGATTAGC
	Reverse: TGGAACGCTTCACGAATTTGCG

2.9. Statistical analysis

Data were analyzed using the SPSS 21.0 and GraphPad Prism 9.0 software. All experiments were performed in at least 3 independent assays, and the data were presented as mean ± standard deviation. One-way ANOVA was used to compare mean data among multiple groups, and a post hoc LSD test was used for inter-group comparisons. $P < .05$ was considered statistically significant.

3. Results

3.1. CSE exposure inhibited MH-S cell viability

To evaluate the effect of CSE exposure on cell viability, MH-S cells were exposed to 0%, 2.5%, 5%, 7.5%, and 10% CSE for 24 hours or 5% CSE for 0, 6, 12, 24, and 48 hours. The MTT assay indicated that cell viability was decreased in a dose- and time-dependent manner (Fig. 1). Interestingly, when the concentration of CSE reached 7.5% and 10%, or when cells were exposed to 5% CSE for 48 hours, the survival rate of MH-S cells was < 50%. A concentration of 5% CSE was used to treat cells for the following experiments, and experimental times were set at 24 hours, as determined by the aforementioned results.

3.2. CSE exposure decreased the phagocytotic ability and miR-155-5p level of MH-S cells

MH-S cells were exposed to 5% CSE for 24 hours, followed by measuring phagocytotic parameters and the expression level of miR-155-5p. As illustrated in Table 2 and Figure 2A, the phagocytotic ability of FITC-labeled *E coli* and the percentage of fluorescence in MH-S cells were decreased compared with the control group. The qRT-PCR assay demonstrated that CSE down-regulated the expression of miR-155-5p in MH-S cells (Fig. 2B). Contrastingly, CSE significantly up-regulated the expression of mTOR and Rictor mRNA and protein and significantly down-regulated that of RhoA and P-RhoA ($P < .05$) (Fig. 2C–E). Taken together, this implies that CSE may induce a decrease in phagocytosis of MH-S cells through the miRNA-155/mTORC2/RhoA pathway.

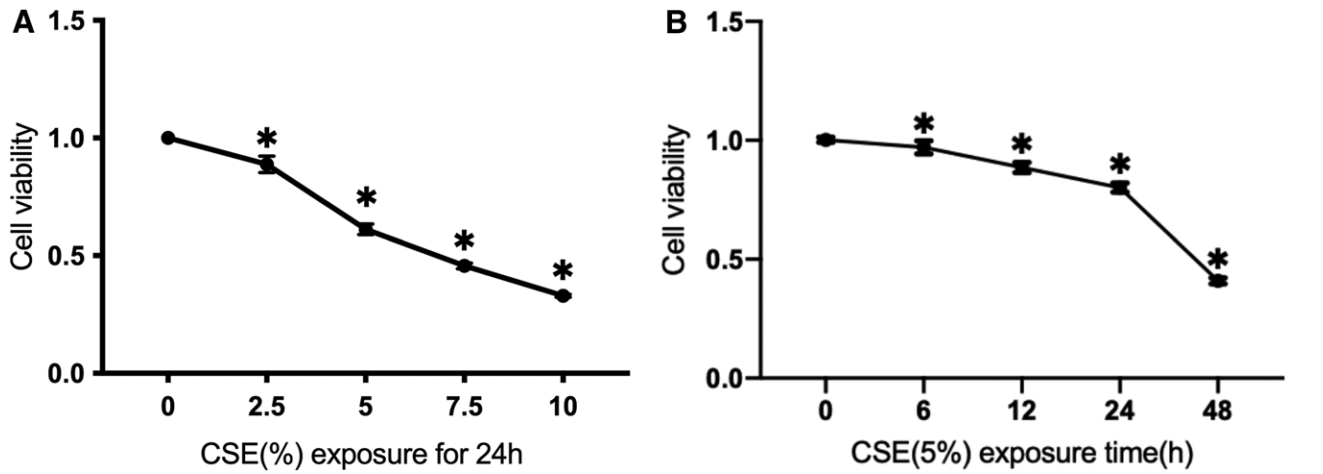

Figure 1. CSE inhibited MH-S cells viability. MH-S cells were treated with 0, 2.5, 5, 7.5 and 10% CSE for 24 hours (A) or 5% CSE for o, 6, 12, 24 and 48 hours (B). MTT assay was measured to evaluate the cell viability. Date were presented as means ± SD (n = 5). *$P < .05$ versus control. CSE = cigarette smoke extract, MTT = methyl thiazolyl tetrazolium.

3.3. Overexpression of miR-155-5p increased the phagocytosis of MH-S cells by targeting Rictor via the mTORC2/RhoA pathway

To clarify the probable functional connection between miR-155-5p and increasing phagocytic activity, predicted target genes of miR-155-5p were analyzed using TargetScan and MiRbase. As depicted in Figure 3A, miR-155-5p had sequences complementary to Rictor. The cytoskeleton is involved in macrophage phagocytosis, and mTORC2/RhoA plays a decisive role in this process. Therefore, further experiments were conducted to verify whether Rictor is a target of miR-155-5p and the role of miR-155-5p and Rictor in macrophage phagocytosis via the mTORC2/RhoA pathway.

Firstly, FAM-labeled NC was transfected into MH-S cells for 24 hours, and fluorescence imaging displayed that the transfection rate was over 80% (Fig. 3B). Secondly, a dual-luciferase reporter assay was performed to investigate the target of miR-155-5p (Fig. 3C). The fluorescence intensity decreased in cells co-transfected with the Rictor-3′UTR-wt vector and miR-155-5p mimic. However, there were no significant differences in fluorescence intensity in cells transfected with the Rictor-3′UTR-mut vector. This result implied that Rictor is a target of miR-155-5p. Finally, MH-S cells were transfected with miR-155-5p mimics, mimic NC, miR-155-5p inhibitor, and inhibitor NC for 24 hours. As displayed in Table 3 and Figure 4, compared with the control group, overexpression of miR-155-5p promoted phagocytosis and increased the percentage of phagocytic fluorescence intensity in MH-S cells, down-regulated the expression of mTOR and Rictor, and increased that of RhoA and p-RhoA. Conversely, inhibition of miR-155-5p expression decreased the phagocytic ability and the percentage of phagocytic fluorescence intensity of MH-S cells, up-regulated the expression of mTOR and Rictor, and down-regulated the expression of RhoA and p-RhoA. As expected, the phagocytic capacity, phagocytic fluorescence intensity percentage, and mTOR, Rictor, RhoA, and P-RhoA mRNA and protein expressions were not significantly increased or decreased in the

Table 2

Phagocytic capacity against FITC-labeled *E coli* in Control and CSE group (mean ± SD, n = 3).

Group	Phagocytosis (%)	MFI
Control	92.86 ± 0.92	41183 ± 350
CSE	71.63 ± 1.36**	21279 ± 404**
P value	<.01	<.01

CSE = cigarette smoke extract, MFI = mean fluorescence intensity.
***P* < .01 compared with the control.

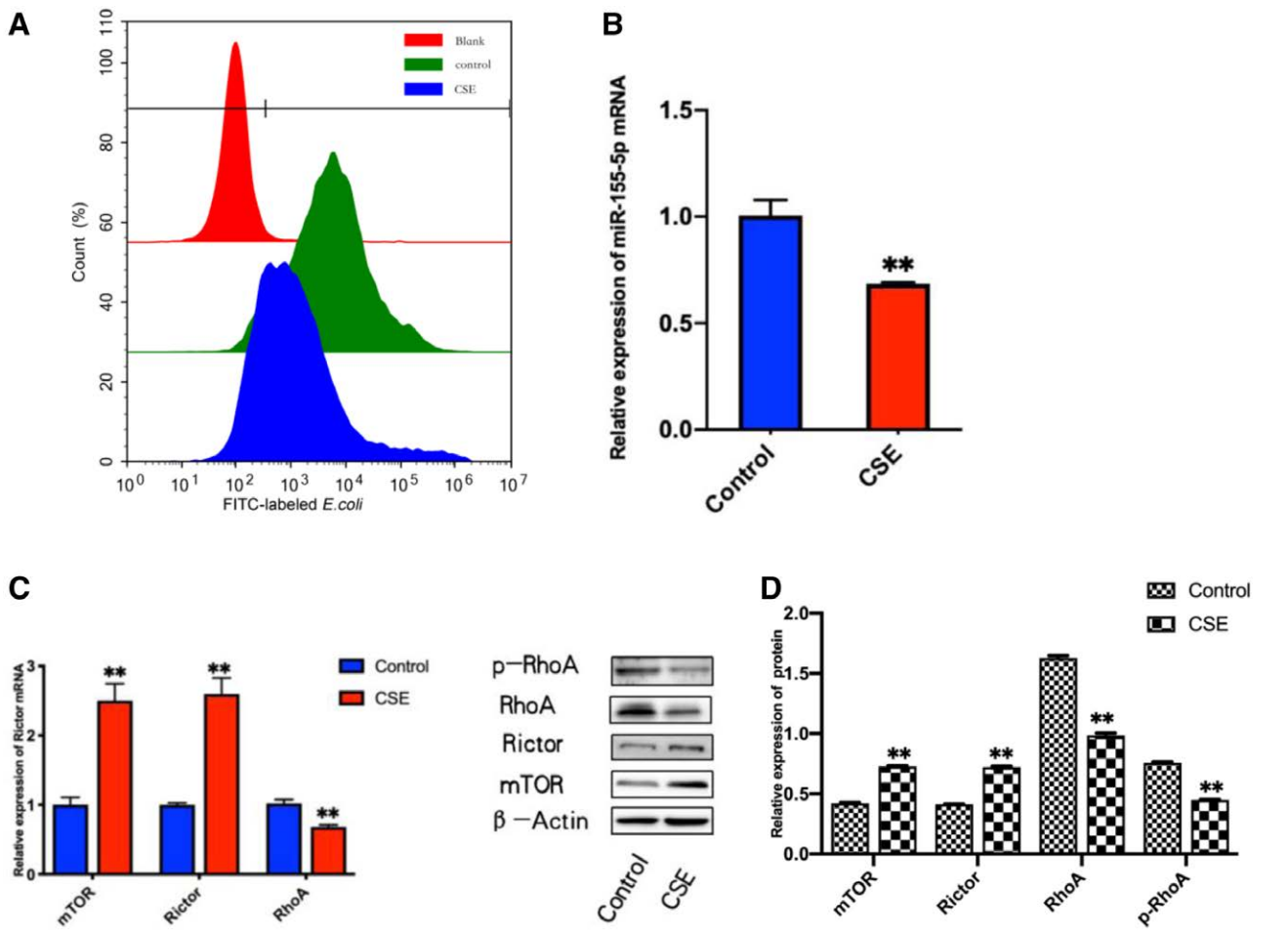

Figure 2. Effects of CSE on phagocytosis and miR-155-5p expression in MH-S cells. A: Phagocytosis of alveolar macrophages by Flow Cytometry. B and C: The expression of miR-155-5p, mTOR, Rictor, and RhoA were assessed by qRT-PCR. D and E: The protein expressions of mTOR, Rictor, RhoA, and p-RhoA were quantified by western blot. Data are presented as means ± SD (n = 3). ***P* < .01 versus control. Blank: Alveolar macrophages without FITC-labeled *E coli*. CSE = cigarette smoke extract, FITC = fluorescein isothiocyanate, miRNA = microRNA, mTOR = Mammalian rapamycin target protein, qRT-PCR = quantitative real time polymerase chain reaction.

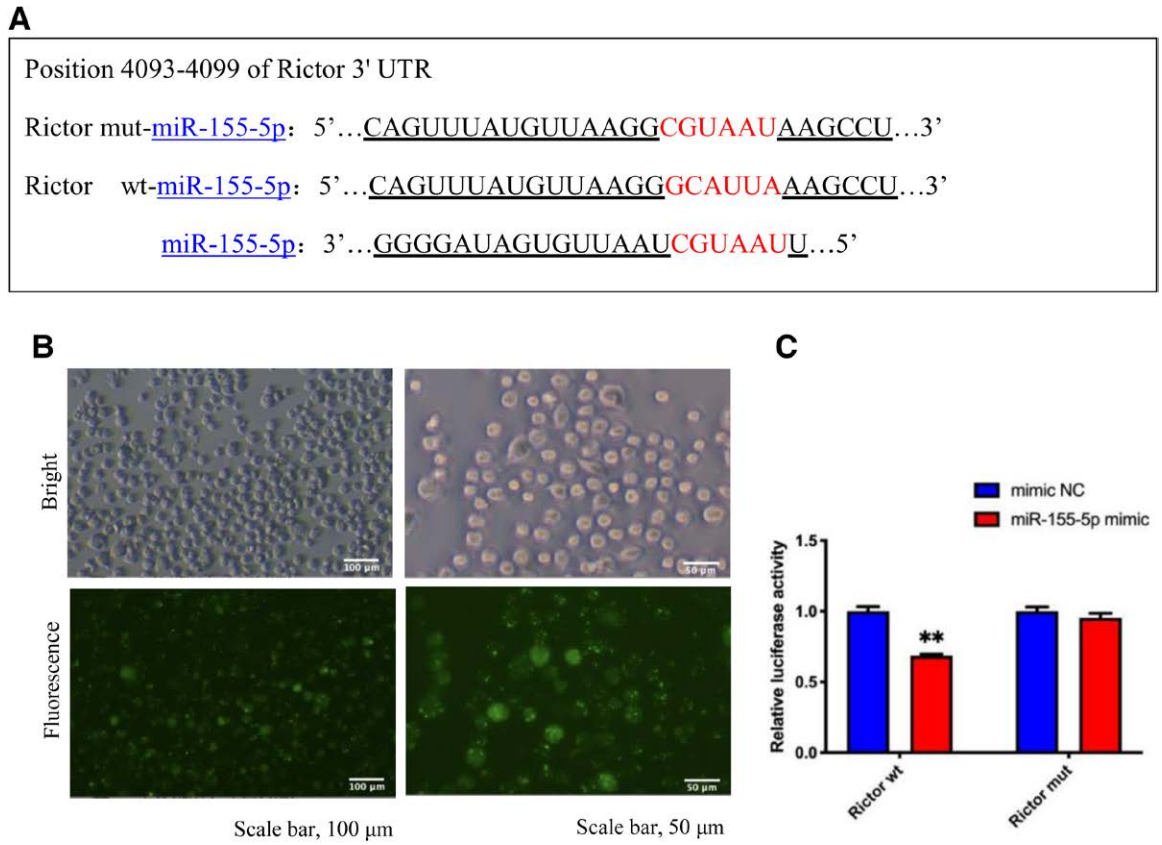

Figure 3. MiR-155-5p targets Rictor and its transfection efficiency. A: The binding sites between miR-155-5p and 3′UTR of Rictor. B: Transfection efficiency (×100 and ×200). FAM-labeled NC was transfected into MH-S cells for 24 hours, and the fluorescence microscope showed that the transfection rate was over 80%. C: Dual-luciferase reporter assay. Data are presented as means ± SD (n = 3). $^{**}P < .01$ versus mimic NC. Transfection efficiency = Number of cells transfected (Green fluorescence)/Total cells in the sample × 100%. Green fluorescence: FAM-labeled NC = 6-carboxyfluorescein-labeled negative control, mut = mutant, wt = wild.

Table 3

Phagocytic capacity against FITC-labeled *E coli* in each group (mean ± SD, n = 3).

Group	Phagocytosis (%)	MFI
Control	92.86 ± 0.92	41183 ± 350
miR-155-5p mimic	95.10 ± 0.67**	62853 ± 707**
mimic NC	91.66 ± 0.65	41241 ± 306
miR-155-5p inhibitor	84.70 ± 0.65**	34006 ± 940**
Inhibitor NC	91.78 ± 0.82	40068 ± 963
F value	80.82	721.68
P value	<.01	<.01

FITC = fluorescein isothiocyanate, MFI = mean fluorescence intensity.
$^{**}P < .01$ compared with the control.

mimic NC and inhibitor NC groups (P>.05). Taken together, the results established that miR-155-5p targeted Rictor to participate in macrophage phagocytosis through the mTORC2/RhoA pathway.

3.4. Cytoskeletal changes in different groups

In the control group, mimic NC group, and inhibitor NC group, alterations in cell morphology were evident. Cell membrane pseudopods protruded more and were denser, membrane folds were visible, and cells that phagocytized a larger number of FITC-labeled *E coli* were relatively larger in volume. Compared with the control group, cells in the CSE group did not exhibit substantial pseudopodia protrusion, and a lower number of FITC-labeled *E coli* was engulfed. In the miR-155-5p mimic group, pseudopodia protrusion was marginally more pronounced, elongated, and dense, with a higher proportion of phagocytosed FITC-labeled *E coli*. Lastly, cells in the miR-155-5p inhibitor group had fewer and shorter pseudopods that protruded and a relatively small amount of phagocytosed FITC-labeled *E coli* (Fig. 5).

4. Discussion

CS exposure is one of the most important risk factors for persistent airway inflammation and chronic obstructive pulmonary disease.[5] The AM function of smokers and patients with chronic obstructive pulmonary disease is impaired, which is characterized by macrophage phagocytosis dysfunction and the persistent existence of pathogens that conjointly promote the occurrence and development of chronic obstructive pulmonary disease.[22] Compared with previous studies, this study noted that CSE intervention suppressed macrophage phagocytosis. However, the specific mechanism remains unclear. Thus, it is critical and necessary to elucidate the mechanism of macrophage phagocytosis dysfunction induced by CS (or CSE).

MicroRNA disorders play a key role in the pathogenesis and development of COPD.[23,24] As is well documented, miRNAs are implicated in the regulation of the phagocytosis of macrophages,[25–29] and phagocytosis can induce a change in the miRNA spectrum of macrophages.[30] It has been found that miR-155 participates in the regulation of numerous cellular and biological processes, is the common target of various inflammatory mediators in macrophages, and engages in the regulation of

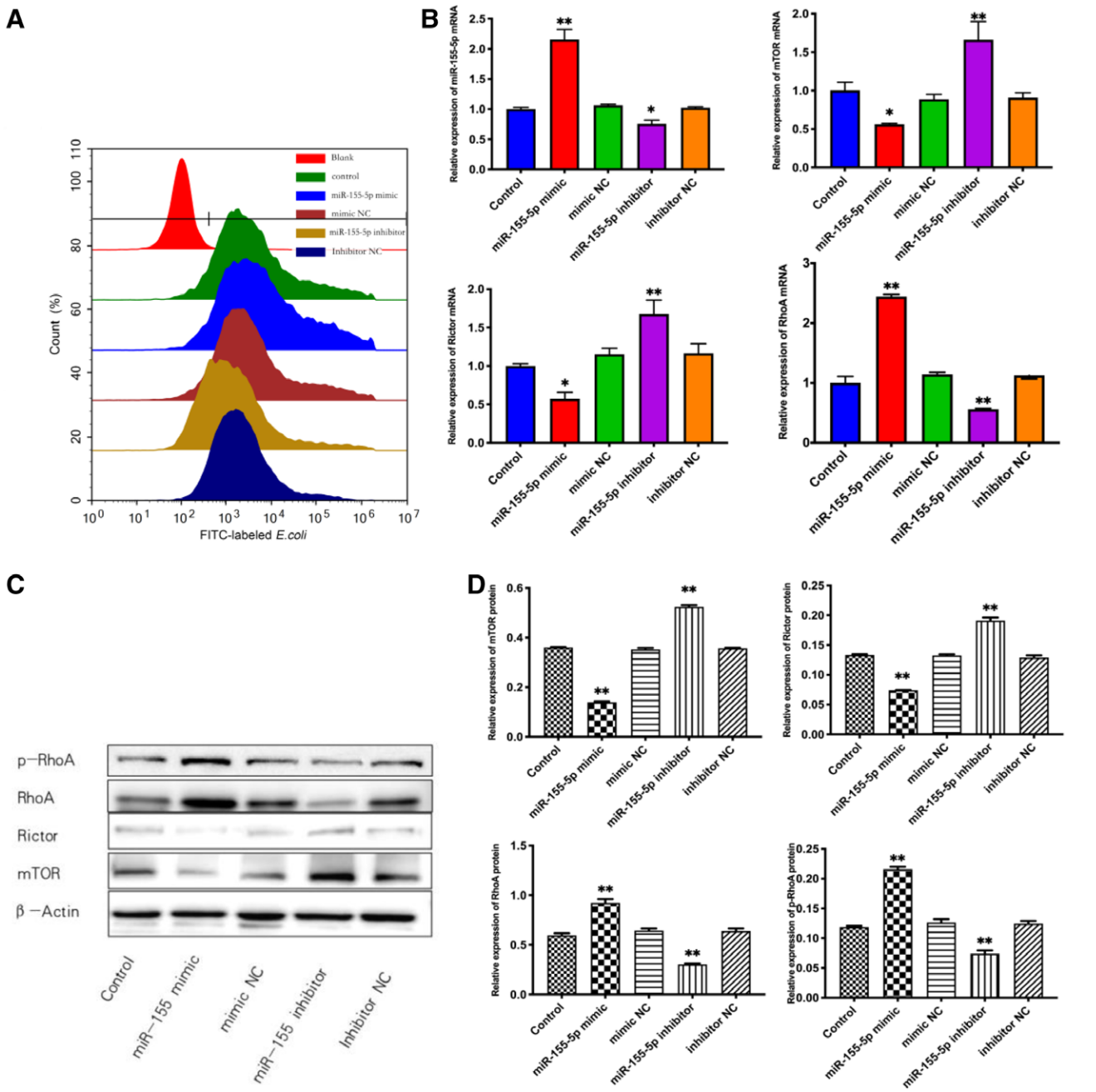

Figure 4. Effects of overexpression or inhibition of miR-155-5p on phagocytosis and related proteins in MH-S cells. A: Phagocytosis of alveolar macrophages by Flow Cytometry. B: The expression of miR-155-5p, mTOR, Rictor, and RhoA was assessed by qRT-PCR. C and D: The protein expressions of mTOR, Rictor, RhoA, and p-RhoA were quantified by western blot. Data are presented as means ± SD (n = 3). $^{**}P < .01$ versus control. Blank: Alveolar macrophages without FITC-labeled *E coli*. CSE = cigarette smoke extract, miRNA = microRNA, mTOR = Mammalian rapamycin target protein, qRT-PCR = quantitative real time polymerase chain reaction.

macrophage-related inflammatory reactions, such as phagocytosis, autophagy, lipid uptake, and polarization. Enhancing or inhibiting miR-155 depends on cell types or stimuli.[9] An earlier study described that different concentrations of PMA could upregulate the expression of miR-155 and increase the phagocytic activity of U937-derived cells in a dose-reversal manner, and NF-kB may be a key factor that governs the expression levels of the miR-155 following PMA treatment.[31] Additionally, miR-155 enhances the phagocytotic and bactericidal abilities of monocytes/macrophages, promotes M1 polarization, and inhibits M2 polarization. Overexpression of miR-155 significantly promoted the phagocytosis of MO/Mφ, while its inhibition reversed this activity.[32] Streptococcus pneumoniae endopeptidase O (PepO) induces up-regulation of miR-155 expression and enhancement of phagocytosis in peritoneal exudate macrophages, thereby decreasing phagocytosis in cells transfected with a miR-155 inhibitor and increasing phagocytosis in cells transfected with miR-155 mimics, which is helpful in enhancing phagocytosis and host defense responses to pneumococcus and Staphylococcus aureus.[33] More importantly, overexpression of miR-155 down-regulated the expression of its target gene CD47 in myeloma cells and promoted the phagocytosis of myeloma cells, thus exerting a tumor-suppressing effect.[34] The above-mentioned findings demonstrate that overexpression of miR-155 can increase macrophage phagocytosis. However, the underlying mechanism is poorly understood. Herein, the expression of miR-155-5p in the AM group

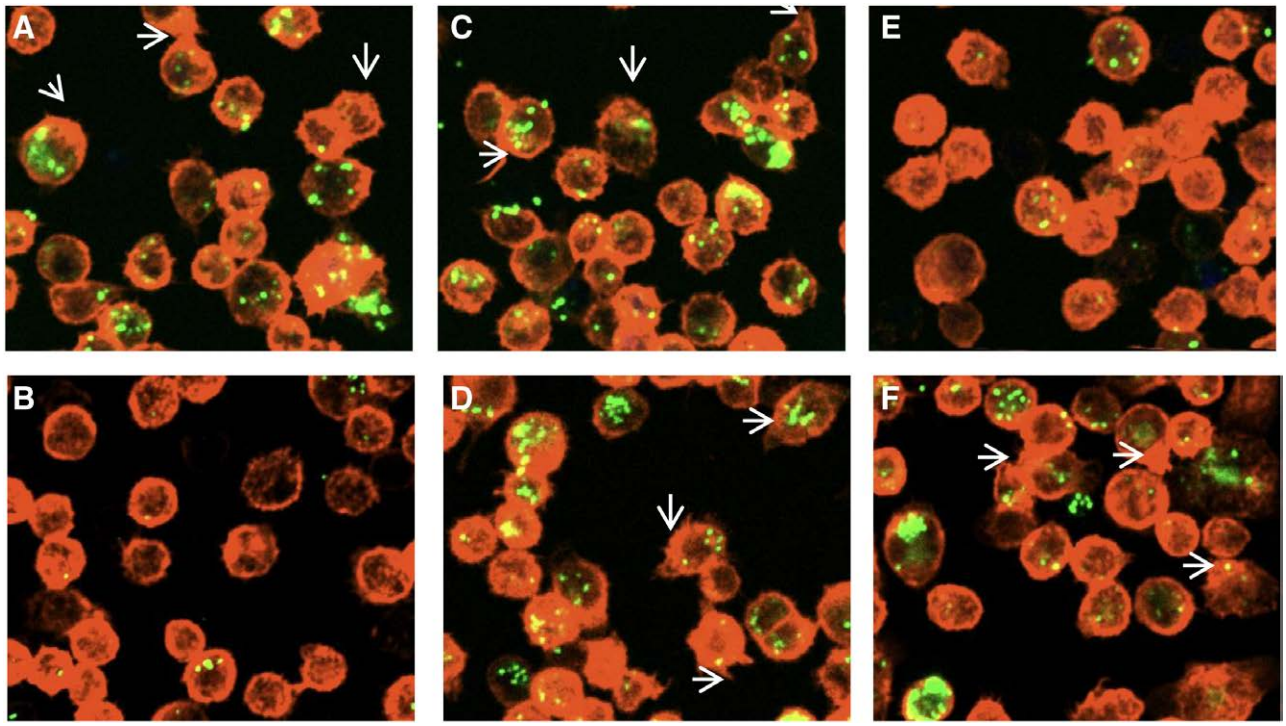

Figure 5. Morphological manifestations of alveolar macrophage cytoskeleton under laser confocal microscopy (×400). A: Control, B: CSE, C: miR-155-5p mimic, D: mimic NC, E: miR-155-5p inhibitor, F: inhibitor NC. A, C, D, and F: The cells are deformed; there are distinct protrusions on one side of the cell, and green fluorescence spots can be visualized in the cells. B and E: Cell deformation and protrusions are not pronounced, and intracellular green fluorescence is relatively small. Red fluorescence: Tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin. Green fluorescence: FITC-labeled *E coli*. White arrow: pseudopodia. CSE = cigarette smoke extract, FITC = fluorescein isothiocyanate.

exposed to CSE was lower than that in the control group, and the phagocytic function of AMs exposed to CSE was decreased. It is hypothesized that miR-155-5p may be involved in the decrease of phagocytosis of AMs exposed to CSE. To corroborate our conjecture, MH-S cells transfected with miR-155-5p mimics induced phagocytosis, whereas transfection with miR-155-5p inhibitor decreased the phagocytosis of AMs, which further confirmed that miR-155-5p was involved in the decreased phagocytosis of AMs exposed to CSE. Next, the role and possible mechanism of miR-155-5p in the decreased phagocytosis of AMs exposed to CSE were also evaluated. This study provided a novel target for the treatment of decreased phagocytosis of alveolar macrophages in COPD patients.

Phagocytosis is critical for immune defense and tissue remodeling/homeostasis of macrophages and requires extensive remodeling of the cell membrane, in which actin polymerization and depolymerization play a major role. Prior studies have found that the PI3K/mTOR/RhoA pathway participates in the regulation of macrophage phagocytosis by affecting cytoskeleton rearrangement. After silencing mTOR expression, the expression level of RhoA and p-RhoA in macrophages was up-regulated, and phagocytosis was enhanced.[12] However, the specific mechanism of mTOR/RhoA causing phagocytic dysfunction remains unknown. More and more evidence shows that mTORC2 and GTPase jointly mediate actin polymerization and play an important role in actin cytoskeleton recombination.[13,14,35–37] Indeed, the activation of mTORC2 is crucial for cytoskeleton rearrangement through F-actin and myosin II. Nevertheless, the deletion of mTOR2 destroys the polarization of the actin cytoskeleton, which controls the actin cytoskeleton by activating Rho GTPase.[18] Activated RhoA can stimulate the formation of stress fibers and the extension of pseudopodia, induce cell deformation,[19] and increase phagocytosis.[38] The kinase-independent effect of mTORC2 limits actin polymerization in the anterior part of cells, and its kinase activity is essential for myosin contractility in the posterior part of cells. Although anterior/posterior regulation is typically highly coordinated, they lose coordination when mTORC 2 is absent.[39]

Rictor is considered a key component of mTORC2, which regulates the actin cytoskeleton. At present, studies on the role of Rictor in regulating actin to participate in macrophage phagocytosis are lacking. The destruction of mTORC2 in neutrophils leads to an increase in membrane tension level caused by elevated actin polymerization.[40] Notably, the loss of Rictor in B cells significantly increases cortical F-actin levels after B cell receptor (BCR) stimulation.[41] In Rictor knockout neutrophil-like dHL-60 cells, the level of F-actin was significantly increased in cell polarization, and a wider range of F-actins was displayed on the front end of cells.[37] In a study conducted by Rosel et al[15] real-time examining alterations in F-actin cytoskeleton between wild-type cells and cells lacking mTORC2 during phagocytosis uncovered that there was no morphological difference in actin polymerization and depolymerization at the phagocytic cup while monitoring the phagocytotic process using a confocal microscope found enhanced phagocytosis in cells deficient in mTORC2. Altogether, these observations signify that mTORC2 and Rictor play an influential role in macrophage phagocytosis by regulating the cytoskeleton.

In this study, it was found that upregulated Rictor expression levels activated mTORC2, inhibited the activity of its downstream RhoA, and decreased the phagocytosis of macrophages. When Rictor expression was down-regulated, the activity of RhoA increased, and the phagocytosis of macrophages increased. Furthermore, our results verified that mTORC2/RhoA is involved in macrophage phagocytosis. However, the specific role of mTORC2/RhoA in macrophage phagocytosis is yet to be determined. Considering that mTORC2/RhoA plays an important role in cytoskeletal rearrangement, Rictor was theorized to participate in the phagocytosis of macrophages through actin skeleton rearrangement. The dual luciferase assay determined that Rictor is a target of miR-155-5p, and transfection of a miR-155-5p mimic can lower the expression of Rictor and increase phagocytosis in macrophages, whereas transfection of

a miR-155-5p inhibitor can increase the expression of Rictor and decrease phagocytosis. Moreover, our results confirmed that miR-155-5p targets Rictor to increase macrophage phagocytosis. After CSE intervention, similar performance and change of phagocytic ability was noted and was accompanied by modifications in the cytoskeleton (the formation of stress fibers and the extension of pseudopodia), which indicated that miR-155-5p could participate in the regulation of macrophage skeleton by regulating the expression of mTORC2/RhoA, and subsequently improve the phagocytosis of alveolar macrophages.

In summary, our results suggest that overexpression of miR-155-5p targets Rictor to improve the reduction of phagocytosis in alveolar macrophages under CSE intervention by modulating the cytoskeleton through the mTORC2/RhoA pathway. The findings offer new ideas and lay a theoritical basis for improving the phagocytic ability of alveolar macrophages in COPD patients and provide potential therapeutic targets for COPD treatment.

Author contributions

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