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Trichostatin A exerts anti-inflammation functions in LPS-induced acute lung injury model through inhibiting TNF-α and upregulating micorRNA-146a expression

T. LING[1], J. XIE[1], Y.-S. SHEN[1], M. QIAO[1], H. YANG[1], D.-Y. SUN[1], K.-J. QIAN[2]

[1]Department of Emergency Medicine, Shenzhen Longgang Center Hospital, Shenzhen, China
[2]Department of Critical Care Medicine, the First Affiliated Hospital of Nanchang University, Nanchang, China

Abstract. – OBJECTIVE: Acute lung disease is characterized by inflammation. This research aimed to investigate effect of trichostatin A (TSA) on microRNA-146a (miR-146a) and tumor necrosis factor α (TNF-α) in lipopolysaccharide (LPS)-induced alveolar macrophage injury model.

MATERIALS AND METHODS: Rat alveolar macrophage, NR8383, was cultured and induced using LPS to establish acute lung injury model *in vitro* level. Cell Counting Kit-8 (CCK-8) assay was used to determine cell viability of NR8383 cells. TSA was administrated to LPS-induced NR8383 cells. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) assay was utilized to evaluate TNF-α and miR-146a mRNA expression in LPS and/or TSA treated NR8383 cells. Enzyme-link immunosorbent assay (ELISA) was used to examine TNF-α levels.

RESULTS: This study selected 1 ng/ml and 10 ng/ml TSA as the optimal concentrations for treating NR8383 cells. LPS-induced acute lung injury model was successfully established. TSA administration significantly enhanced accounts of LPS-stimulated NR8383 cells. LPS induction significantly increased miR-146a mRNA expression in NR8383 cells compared to NR8383 cells ($p<0.05$). TSA administration significantly reduced the levels of TNF-α in LPS-induced NR8383 cells compared to those in LPS-induced NR8383 cells ($p<0.05$). TSA administration significantly enhanced miR-146a expression in LPS-induced NR8383 cells compared to that in LPS-induced NR8383 cells ($p<0.05$).

CONCLUSIONS: TSA administration exerted anti-inflammation functions in LPS-induced acute lung injury model *in vitro*, which might be triggered by inhibiting TNF-α molecule and upregulating miR-146a expression. The present data hint that TSA could be considered as a potential therapeutic agent for treating acute lung injury.

Key Words:
Acute lung disease, MicroRNA-146a, Inflammation, Trichostatin A, Tumor necrosis factor α.

Introduction

Clinically, acute lung diseases or chronic lung disorders are characterized by inflammations, which could further induce the pulmonary tissue injury[1,2]. Moreover, inflammation also involves other lung disorders, including interstitial lung disease, chronic obstructive pulmonary disorder, asthma, acute-respiratory distress syndrome (ARDS)[1,3-5]. In the pathological or inflammatory processes of lung diseases, the particles (or micro-particles) and pathogens in patients' bronchoalveolar lavage fluid play critical roles in progression of lung disorders[6]. However, until now, there are also no studies investigating the clearance of particles or pathogens, which are associated with inflammation resolution.

The development of acute ARDS or lung injury is usually characterized by the injury to lung alveolar macrophages, which account for about 95% of all the airspace leukocytes[7]. The alveolar macrophages could affect the progression of acute lung injury post the stimuli of infection or the other factors, by releasing or synthesizing multiple inflammatory mediators[8]. Meanwhile, the alveolar macrophages, as primary phagocytes, play crucial roles in clearing pathogens or particles in lung tissues[1]. In recent years, the alveolar macrophages have been proven to facilitate clearance inflammatory cells in the process of inflammation resolution[9]. Therefore, the therapeutic administration focusing on al-

Corresponding Author: Dayong Sun, MD; e-mail: huangzyungz@sina.com

veolar macrophages death signaling pathways might act as a treating strategy for acute lung injury or ARDS.

Nowadays, several microRNAs (miRs), such as miR-146a, miR-155, miR-125, miR-18b, have been proven to participate in modulation for inflammatory response through the particles or antigens[10,11]. Among the above miRs, miR-146a is mainly induced by the endotoxin *via* stimulation of the toll-like receptor 4 (TLR4) to distinguish cell lines[12]. Moreover, miR-146 could regulate the immune response, negatively affect innate immune response, and protect against the ischemic reperfusion injury[13-15]. The tumor necrosis factor α (TNF-α) has been extensively proven to be at the center for inflammatory responses and progression of tumor or immune diseases[16]. Wu et al[17] showed that miR-146 is considered the most differentially and highly expressed microRNA post the administration of TNF-α. However, the roles of miR-146a in lung injury associated inflammation have not been clarified until now.

In this study, we speculated that trichostatin A (TSA) in alveolar macrophages plays critical roles in preventing inflammation in lipopolysaccharide (LPS)-induced acute lung injury model *in vitro* level. Meanwhile, by using the *in vitro* model, we also proved that TSA could downregulate TNF-α, which triggers inflammation of alveolar macrophages.

Materials and Methods

Cell Culture

The rat alveolar macrophage, NR8383, was obtained from Shanghai Life and Science Institute of Chinese Academy of Science (CAS; Shanghai, China). NR8383 cells were cultured in F12K medium (Gibco, Grand Island, NY, USA) containing 20% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and supplemented with 1% penicillin-streptomycin (Beyotime Biotechnology, Shanghai, China) at 37°C with 5% CO_2.

Cell Counting Kit-8 (CCK-8) Assay

In this research, the growth of NR8383 cell undergoing TSA administration was evaluated using CCK-8 Kit (Cat. No. 96992, Sigma-Aldrich, St. Louis, MO, USA) due to protocol of manufacturer. Firstly, NR8383 cells were treated with TSA (at dosage of 50, 30, 10, 3, and 1 ng/ml medium) and 10% dimethyl sulfoxide (DMSO, as positive control, Sigma-Aldrich, St. Louis, MO, USA) and cultured in 96-well plates (Corning-Costar, Corning, NY, USA) for 48 h. While, the blank NR8383 cells were employed as the negative control. Then, the above NR8383 cells were incubated with 10% CCK-8 at final concentration of 4 h at 37°C. The absorbance values were determined using the microplate reader (Model: Multiskan Spectrum, Thermo Scientific Pierce, Rockford, IL, USA) at a wavelength of 450 nm. Here, the higher optical density represented the higher cell viability.

Establishment of Acute Lung Injury Model and TSA Intervention

The NR8383 cells were seeded onto 6-well plates, adjusted to density of 5×10^5 cells/ml and incubated with lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO, USA) at final concentration of 1 μg/ml for 6 h, 12 h, 18 h and 24 h, to establish acute lung injury model. For the TAS intervention (MedChemExpress, Momouth Junction, NJ, USA), NR8383 cells were divided into 4 groups, including blank control group (treating with PBS only), LPS model group (treating with 1 μg/ml LPS), 1 ng/ml TSA plus LPS group (treating with 1 ng/ml TSA and 1 μg/ml LPS), and 10 ng/ml TSA plus LPS group (treated with 10 ng/ml TSA and 1 μg/ml LPS).

Quantitative Reverse Transcription-PCR (qRT-PCR) Assay

Total RNAs in NR8383 cells were extracted using TRIzol associated reagents (TaKaRa, Dalian, China). The complementary DNA (cDNA) was generated with First cDNA-Synthesis Kit (Western BioTechnology Co. Ltd., Chongqing, China) according to the protocol of manufacturer. The qRT-PCR assay was performed using the specific primers (Table I) and ABI StepOne plus PCR system (ABI, Foster City, CA, USA). Here, U6 gene was employed as the endogenous control for normalizing miR-146a gene expression. By contrast, GAPDH gene was used as endogenous control for the TNF-α mRNA expression. In this study, the gene expression was normalized to U6 or GAPDH gene expression and analyzed based on $2^{\Delta\Delta\text{-Ct}}$ method[18].

Enzyme-Link Immunosorbent Assay (ELISA)

The medium in NR8383 culture in 96 well-plates was centrifuged to harvest cell-supernatants, for ELISA examination. The levels

Table I. The primers for qRT-PCR assay.

Genes		Sequences (5'-3')
miR-146a	Forward	AGCGGGCTGAACTGAA
	Reverse	CAGTGCAGGGTCCGAGGTAT
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT
TNF-α	Forward	TGGCATGGATCTCAAAGAC
	Reverse	GGTATGAAGTGGCAAATCG
GAPDH	Forward	GGCAAGTTCAACGGCACAG
	Reverse	CGCCAGTAGACTCCACGACAT

of TNF-α were examined using the ELISA Detection Kit (Likechuangxin Biotech. Co. Ltd., Beijing, China) due to protocol of manufacturer.

Statistical Analysis

Data were assigned as mean ± standard deviation (SD) and analyzed using SPSS software (version: 20.0, IBM Corp., Armonk, NY, USA). The differences between the groups were analyzed with the post-hoc Tukey test validated analysis of variance (ANOVA) analysis. The $p<0.05$ was defined as the statistically significant.

Results

Effects of Lower Concentration of TSA (1 ng/ml) on NR8383 Cell Viability

In this research, the optimal concentration of TSA for treating NR8383 cells was verified by administrating cells with 50, 30, 10, 3, and 1 ng/ml TSA. The graphs of cell culture showed that the positive control 10% DMSO damaged the morphology of NR8383 cells, while the amounts of cells were also decreased and damaged in both 50 ng/ml and 30 ng/ml TSA treating groups (Figure 1A). However, there were no significant effects of TSA on NR8383 cell viability among 10 ng/ml, 3 ng/ml, and 1 ng/ml TSA treating groups (Figure 1A). Moreover, the statistical analysis results also indicated that both 50 ng/ml and 30 ng/ml TSA treatment significantly decreased the optical density (OD) values of NR8383 cells compared to that in the blank NR8383 cells (Figure 1B, $p<0.05$). However, there were no significant differences for OD values of NR8383 cells among 10 ng/ml, 3 ng/ml, and 1 ng/ml TSA treating groups (Figure 1B, $p<0.05$).

LPS-Induced Acute Lung Injury Model was Successfully Established

The LPS (1 μg/ml) was administrated to NR8383 cells to induce the acute lung injury model. Meanwhile, 4 time-points, including 6 h, 12 h, 18 h, and 24 h, were selected as candidates for inducing *in vitro* injury model. Our experimental data indicated that the NR8383 cells showed the vacuolation, aggregation, and rupture even at 6 h post LPS administration (Figure 2A). Therefore, we selected 1 μg/ml LPS treating NR8383 cells for 6 h as the optimal inducible time point for generating acute lung injury model.

TSA Administration Enhanced Accounts of LPS-Stimulated NR8383 Cells

In order to clarify the effects of TSA on the LPS-stimulated NR8383 cells, the cell morphologies of cells were captured and imaged. The results illustrated that 1 μg/ml LPS treatment significantly decreased the NR8383 cells accounts compared to that in normal NR8383 cells (Figure 2B). However, both 1 ng/ml and 10 ng/ml TSA treatment remarkably enhanced NR8383 cell accounts compared to those in 1 μg/ml LPS group, especially for 10 ng/ml TSA exhibiting more cell accounts (Figure 2B).

LPS Induction Increased MiR-146a Expression in NR8383 Cells

The qRT-PCR assay findings showed that LPS treatments at different time-points (6 h, 12 h, 18 h, and 24 h) could significantly increase miR-146a mRNA expression compared to that in the blank NR8383 cells (Figure 3, $p<0.05$). Meanwhile, miR-146a mRNA levels were increased following the increased treating time of LPS; however, they were decreased at 24 h post LPS treatment (Figure 3).

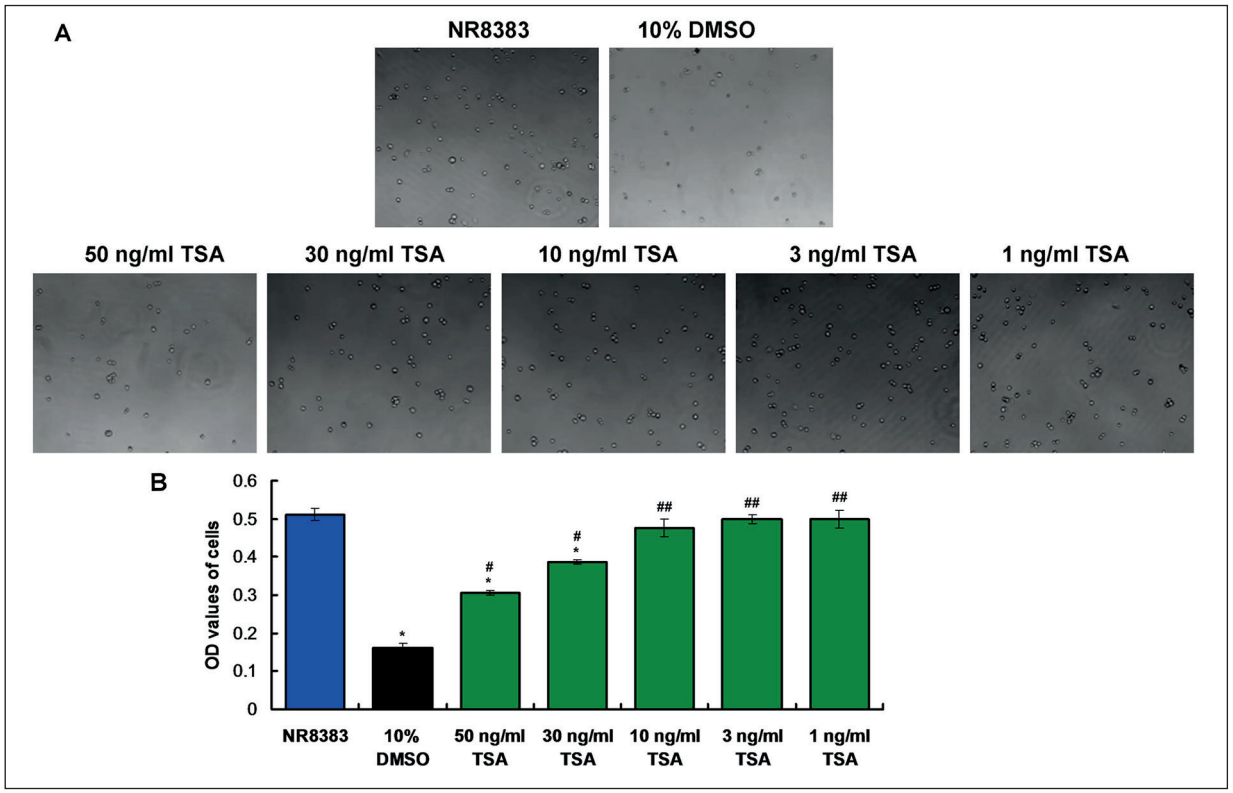

Figure 1. Effects of TSA administrations on the cell viability of NR8383 cells. **A,** The morphology images for the cultured NR8383 cells. Magnification, 100×. **B,** Statistical analysis for the optical density (OD) values of NR8383 cells. *p<0.05 *vs.* NR8383 group, $^{\#}p$<0.05, $^{\#\#}p$<0.01 *vs.* 10% DMSO group.

TSA Administration Reduced Levels of TNF-α in LPS-Induced NR8383 Cells

According to the results, the LPS induction remarkably enhanced TNF-α levels (Figure 4) and TNF-α mRNA expression (Figure 5A) compared to those in PBS treated NR8383 cells (p<0.01). The TSA treatments (1 ng/ml and 10 ng/ml) significantly reduced the TNF-α levels (Figure 4) and TNF-α mRNA expression (Figure 5A) compared to those in the LPS induced NR8383 cells (p<0.05). Meanwhile, 10 ng/ml TSA demonstrated more inhibitive effects on TNF-α expression (Figure 4, Figure 5A).

TSA Administration Enhanced MiR-146a Expression in LPS-Induced NR8383 Cells

The results showed that LPS induction significantly increased miR-146a mRNA expression compared to that in PBS treated NR8383 cells (Figure 5B, p<0.05). However, the TSA administrations significantly enhanced the miR-146a mRNA expressions compared to that in LPS induced NR8383 cells (Figure 5B, p<0.05), and 10 ng/ml TSA demonstrated more enhancive effects on miR-146a expression (Figure 5B).

Discussion

Acute lung injury is considered to be a severe pulmonary disorder accompanying acute inflammation and leading to respiratory system failure[19]. Although plenty of efforts have been carried out, the morbidity rates of acute lung injury patients are still higher[20]. Therefore, new investigations associated to lung injury are urgently needed to bring potential insight into acute lung injury treatment. The present data revealed that TSA administration could reduce the levels of TNF-α and enhanced miR-146a expressions in LPS-induced NR8383 cells, which would be benefit to the prevention of inflammation in progression of LPS-induced acute lung injury.

LPS, as a component for the Gram-negative bacteria, could induce the systemic inflammation and generate the lung injury model[21]. In this study, we established the LPS-induced acute lung

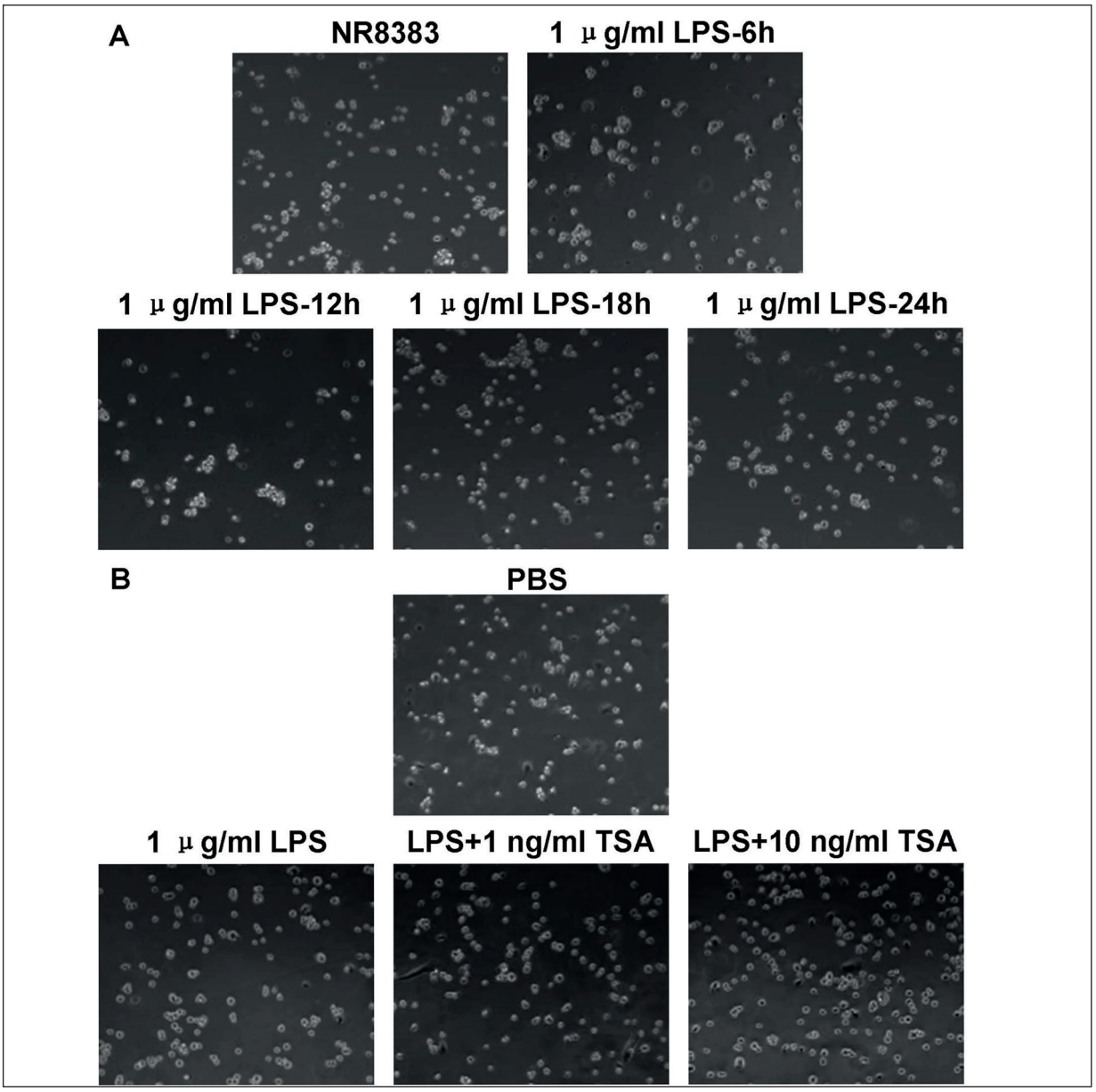

Figure 2. Evaluation for effects of LPS induction and TSA treatment on the NR8383 cells. **A,** Effects of LPS induction on NR8383 cells at 6 h, 12 h, 18 h and 24 h post the treatment. Magnification, 100×. **B,** Effects of TSA administrations on the LPS-induced NR8383 cells. Magnification, 100×.

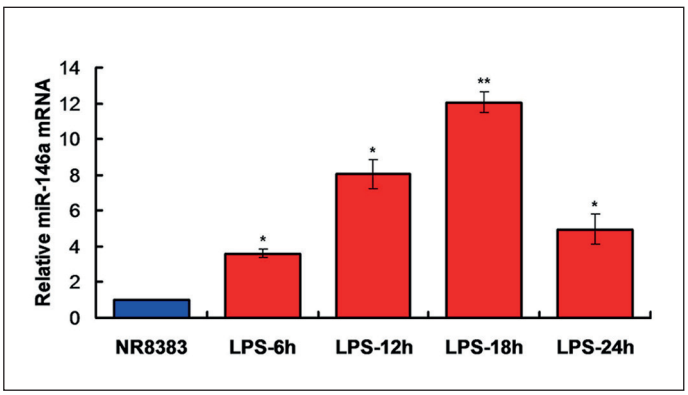

Figure 3. LPS induction increased the miR-146a mRNA expression in the NR8383 cells. This image presented the statistical analysis for the miR-146a mRNA expression in LPS-induced NR8383 cells. *$p<0.05$, **$p<0.01$ *vs.* NR8383 group.

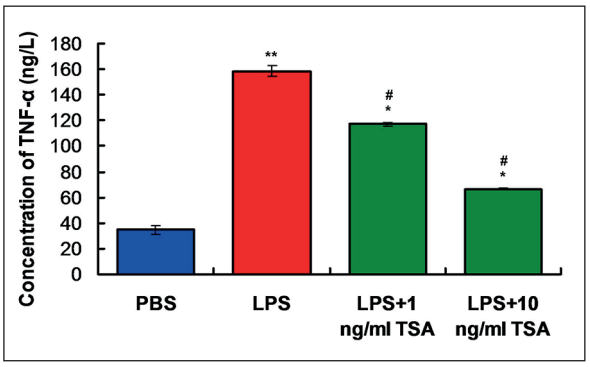

Figure 4. Determination for effects of TSA administration on TNF-α levels using ELISA test. This image presented the statistical analysis for the TNF-α levels in LPS-induced NR8383 cells. *p<0.05, **p<0.01 *vs.* PBS group, $^{\#}p$<0.05 *vs.* 10% LPS group.

injury model *in vitro* levels and examined the miR-146a expression in these NR8383 cell models. Our results showed that LPS induction cause the vacuolation, aggregation, and rupture; therefore, we selected 1 μg/ml LPS treating NR8383 cells for 6 h as the optimal inducible time point for generating acute lung injury model. Moreover, in order to exclude the additional effects of TSA on the growth of NR8383 cells, the CCK-8 assay was conducted to evaluated cell viability. The results indicated that 30 ng/ml and 50 ng/ml TSA treatment could significantly reduce the cell viabilities of NR8383 cells compared to that in the NR8383 group. However, both 1 ng/ml and 10 ng/ml TSA demonstrated no effects on NR8383 cell viabilities. Therefore, we selected the 1 ng/ml and 10 ng/ml TSA as the optimal concentrations for treating the NR8383 cells in the following experiments.

Zhao et al[22] reported that miR-146a could be activated by LPS by triggering the associated signaling pathway. In our study, the LPS induction also caused the remarkable increase of miR-146a, which also suggests that LPS successfully stimulated NR8383 cells. Targeting the TNF-α has been proven to be the promising therapeutic strategy for many diseases, such as diabetes, cancers, and cardiovascular diseases[23,24]. The present findings also showed that TSA administration significantly reduced the levels of TNF-α in LPS-induced NR8383 cells, which suggest that TSA might inhibit the LPS-caused inflammatory responses in NR8383 cells. Furthermore, according to Wu et al[17], miR-146a demonstrated the most drastic increasing expressions in NR8383 cells undergoing the TNF-α treatments. Meanwhile, El Gazzar et al[25] showed that miR-146a could target the molecules in lipopolysaccharide/Toll-Like receptor 4/nuclear factor kappa-B (LPS/TLR4/NF-kB) signaling pathway, which is a classic pathway for inflammatory response. Therefore, we also examined the miR-146a expression in the LPS-induced NR8383 cells. The data illustrated that TSA administration significantly enhanced miR-146a expression in the LPS-induced NR8383 cells. This result suggests that the TSA administration might prevent the inflammation by inhibiting TNF-α and triggering miR-146a expression in the LPS-induced NR8383 cells.

Conclusions

The above results demonstrated that TSA exerted the anti-inflammation functions in the LPS-induced acute lung injury model *in vitro*, which might be triggered by inhibiting TNF-α molecule and upregulating miR-146a expression. Therefore, TSA could be considered to be a potential therapeutic agent for treating acute lung injury.

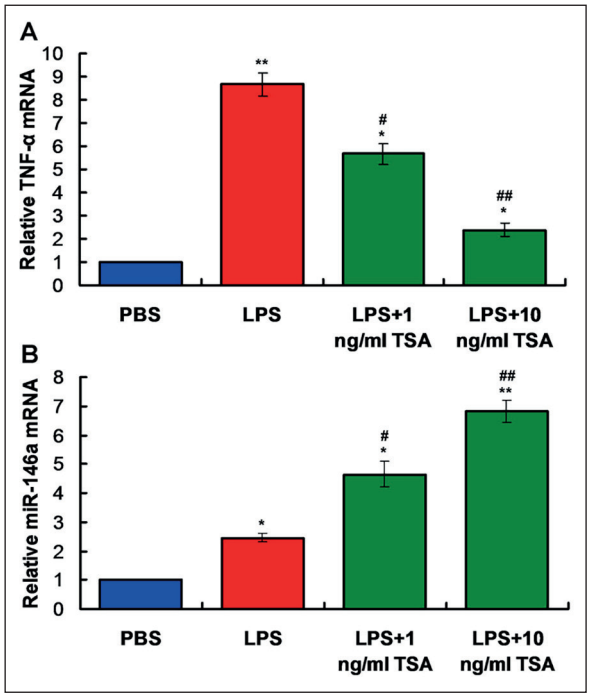

Figure 5. TSA administrations reduced TNF-α mRNA expression and miR-146a mRNA expression in the LPS-induced NR8383 cells using qRT-PCR assay. **A,** Statistical analysis for the TNF-α mRNA expression. **B,** Statistical analysis for the miR-146a mRNA expression. *p<0.05, **p<0.01 *vs.* PBS group, $^{\#}p$<0.05, $^{\#\#}p$<0.01 *vs.* 10% LPS group.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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