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MicroRNA-155 regulates lipopolysaccharide-induced mucin 5AC overproduction via a suppressor of cytokine signaling 1-mediated mechanism in human bronchial epithelial cells

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ABSTRACT

Chronic inflammatory lung diseases accompanied by Gram-negative bacteria infection are characterized by excessive mucin production. Lipopolysaccharide (LPS), the major endotoxin released from Gram-negative bacteria, is a potent inflammatory agonist for mucin overproduction. In this study, we sought to examine whether the toll-like receptor (TLR)-responsive microRNA miR-155 plays a role in LPS-provoked induction of mucin 5AC (MUC5AC) and the potential role of suppressor of cytokine signaling 1 (SOCS1) involved in this process. We found that LPS increased the expression of MUC5AC in association with TLR4-dependent miR-155 induction. The suppression of miR-155 by antagomir led to an excessive production of SOCS1, thereby downregulation of MUC5AC production. Collectively, these data imply that miR-155 is involved in LPS-induced MUC5AC overproduction through a TLR4-dependent manner and thereby the downregulation of SOCS1.

1. Introduction

Mucin overproduction is a remarkable characteristic of chronic inflammatory lung diseases accompanied by Gram-negative bacteria infection. Excessive mucin 5AC (MUC5AC), which is the major respiratory secreted mucin, accumulates in inflamed airways and contributes to symptoms, such as cough, sputum production, persistent inflammation, mucus obstruction, serious hypoxia and even increased mortality (Curran and Cohn, 2010; Rubin et al., 2014). The endotoxin of Gram-negative bacteria lipopolysaccharide (LPS) challenge of the airway is common in chronic inflammatory lung diseases and, to a certain extent, acts as a trigger, leading to upregulation MUC5AC expressions (Gupta et al., 2015; Li et al., 2013; Ma and Ma, 2018; Oliviero et al., 2016). Thus, an in-depth exploration of the regulatory mechanisms of LPS challenge that mediate MUC5AC overproduction in patients with these diseases is helpful to clinical therapy.

MicroRNAs (miRNAs) are small endogenous noncoding RNA molecules of ~22 nucleotides that regulate gene expression by binding to the 3′-untranslated region of their target gene and modulating mRNA degradation or translational inhibition (Ambros, 2004; Battel, 2009). Many studies have reported the profound impact of specific miRNAs on normal immune function as well as on inflammatory responses (Lu and Rothenberg, 2013; O'Connell et al., 2013; Xiao et al., 2017). For example, several studies found that *miR-155*$^{-/-}$ mice are immunodeficient and that miR-155 is required for immune responses by T-cells, B-cells and dendritic cells (O'Connell et al., 2007; Rodriguez et al., 2007; Thai et al., 2007). Xiao et al. (2017) implied that miR-155 features a common denominator in treating a wide spectrum of inflammatory responses. Zhang et al. (2017) also indicated that miR-155/suppressor of cytokine signaling 1 (SOCS1) is involved in oxidized low density lipoprotein Ox-LDL-induced inflammation. Additionally, miR-155 has emerged as a critical regulator of toll-like receptors (TLRs) signaling. It can be highly inducible in response to TLR4 ligands such as LPS (Olivieri et al., 2013; Quinn and O'Neill, 2011). Thus, we speculated that LPS elevates miR-155 via the TLR4-pathway, which in turn, is required for MUC5AC overproduction.

Functional studies have implicated that miR-155 targets SOCS1 to block the negative feedback loop, leading to prolonged and maximized the inflammatory processes. SOCS1 is well-known as a negative regulator of LPS-induced inflammation by directly inhibiting TLR-4 signaling (Chen et al., 2013; Inagaki-Ohara et al., 2013; Linossi et al., 2013; Strebovsky et al., 2012; Yoshimura et al., 2012). Based on the

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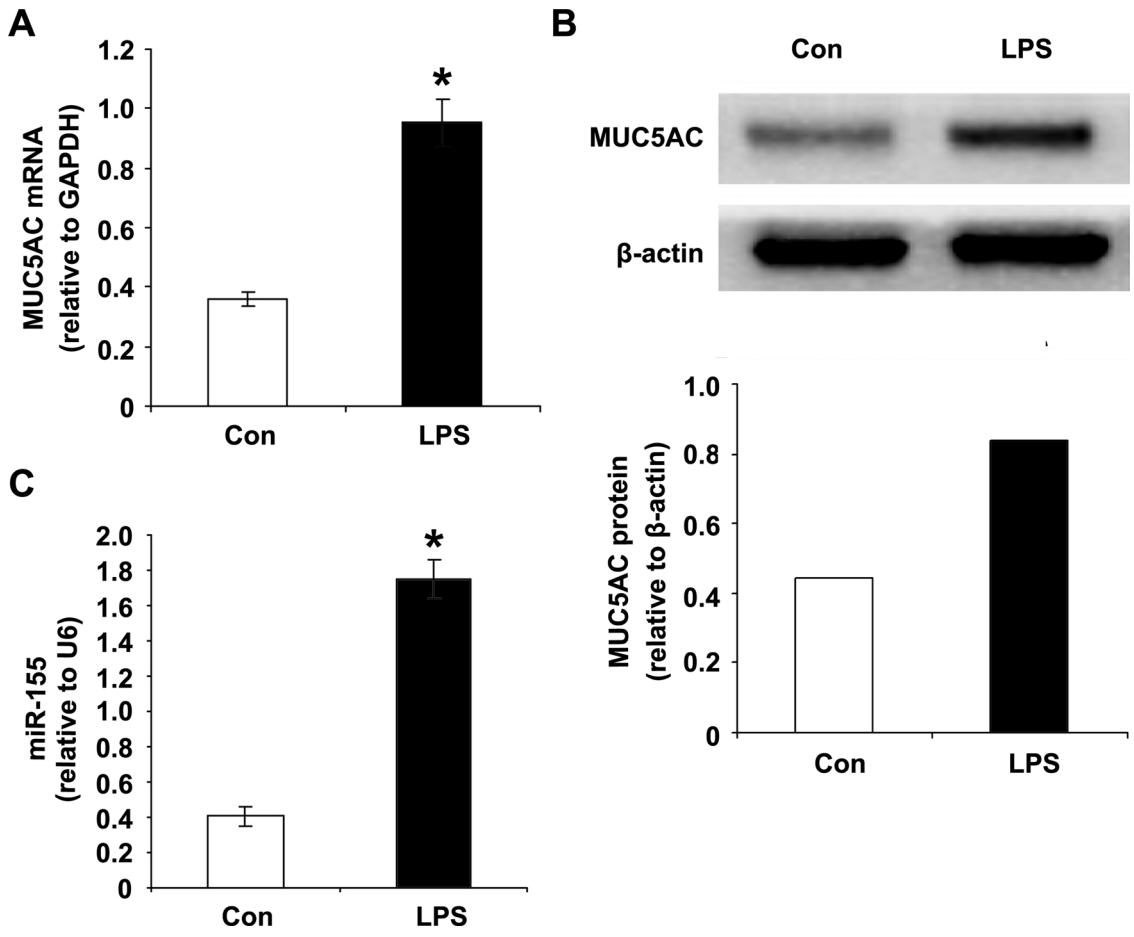

Fig. 1. LPS increases the expressions of MUC5AC and miR-155 in 16HBE cells. Confluent serum-starved cells were exposed to 1 μg/ml of LPS for the period of 6 h. The mRNA level of MUC5AC (**A**) and miR-155 (**C**) were analyzed by quantitative real-time PCR and were normalized to GAPDH and U6, respectively. MUC5AC protein production was analyzed by western blotting (**B**). These data are representative of three independent experiments and are presented as the means ± SD (n = 3). *$p < 0.01$ vs. the control group.

direct link between miR-155 and SOCS1 in LPS-promoted MUC5AC overproduction, the goals of this study were as follows: first, to explore the hypothesis that LPS-induced the activation of TLR4 could lead to the upregulation of miR-155 and participate in the overproduction of MUC5AC. Second, to assess the hypothesis that the loss of SOCS1 enhanced MUC5AC production.

2. Materials and methods

2.1. Materials

Human bronchial epithelial 16HBE, a post-crisis SV40 large T-antigen-transformed polarized immortal epithelial cell line, was purchased from National Infrastructure of Cell Line Resource (Shanghai, CHN). Bronchial epithelial growth medium (BEGM) and fetal bovine serum (FBS) were purchased from Gibco (Thermo, Suzhou, Jiangsu, CHN). LPS was purchased from Sigma (St Louis, MO, USA). The TLR4 inhibitor Polymyxin B (PMB) was purchased from InvivoGen (San Diego, CA, USA). Primary antibodies were all purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jinqiao Biotech (Beijing, CHN). All chemicals for real-time PCR were purchased from Takara (Dalian, Liaoning, CHN). The synthetic miR-155 antagomir and negative control miRNA (NC-miRNA) were all purchased from GenePharma (Shanghai, CHN). All primers, SOCS1-siRNA and non-targeted siRNA (NT-siRNA) were synthesized by Sangon (Shanghai, CHN).

2.2. Cell culture and treatments

16HBE cells (1×10^5/ml) were cultured in BEGM supplemented with 10% (v/v) FBS in a 37 °C humidified incubator with 5% CO_2. Before the experiments, the confluent cells were serum-starved for an additional 24 h to maintain the low basal levels of MUC5AC production. In the LPS experiments, serum-starved cells were exposed to 1 μg/ml concentration of LPS for 6 h. In the reagent experiments, TLR4 inhibitor (PMB; 100 μM) was added to the medium for 1 h prior to exposure to LPS. The equivalent amount of medium was used as the vehicle control.

2.3. Cell transfection

For transfection, cells (1×10^5/ml) were seeded in 6-well plates and were maintained in BEGM without antibiotics for 24 h before transfection. 5 μl of lipofectamine 2000 was diluted with Opti-MEM to 250 μl for 5 min, and then further incubated with the mixture of 10 μl of oligonucleotide (SOCS1-siRNA, 40 nM; miR-155 antagomir, 200 nM) and 240 μl of Opti-MEM for another 20 min at room temperature. Each wells were added 100 μl of the mixtures and kept at 37°C in a 5% CO_2 incubator for 6 h before replaced the fresh media. Keeping on incubating for another 48 h or 72 h, transfected cells were harvested for further examination. Non-transfected (Non), non-targeted siRNA-transfected (NT-siRNA) or negative-control miRNA-transfected (NC-miRNA) cells were established as the matched controls, respectively. The sequences of siRNA and miR-155 antagomir were as follows: SOCS1-siRNA (5′-GACAAUGCAGUCUCCCACG-3′), NT-siRNA (5′-UUC

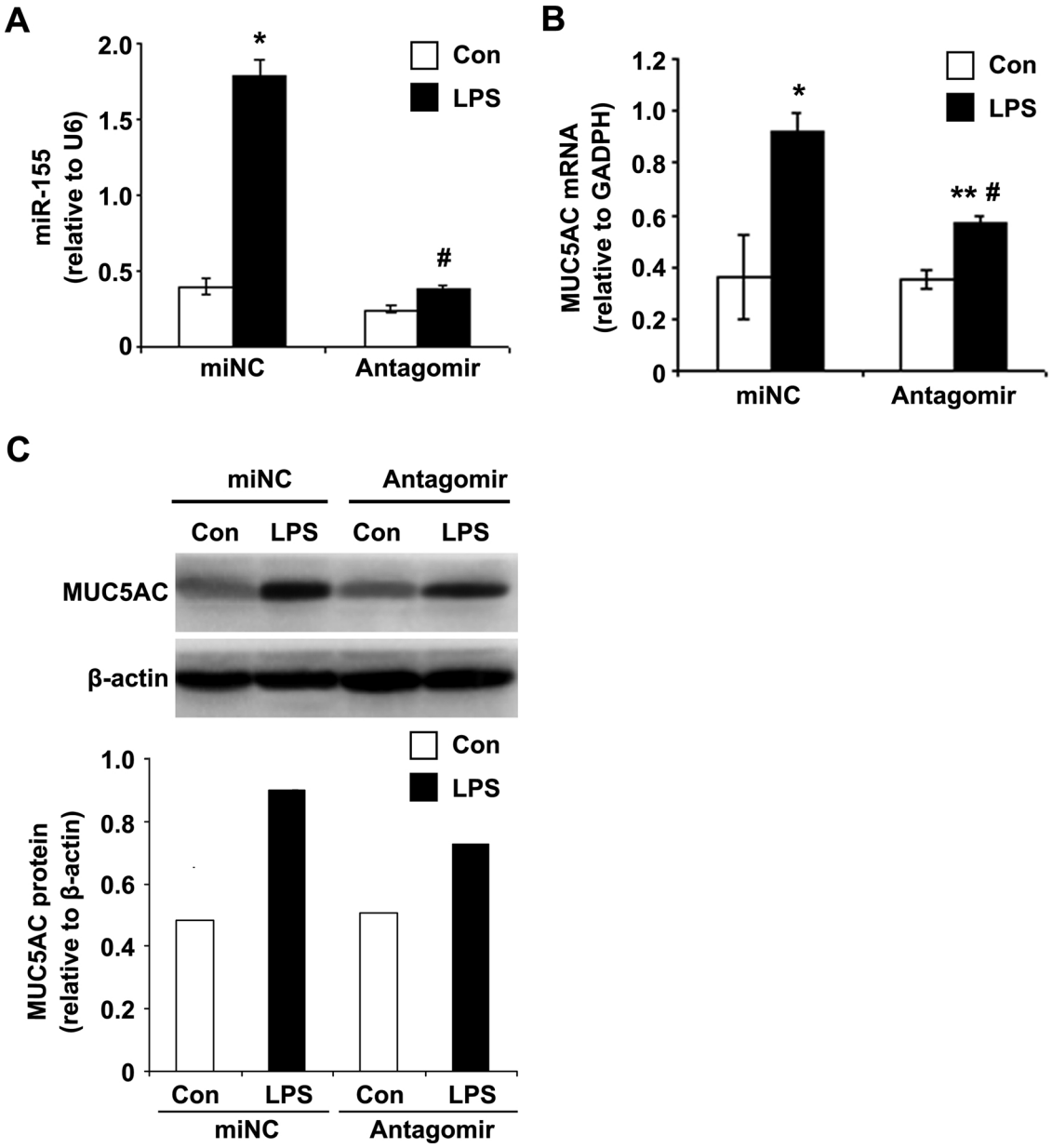

Fig. 2. miR-155 is involved in LPS-induced MUC5AC mRNA expression and protein production in 16HBE cells. Cells were transfected with miR-155 antagomir, or negative control miRNA (miNC) as the control. The cells were further incubated with LPS (1 μg/ml) for 6 h. **A.** The suppression of miR-155 by its specific antagomir was confirmed by real-time PCR. The results were expressed as the relative ratio to U6 expression. **B–C.** The levels of MUC5AC mRNA (**B**) and protein (**C**) were analyzed by real-time PCR and western blotting, respectively. These data are representative of three independent experiments and are presented as the means ± SD (n = 3). $*p < 0.01$ and $**p < 0.05$ vs. the miNC-transfected group with control treatment, $\#p < 0.01$ vs. the miNC-transfected group with LPS treatment.

UCCGAACGUGUCACGU-3′), miR-155 antagomir (UGUUAAUGCUAAU AUGUAGGAG) and NC-miRNA (UCUACUCUUUCUAGGAGGUUGUGA).

2.4. RNA extraction and quantitative real-time PCR

Total RNA was isolated using Trizol solution. The first-strand cDNA was synthesized from total RNA (1 μg), primer (1 μl), avian myeloblastosis virus (AMV) reverse transcriptase (3.33 U/μl), RNase inhibitor (0.25 U/μl) and 1 × RT buffer in a final volume of 20 μl. The mixture was incubated at 16 °C for 15 min, 42 °C for 15 min and 85 °C for 5 min. Subsequent PCR reactions (20 μl) contained cDNA (1 μl), primer mix (1 μl) and 1 TaqMan Master Mix. The reaction conditions consisted of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. MUC5AC mRNA levels were determined using the forwards primer: 5′-CAGCCGAGAGGAGGGTTTGATCT-3′ and the reverse primer 5′-AGTCTCTCTCCGCTCCTCTCAAT-3′, with GAPDH (forwards primer 5′-AACGACCCCTTCATTGACCTC-3′ and the reverse primer 5′−CCTT GACTGTGCCGTTGAACT-3′) as the internal control. MiR-155 levels were detected by the forward primer: 5′-ACACTCCAGCTGGGTTAATG CTAATCGTG-3′ and the reverse primer: 5′-CTCAACTGGTGTCGTGG AGT-3′, with U6 (the forward primer: 5′-CTCGCTTCGGCAGCACA-3′ and the reverse primer 5′-AACGCTTCACGAATTTGCGT-3′) as the internal control.

2.5. Western blotting

Cells were lysed with lysis buffer containing a protease inhibitor cocktail (1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin)

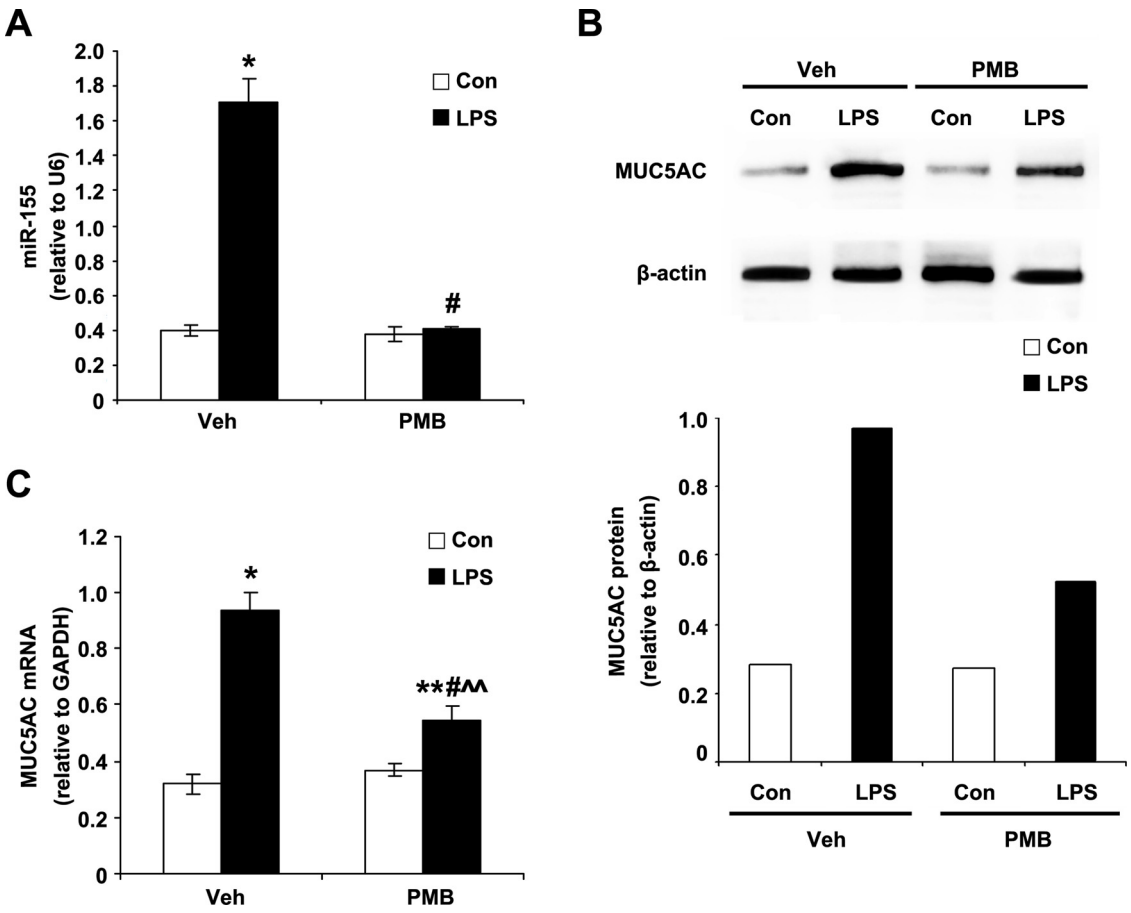

Fig. 3. TLR4 is involved in the LPS-mediated overproduction of miR-155 and MUC5AC expression in 16HBE cells. Cells were exposed to the TLR4 inhibitor PMB, and the equivalent amount of medium was used as the vehicle controls (Veh). The cells were further incubated with LPS (1 μg/ml) for 6 h. **A.** The expression of miR-155 was confirmed by real-time PCR. The results were expressed as the relative ratio to U6 expression. **B–C.** The levels of MUC5AC mRNA (**B**) and protein (**C**) were analyzed by real-time PCR and western blotting, respectively. These data are representative of three independent experiments and are presented as the means ± SD (n = 3). *$p < 0.01$ and **$p < 0.05$ vs. the Veh group with control treatment, #$p < 0.01$ vs. the Veh group with LPS exposure, ^^$p < 0.05$ vs. the PMB group with control treatment.

on ice after various treatments. The total protein in the cell lysates was measured using the BCA assay. 20 μl of the lysates were separated by 6% or 10% SDS-PAGE and transferred onto a PVDF membrane. After blocking with 5% non-fat milk, the PVDF membranes were probed with appropriate primary antibodies (1:10 000) at 4 °C overnight, followed by secondary antibodies conjugated with HRP (1:5 000) at 37 °C for 1 h. The immunoreactive bands were visualized by enhanced chemiluminescence. The relative amount of the proteins of interest was normalized to β-actin. Densitometric quantification of the bands was performed with Quantity one.

2.6. Statistical analysis

Values are presented as the mean ± SD. The data were analyzed using the SPSS 10.0 statistical package (SPSS, Inc., USA). Differences were evaluated for statistical significance using Student's *t* test for paired comparisons or one-way ANOVA for multiple comparisons followed by Bonferroni's post hoc test after testing for normality with W test. *p* values of less than 0.05 were considered statistically significant.

3. Results

3.1. LPS induces the excessive expression of MUC5AC and miR-155

Fig. 1A showed that MUC5AC mRNA expression significantly increased at the dose of 1 μg/ml for 6 h. Meanwhile, exposure of the cells to LPS (1 μg/ml, 6 h) also led to a significant increase in MUC5AC protein production (Fig. 1B). Both of MUC5AC mRNA and protein were not upregulated in the control group without LPS challenge.

To explore the role of miR-155 in the LPS-induced MUC5AC responses, we initially determined whether miR-155 was changed in the cells in response to LPS challenge. In the LPS-treated group, a significant increase in miR-155 expression was observed at a 1 μg/ml and 6 h LPS challenge (Fig.1C). Therefore, the dose of 1 μg/ml and the time point of 6 h were selected as the optimal response conditions in the ensuing LPS experiments. Collectively, these results demonstrated that LPS induced an excessive expression of MUC5AC and miR-155.

3.2. Inhibition of miR-155 function attenuates LPS-induced MUC5AC expression

We further investigated the potential functional role of miR-155 in regulating LPS-induced MUC5AC responses by loss-of-function experiments using a specific miR-155 antagomir. As anticipated, cells transfected with the miR-155 antagomir effectively reduced miR-155 expression to ˜60% of the initial level and ˜21.56% of the induced level (Fig. 2A). Upon reduction of miR-155 with the antagomir, we observed a significantly but incompletely inhibition of LPS-induced MUC5AC gene expression (Fig. 2B) and protein production (Fig. 2C) compared with the LPS-treated negative control miRNA (miNC). Meanwhile, it is worth noting that antagomir significantly reduced the basal expression of miR-155 but failed to inhibit the basal production of MUC5AC

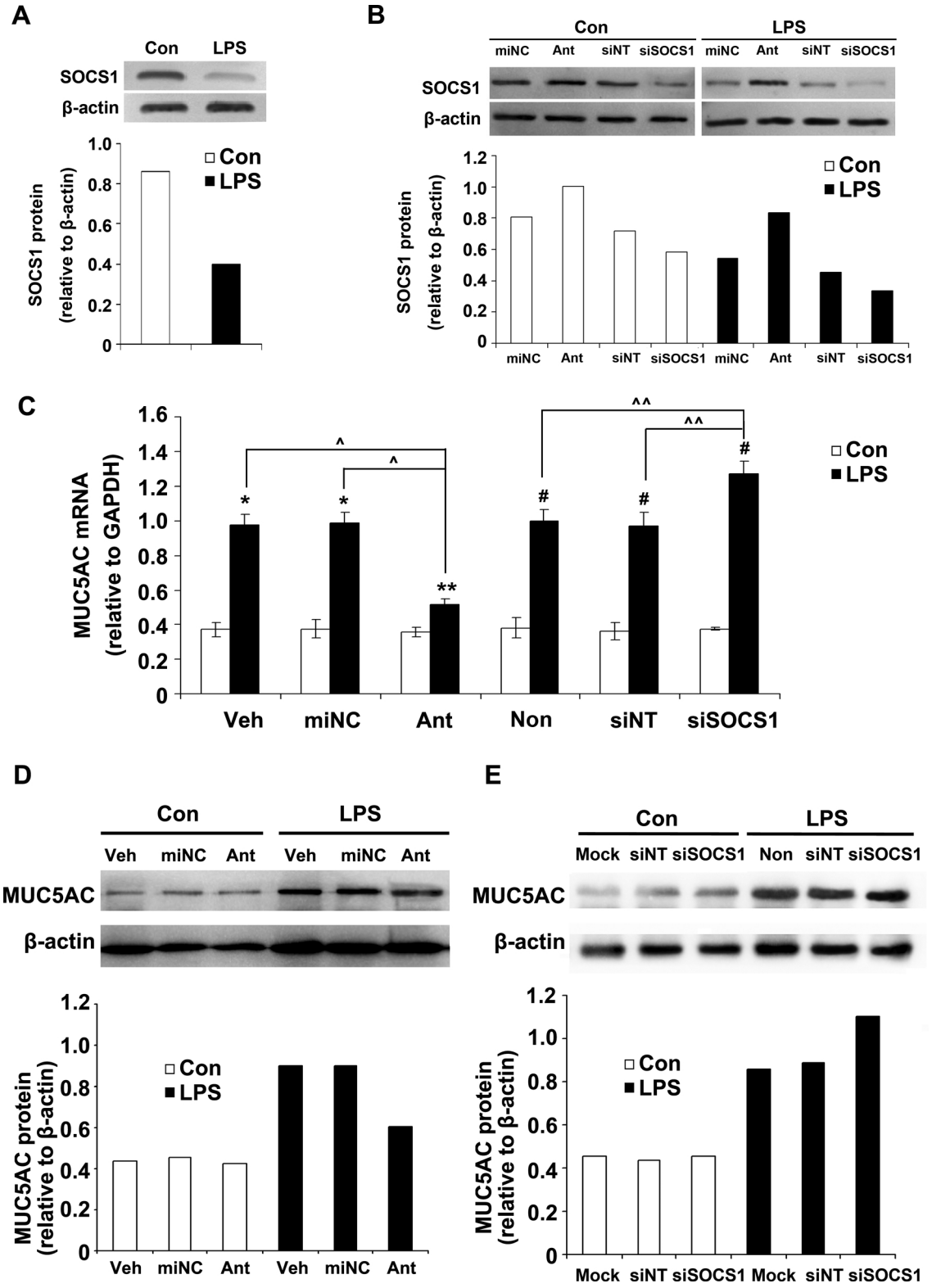

Fig. 4. miR-155 and its target protein SOCS1 regulate MUC5AC expression in 16HBE cells. Cells were transfected with SOCS1-siRNA (siSOCS1), the miR-155 antagomir (Ant), and the non-targeted siRNA (siNT) or negative control miRNA (miNC) as the matched controls, the vehicle (Veh) and non-transfected (Non) as the blank controls, respectively. The cells were further incubated with LPS (1 μg/ml) for 6 h. **A–B.** The expression of SOCS1 was examined by western blotting. **C–E.** MUC5AC mRNA expression and protein production were measured by real-time PCR (**C**) and western blotting (**D** and **E**), respectively. These data are representative of three independent experiments and are presented as the means ± SD (n = 3). $*p < 0.01$ and $**p < 0.05$ vs. the vehicle control group (veh) without LPS treatment, $\#p < 0.01$ vs. the mock control group without LPS challenge, $\hat{}p < 0.01$ and $\hat{}\hat{}p < 0.05$ the marked columns.

production. This result may explain that the basal MUC5AC production is independent of miR-155. Taken together, the data demonstrate that the MUC5AC response to LPS is partly driven by a miR-155 pathway but suggest that other pathways also contribute to this LPS challenge.

3.3. The induction of MUC5AC by LPS is mediated by TLR4-dependent miR-155 expression

Among the multiple signaling pathways previously linked to LPS, TLR4 has been widely implicated in LPS-induced MUC5AC responses. Therefore, we initially hypothesized that TLR4 is involved in the LPS-mediated regulation of miR-155 and MUC5AC expression. As illustrated, PMB, an inhibitor of TLR4, completely attenuated LPS-induced miR-155 expression (Fig. 3A), while the inhibition of MUC5AC expression is significant but not complete (Fig. 3B and 3C). This indicated that LPS-induced miR-155 is TLR4 dependent; however, induction of MUC5AC by LPS is partially mediated by TLR4-triggered signaling cascades.

3.4. SOCS1 is a negative regulator of MUC5AC expression

MiR-155 is predicted to target SOCS1, a negative regulator of inflammatory responses and a mediator of homeostasis (Chen et al., 2013; Inagaki-Ohara et al., 2013; Rao et al., 2014). Therefore, we hypothesized that miR-155 might induce MUC5AC expression via the loss of SOCS1 function. Thus, we performed experiments using a miR-155 antagomir or SOCS1-siRNA. As indicated in Fig. 4A, the expression of SOCS1 decreased significantly upon LPS stimulation, reciprocal to the increase in miR-155 expression (Fig. 2A). Suppression of miR-155 with an antagomir increased SOCS1 expression (Fig. 4B, Ant, Con and LPS), while the expression of MUC5AC was reduced simultaneously (Fig. 4C and D, Ant, Con and LPS).

For further explore the role of SOCS1 in LPS-increased MUC5AC expression, we inhibited the expression of SOCS1 with its specific siRNA (SOCS1-siRNA). We found that knockdown of SOCS1 with SOCS1-siRNA reduced both of the basal levels of SOCS1 (Fig. 4B, Con, siSOCS1) and the induced levels of SOCS1 (Fig. 4B, LPS, siSOCS1), and there were detectable effects on the levels of MUC5AC (Fig. 4E, LPS, siSOCS1). These results firmly show a close relationship between the contradict expression of SOCS1 and miR-155 and the following changing of MUC5AC expression.

4. Discussion

Understanding the molecular mechanisms underlying mucin overproduction is crucial for the development of better clinical therapy for patients with airway mucin overproduction. Recently, considerable attention has been concentrated on the roles of specific miRNAs in immunological processes (Chen et al., 2017; Lu and Rothenberg, 2013; O'Connell et al., 2012; Pourteimoor et al., 2018). However, the contributions of miRNAs to inflammatory disease with mucin overproduction remain unknown. In this study, we demonstrated a specific role for miR-155, which is closely associated with inflammation (O'Connell et al., 2007; Rodriguez et al., 2007; Thai et al., 2007; Xiao et al., 2017; Zhang et al., 2017), in the regulation of mucin overproduction in chronic airway inflammatory diseases. We found that the activation of TLR4 by LPS induces the massive expression of miR-155 and the subsequent knockdown of SOCS1. This leads to the increased expression of MUC5AC mRNA and protein.

MiR-155 was the first oncogenic miRNA to be discovered. Many studies have reported that miR-155 is a promoter and biomarker of several types of B cell lymphoma, especially Hodgkin's lymphoma (Eis et al., 2005; Kluiver et al., 2005; Yao et al., 2012). Previous studies and our present study further demonstrated the involvement of miR-155 in other numerous biological processes, such as inflammation and immunity. For example, O'Connell et al. (2007) characterized miR-155 as a component of macrophage responses to a broad range of inflammatory mediators, and further identified it as a potential link between inflammation and cancer. Stanczyk et al. (2008) also described miR-155 as a typical TLR-responsive miRNA that is upregulated after stimulation with TLR ligands, such as LPS. However, Ceppi et al. (2009) noted that miR-155 exerts a negative influence on inflammatory cytokine production in response to microbial stimuli in human dendritic cells. Xiao et al. (2009) also reported that increased expression of miR-155 functions as a negative regulator of inflammatory signaling. Both the positive and negative regulatory roles of miR-155 in inflammation implicate it as an active player in downstream inflammatory pathways.

We sought to investigate how miR-155 promotes MUC5AC expression. SOCS1, which is a repressor of cytokine production and inflammatory responses, is predicted to be a target of miR-155 (Chen et al., 2013; Inagaki-Ohara et al., 2013; Rao et al., 2014; Xiao et al., 2017; Zhang et al., 2017). In our report, LPS triggers a MUC5AC response, primarily caused by the upregulation of miR-155 via TLR-4-pathway, leading to significant suppression of SOCS1. As an important regulator of inflammatory reaction, miR-155 has multiple potential targets involved in inflammatory response. In addition to SOCS1, miR-155 also represses Src homology 2 domain-containing inositol-5-phosphatase 1 (SHIP1) in promoting IL-8 hyperexpression in cystic fibrosis lung epithelial cells (Bhattacharyya et al., 2011). However, we failed to detect the expression of SHIP1 in our mucin-secreted lung epithelial cells, including 16HBE cell in this study, A549 and NCI-H292. SHIP1 is widely known to be expressed almost exclusively in hematopoietic cells. Perhaps the mucin-secreted lung epithelial cells are not good models for miR-155/SHIP1 experiments.

5. Conclusions

This study highlights a novel role for miR-155 in MUC5AC gene expression and protein production. LPS induced miR-155 expression in a TLR4-dependent manner. This expression leads to the downregulation of SOCS1, and in turn, the induction MUC5AC overproduction. With an increasing number of studies being performed on modulating miRNAs for therapeutic use, our study could provide new therapeutic approaches to mitigate excessive mucus secretion in chronic inflammatory diseases.

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