The effects of perfluorocarbon on ICAM-1 expression in LPS-induced A549 cells and the potential mechanism

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Abstract. Acute lung injury (ALI)/ARDS is a critical clinical syndrome with high mortality, and the effective therapeutic methods for the treatment remain limited. Previous studies have indicated that liquid ventilation with perfluorocarbon (PFC) may be advantageous over conventional mechanical ventilation in the treatment of ALI/ARDS. Additionally, PFC inhibits the inflammatory response caused by ALI/ARDS. However, the anti-inflammatory mechanism remains to be completely elucidated. In the present study, the aim was to determine the anti-inflammatory mechanism of PFC and the association with microRNA (miR). PFC was used to modulate LPS-induced A549 cells, with the cells divided into four groups: Untreated control group; LPS group, treated with 10 μg/ml LPS; LPS+PFC group, treated with 10 μg/ml LPS and PFC; and PFC group, treated with PFC alone. The intercellular adhesion molecule-1 (ICAM-1) mRNA and protein expression levels of each group were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting, respectively. A549 cells were transfected with miR-17-3p mimics, miR-17-3p inhibitors or negative controls to observe the alterations in the anti-inflammatory effects of PFC. A dual luciferase reporter gene assay was used to determine whether ICAM-1 is a target gene of miR-17-3p. PFC was observed to attenuate the mRNA and protein expression levels of ICAM-1 in LPS-induced A549 cells, with no significant effect on the untreated A549 cells. miR-17-3p was demonstrated to be regulated by PFC. Transfection with miR-17-3p mimics enhanced the anti-inflammatory effects of PFC, whereas the miR-17-3p inhibitor weakened the anti-inflammatory effects of PFC at early time points. To conclude, the current study indicates that ICAM-1 was a target gene of miR-17-3p, and PFC has anti-inflammatory effects. Additionally, the present study is the first report, to the best of our knowledge, that PFC is able to attenuate ICAM-1 expression in LPS-induced A549 cells by increasing miR-17-3p expression.

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Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are life-threatening diseases that present with progressive dyspnea, refractory hypoxemia and a marked increase in difficulties breathing (1,2). Since the initial description of ARDS in 1967 (3), there have been advances in the pathogenesis and treatment of this disease, however, challenges remain. Epidemiological investigations suggest that ARDS remains a significant health burden with substantial morbidity and mortality (4). The majority of patients require endotracheal intubation and positive pressure ventilation (1,2) and account for millions of days spent in intensive care units (5). In recent years, treatments of ALI/ARDS with improved efficacy have been sought, and studies have indicated that liquid ventilation with perfluorocarbon (PFC) compounds is a promising therapeutic approach (6-14). PFC is of interest for the treatment of ALI/ARDS due to the unique physicochemical properties, such as high solubility for oxygen, and the anti-inflammatory effects. Liquid ventilation or aerosolized PFC has been demonstrated to improve gas exchange and improve pulmonary compliance compared with conventional mechanical ventilation in a variety of animal models of ALI/ARDS (6-8). Specifically, PFC has been shown to reduce levels of cytokines, chemokines and other mediators of pulmonary inflammation in both *in vivo* and *in vitro* models (9-14). However, the understanding of the underlying mechanisms remains poor, in particular of the mechanisms associated with the PFC-induced anti-inflammatory effects. A limited number of mechanistic studies have indicated that PFC can reduce the activation of nuclear factor-κB and/or the Syk-phosphorylation pathway (15-17). Considering that ARDS is a complex inflammatory disease, PFC may serve an anti-inflammatory role through multiple mechanisms.

In the pathogenesis of ARDS, the early inflammatory responses, including the expression of adhesion molecules and cytokines in the lung with subsequent activation of neutrophils, serve a pivotal role. Intercellular adhesion molecule-1 (ICAM-1) is a cell surface glycoprotein that

is expressed on alveolar epithelial cells and vascular endothelium (18,19). Injury to the alveolar epithelium and vascular endothelium is of central importance in the pathogenesis of ALI/ARDS (20), and in lung injury, the expression of ICAM-1 in lung tissue was increased which is thought to serve an important role in neutrophil recruitment and trafficking into the lung (21). ICAM-1 may be a useful biomarker of ALI/ARDS. Clinical trials have demonstrated increased ICAM-1 expression in the setting of acute lung injury (22,23), and plasma and edema fluid levels of ICAM-1 are higher in patients with ALI compared with patients with hydrostatic pulmonary edema. Additionally, elevated plasma levels of ICAM-1 were associated with poor outcomes in patients with acute lung injury (24,25). In the present study, the effects of PFC on the expression of ICAM-1 were investigated in an *in vitro* model of ARDS.

The anti-inflammatory mechanism of action of PFC remains unclear. In recent years, the role of microRNAs (miRNAs) in inflammation have received increased interest. miRNAa are non-coding RNA molecules of approximately 22 nucleotides, which modulate gene expression at the post-transcriptional level in eukaryotic organisms. miRNAs control gene expression by pairing with partially complementary target sites in mRNA 3' untranslated regions (UTRs), resulting in translational repression and/or mRNA destabilization (26-28). With miRNA roles in developmental timing, cell apoptosis and cell proliferation, evidence is mounting that their regulatory effect is more prevalent than was previously suspected (26). Recent studies have indicated that miRNAs regulate inflammatory responses (29), and serve critical roles in inflammatory lung diseases including ALI/ARDS. Given their particularly recognized role in the regulation of immune and inflammatory responses, the present study hypothesized that PFC attenuates the expression of ICAM-1 in injured alveolar epithelial cells, a potential anti-inflammatory mechanism of PFC, through the influence of one or a number of miRNAs. A previous study has indicated that ICAM-1 was a target of tumor necrosis factor (TNF)-induced miR-17-3p; with specific antagonism of miR-17-3p increasing neutrophil adhesion to cultured endothelial cells. Conversely, transfection with mimics of miR-17-3p reduced neutrophil adhesion to endothelial cells (30). Therefore, the present study speculates that miR-17-3p may serve a key role in the mechanism of PFC attenuation of ICAM-1 expression in injured alveolar epithelial cells. To evaluate the hypothesis, the present study used A549 cells stimulated with lipopolysaccharide (LPS) as an *in vitro* model of ARDS. Due to the limited purity and viability of primary human alveolar epithelial cells, and the alterations in the morphological and biochemical characteristics of primary cells over time, a human pulmonary alveolar cell carcinoma cell line (A549) with epithelial type II cell properties was utilized instead of primary human alveolar epithelial cells. Bacterial LPS is a component of the outer envelope of all gram-negative bacteria, and therefore is a highly proinflammatory molecule. LPS is able to induce excessive inflammatory responses in tissue and cells, resulting in a series of pathophysiological alterations, including tissue functional disorders, disorganization, apoptosis and even cell necrosis. The present study observed the effects of PFC on the expression of ICAM-1 in LPS-induced A549 cells and aimed to determine the potential mechanism associated with miRNAs.

Materials and methods

Cell culture. The A549 human pulmonary alveolar cell carcinoma cell line with epithelial type II cell properties was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were grown as a monolayer on 6-well culture plates under conditions of 100% humidity and 5% CO_2 at 37˚C. Cells were cultured in high glucose-Dulbecco's modified Eagle's medium (HG-DMEM) (GE Healthcare Life Sciences, Logan, UT, USA) with 10% fetal calf serum (FCS; GE Healthcare Life Sciences), 100 U/ml penicillin, and 100 μg/ml streptomycin (both purchased from Beyotime Institute of Biotechnology, Haimen, China). The cells were harvested with 0.25% trypsin-ethylenediaminetetraacetic acid (GE Healthcare Life Sciences) to induce detachment. Following washing with DMEM containing 10% FCS, cells were centrifuged at 200 x g for 4 min at 25˚C and resuspended in fresh medium. The 293T human embryonic kidney cell line (American Type Culture Collection) was also used, and the culture conditions were the same as with the A549 cells.

PFC-in-DMEM suspension. Perfluorooctane (C_8F_{18}) (a type of perfluorocarbon) was purchased from Huajieshi Medical Treatment Facility Co., Ltd. (Shanghai, China). Perfluorooctane is a clear, colorless and odorless liquid and has a molecular weight of 438.06. At room temperature, its characteristics are the following: vapor pressure is 61 mmHg; surface tension is 12 mN/m; boiling point is 102.5˚C; and density 1.75 g/ml (31). As PFCs are water insoluble and cannot be used for cellular incubation, a PFC-DMEM suspension was used for subsequent experiments (32). DMEM containing 10% FCS was mixed with PFC at a ratio (v/v) of 9:1. The mixture was exposed to ultrasonic energy for 10 sec on ice at 21 kHz and 350 W in a transonic analogous ultrasonic unit (JY92-2D; Xinzhi Scientz Biotechnology Co., Ltd., Ningbo, China). Following mixing, the suspension appeared to be an emulsion. The number of droplets and size distribution were stable in the PFC-DMEM suspension (data not shown), however, two distinct liquid phases could be separated after several hours. To avoid this phase separation, a mini shaker was used (WuXiang Instrument and Meter Co., Ltd., Shanghai, China) to shake the culture plate continuously, which was necessary to guarantee the temporary mixing of the PFC-DMEM suspension and thus contact of PFC and DMEM with the cells.

LPS. LPS was extracted from *Escherichia coli* 055:B5 (Sigma-Aldrich, St. Louis, MO, USA); the concentration in the media and reagents used was 10 μg/ml.

Experimental protocol. A549 cells were divided into four groups as follows: i) Untreated control group; ii) LPS group, incubated with LPS at a final concentration of 10 μg/ml; iii) LPS+PFC group, incubated with LPS and PFC-DMEM suspension at the above mentioned concentrations; and iv) PFC group, incubated with the PFC-DMEM suspension.

mRNA and miRNA quantification by reverse transcription-quantitative polymerase chain reaction (RT-qRCR) analysis. RT-qPCR was used to assess ICAM-1 mRNA and miR-17-3p expression in A549 cells. Following

treatment, the cells of each group were harvested at 2, 4, 6 and 8 h (for analysis of mRNA expression) or 1, 2, 3, 4, 5, 6, 7 and 8 h (for analysis of miRNA expression). Total RNA was extracted from A549 cells using RNAiso Plus (Takara Biotechnology Co., Ltd., Dalian, China). For mRNA expression analysis, total RNA was reverse transcribed into cDNA using the PrimeScript® RT Reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd.). SYBR® Premix Ex TaqTM II (Takara Biotechnology Co., Ltd.) was used for qPCR for the mRNA quantification in A549 cells according to the manufacturer's instructions. β-actin served as the housekeeping gene. The following primers were used for RT-qPCR: β-actin (product size 215 bp), forward 5'-CAAAGACCTGTACGCCAACAC AGT-3' and reverse 5'-ACTCCTGCTTGCTGATCCACA TCT-3'; ICAM-1 (product size 151 bp), forward 5'-GCCCGA GCTCAAGTGTCTAA-3' and reverse 5'-GGAGAGCACATT CACGGCA-3'. For miRNA analysis, total RNA was reverse transcribed into cDNA using the miRcute miRNA First-Stand cDNA Synthesis kit [poly(A) polymerase method] (Tiangen Biotech Co., Ltd., Beijng, China). The miRcute miRNA qPCR Detection kit (SYBR Green; Tiangen Biotech Co., Ltd.) was used for qPCR. U6 served as the small RNA reference housekeeping gene. The kits and specific primers for miR-17-3p and U6 were purchased from Tiangen Biotech Co., Ltd.. The primer sequences were as follows: miR-17-3p (product size 22 bp), forward 5'-TGCGCACTGCAGTGAAGGCACT-3' and reverse 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACCTACA-3'; U6 (product size 101 bp), forward 5'-CGCTTCGGCAGCACATATAC-3' and reverse 5'-AATATGGAACGCTTCACGA-3'. The cDNA was amplified by PCR in an iQ5 Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The thermal cycling conditions of the mRNA PCR reaction were as follows: 5 Sec at 95˚ and 20 sec at 60˚C for 40 cycles; the thermal cycling conditions of the miRNA PCR reaction were as follows: 5 Sec at 95˚C and 20 sec at 60˚C for 45 cycles. All reactions were performed in triplicate, and relative expression of the RNAs was calculated using the $2^{-\Delta\Delta Cq}$ method (33,34).

Western blot analysis. Western blotting was used to assess ICAM-1 protein expression in A549 cells. Following treatment, cells of each group were harvested at 2, 4, 6 and 8 h for protein extraction. The protein content of each sample was determined using a bicinchoninic acid assay (Applygen Technologies, Inc., Beijing, China). A total of 30 μg protein were mixed with loading buffer, heated at 95˚C for 5 min for protein denaturation, and separated by 8% sodium dodecyl sulfate-polyacrylamide gel (Beyotime Institute of Biotechnology) electrophoresis. Separated proteins were transferred onto polyvinylidene fluoride membranes (Beyotime Institute of Biotechnology). Non-specific binding sites were blocked with 5% skimmed milk in Tris-buffered saline with 0.1% Tween-20. Protein expression was normalized to the housekeeping gene β-actin. Monoclonal mouse anti-human ICAM-1 IgG antibody (dilution, 1:1,000; sc-8439; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse anti-human β-actin IgG antibody (dilution, 1:10,000; ab1801; Abcam, Cambridge, MA, USA) were used for primary detection. Horseradish-conjugated goat anti-mouse IgG antibody (dilution, 1:5,000; sc-2005; Santa Cruz Biotechnology, Inc.) was used for secondary detection. Protein bands were visualized by enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA).

A549 cell transfection. A549 cells were transfected with 50 nM hsa-miR-17-3p mimic or with 100 nM hsa-miR-17-3p inhibitor (Guangzhou RiboBio Co., Ltd., Guangzhou, China) using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Control samples were transfected with a miRNA mimic negative control or miRNA inhibitor negative control (Guangzhou RiboBio Co., Ltd.). The cells were transfected for 6 h followed by two washes, then culturing continued with complete growth medium for 48 h. The effects of transfection were assessed by RT-qPCR. Following transfection, cells were divided into the following 4 groups: i) Control group; ii) LPS group; iii) LPS+PFC; and iv) PFC group, which were processed as detailed in the experimental protocol and harvested at 2, 4, 6 and 8 h after treatment for protein expression analysis.

Luciferase reporter gene assays. The pcDNA-LUC-ICAM-1 vector (containing the 3'UTR region of ICAM-1 downstream of the luciferase gene), pcDNA-LUC (empty vector) and *Renilla* vector (reference) were provided by Dr Yajaira Suárez (New York University, New York, NY, USA). Hsa-miR-17-3p mimic or miR negative control (50 nmol/l), and pcDNA-LUC-ICAM-1 or pcDNA-LUC (200 ng) and *Renilla* vector (10 ng) were co-transfected into 293T cells using Lipofectamine™ 2000 for 6 h, then cultured with complete growth medium for 48 h. The cells were divided into the following groups: i) Group A, co-transfected with pcDNA-LUC, miR-17-3p and *Renilla* vector; Group B, co-transfected with pcDNA-LUC, miR negative control and *Renilla* vector; Group C, co-transfected with pcDNA-LUC-ICAM-1, miR negative control and *Renilla* vector; and Group D, co-transfected with pcDNA-LUC-ICAM-1, miR-17-3p and *Renilla* vector. Luciferase activity analysis was performed using the Dual-Luciferase® Assay system (Promega Corporation, Madison, WI, USA). Relative luciferase activity was obtained by normalizing the *Renilla* luciferase activity to the firefly luciferase activity.

Statistics. Data are presented as the mean ± standard deviation. Significant differences were analyzed by employing Student's *t*-test; the comparison of multiple groups was performed using one-way analysis of variance. SPSS Statistics 17.0.1 software (SPSS, Inc., Chicago, IL, USA) was used for analysis. Data are expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of PFC on ICAM-1 expression in LPS-induced A549 cells. Considering that ICAM-1 serves an important role in ALI/ARDS, the effects of PFC on ICAM-1 expression were studied. ICAM-1 mRNA expression levels in A549 cells from each group at 2, 4, 6 and 8 h following treatment were assessed by RT-qPCR (Fig. 1). The mRNA expression of the control group at 2 h was used as a baseline to assess the relative mRNA expression of ICAM-1 of the other time pointes. Following stimulation with LPS, the mRNA

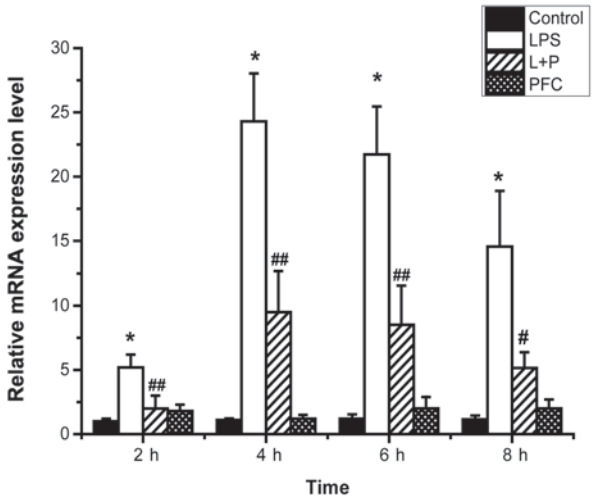

Figure 1. Alterations in the relative ICAM-1 mRNA expression in A549 cells in each group. The expression of mRNA in each group at 2, 4, 6 and 8 h following treatment was assessed by reverse transcription-quantitative polymerase chain reaction. The mRNA expression of the control group at 2 h was used as a baseline to assess the relative mRNA expression of ICAM-1 of the other groups. ICAM-1 mRNA expression was significantly increased in the LPS group compared with the control group at the different time points. The LPS+PFC group exhibited significantly lower ICAM-1 mRNA expression than the LPS group at 2, 4, 6 and 8 h after treatment. No effect of PFC alone was observed on the expression of ICAM-1. Values are presented as the mean ± standard deviation, n=3. *P<0.01 vs. the control group; #P<0.05, ##P<0.01 vs. the LPS group. ICAM-1, intercellular adhesion molecule-1; LPS, lipopolysaccharide; PFC, perfluorocarbon; L+P, LPS and PFC group.

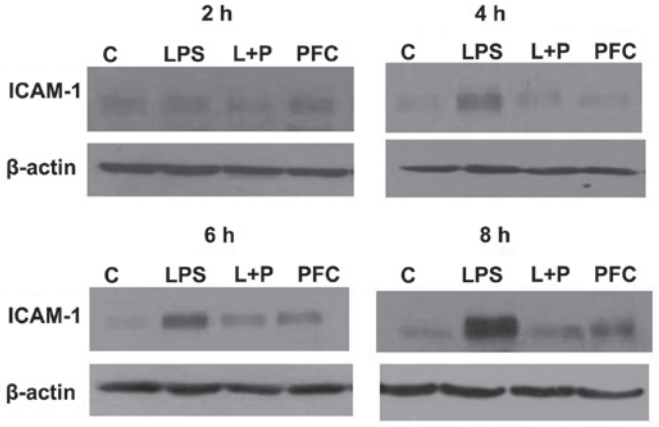

Figure 2. Alterations in ICAM-1 protein expression in A549 cells in each group. Western blotting was used to assess ICAM-1 protein expression of each group at 2, 4, 6 and 8 h following treatment. ICAM-1 protein expression was increased in the LPS group compared with the control group at 4, 6 and 8 h following treatment. The LPS+PFC group exhibited lower ICAM-1 protein expression than the LPS group at 4, 6 and 8 h. ICAM-1, intercellular adhesion molecule-1; LPS, lipopolysaccharide; PFC, perfluorocarbon; L+P, LPS and PFC group.

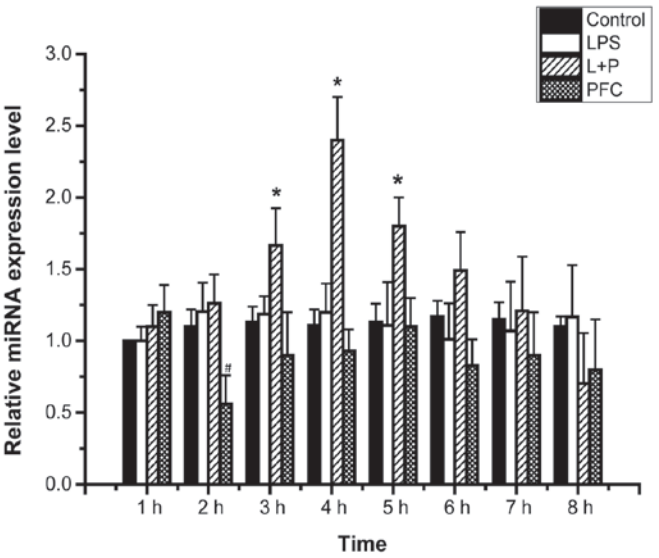

Figure 3. Alterations in miR-17-3p expression in A549 cells in each group. The expression miRNA in each group from 1-8 h following treatment was assessed by reverse transcription-quantitative polymerase chain reaction. The expression of miR-17-3p at 1 h in the control group served as the baseline to assess the relative mRNA expression of miR-17-3p in the other groups. The LPS+PFC group exhibited significantly higher miR-17-3p expression compared with the LPS group at 3, 4 and 5 h following treatment. The PFC group exhibited significantly lower miR-17-3p expression than the control group 2 h after treatment. Values are presented as the mean ± standard deviation, n=3. *P<0.05 vs. the LPS group. miR, microRNA; LPS, lipopolysaccharide; PFC, perfluorocarbon; L+P, LPS and PFC group.

expression of ICAM-1 was significantly increased in the LPS group compared with the control group (P<0.01). The expression of ICAM-1 mRNA peaked at 4 h after stimulation and then gradually reduced in the LPS and LPS+PFC groups. The LPS+PFC group exhibited significantly lower ICAM-1 expression levels than the LPS group at 2, 4, 6, (P<0.01) and 8 h (P<0.05) following treatment. There was no difference observed between the PFC group and the control group (P>0.05) in ICAM-1 mRNA levels.

Western blotting was used to assess ICAM-1 protein expression in A549 cells from each group at 2, 4, 6 and 8 h after treatment (Fig. 2). ICAM-1 protein expression was near undetectable in the control group, however, following stimulation by LPS, the expression of ICAM-1 was increased in the LPS group compared with the control group at 4, 6 and 8 h after treatment. The LPS+PFC group exhibited lower protein expression of ICAM-1 than the LPS group at 4, 6 and 8 h after treatment. There was no difference observed between the PFC group and control group in ICAM-1 protein levels.

In summary, LPS induces the expression of ICAM-1 in A549 cells. PFC attenuates ICAM-1 mRNA and protein expression levels in LPS-induced A549 cells, with no significant effect on untreated A549 cells.

Effects of PFC on miR-17-3p expression in LPS-induced A549 cells. A previous study reported that ICAM-1 was a target of TNF-induced miR-17-3p (30), therefore miR-17-3p may serve a key role in the mechanism of PFC attenuation of ICAM-1 expression in LPS-induced A549 cells. To evaluate this hypothesis, the effect of PFC on miR-17-3p expression in LPS-induced A549 cells was investigated. The expression levels of miRNA in A549 cells of each group from 1-8 h following treatment were assessed by RT-qPCR (Fig. 3). The expression level of miR-17-3p at 1 h in the control group served as the baseline to assess the relative mRNA expression of miR-17-3p of the other groups. The LPS+PFC group exhibited significantly higher miR-17-3p expression compared with the LPS group at 3, 4 and 5 h following

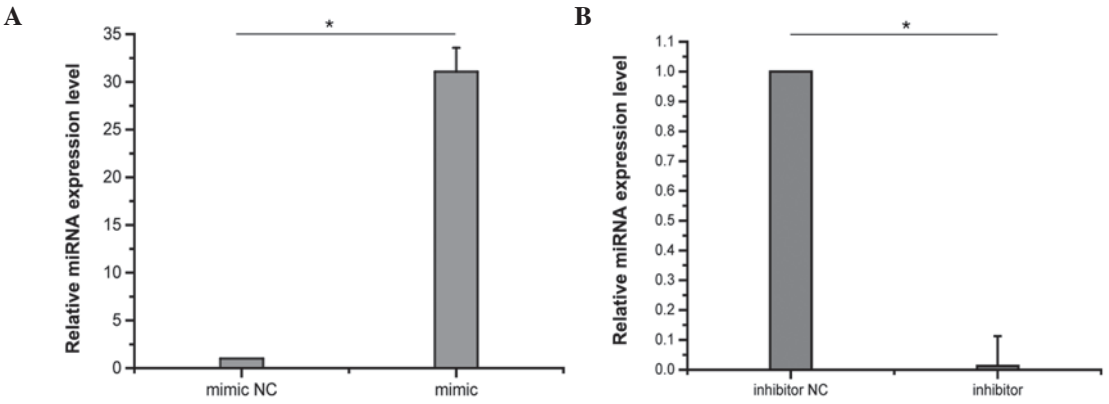

Figure 4. Alterations in miR-17-3p expression in A549 cells following 48 h of transfection, assessed by reverse transcription-quantitative polymerase chain reaction. (A) A549 cells were transfected with miR-17-3p mimics or negative control. The levels of miR-17-3p increased following 48 h of transfection compared with the negative control. (B) A549 cells were transfected with miR-17-3p inhibitors or the negative control. The levels of miR-17-3p were reduced following 48 h of transfection compared with the negative control. Values are presented as the mean ± standard deviation, n=3. *P<0.01. miR, microRNA; NC, negative control.

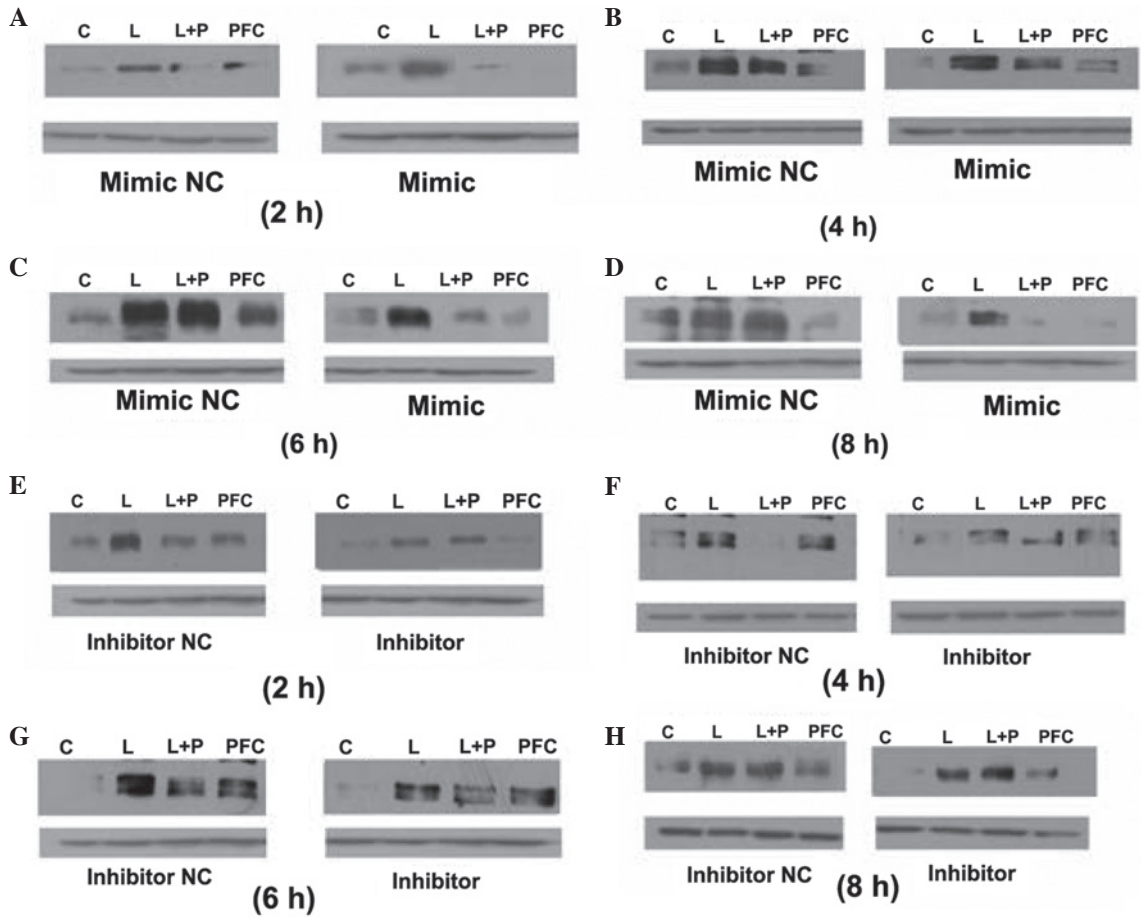

Figure 5. The effects of PFC on ICAM-1 expression in LPS-induced A549 cells following miR-17-3p transfection. Western blotting was used to assess protein expression in A549 cells. (A-D) A549 cells were transfected with 50 nM miR-17-3p mimics or mimic negative control. The anti-inflammatory effects of PFC were greater in the mimic group than the negative control group at each time point. (E-H) A549 cells were transfected with 100 nM miR-17-3p inhibitors or inhibitor negative control. The anti-inflammatory effects of PFC were greater in the inhibitor negative control group compared with the inhibitor group at 2 and 4 h, however, not at 6 and 8 h. PFC, perfluorocarbon; ICAM-1, intercellular adhesion molecule-1; miR, microRNA; C, control; L, lipopolysaccharide; L+P, lipopolysaccharide and PFC group; NC, negative control.

treatment (P<0.05). This indicates that PFC increases the expression of miR-17-3p in LPS-induced A549 cells. There was no difference observed between the PFC group and the control group in the expression levels of miR-17-3p (P>0.05).

Effects of PFC on ICAM-1 expression in LPS-induced A549 cells following miR-17-3p transfection. To determine the role of miR-17-3p in the anti-inflammatory effects of PFC, the effects of PFC on ICAM-1 expression in LPS-induced A549 cells

after miR-17-3p transfection were investigated. A549 cells were transfected with 50 nM miR-17-3p mimic or with 100 nM miR-17-3p inhibitor. Control samples were transfected with miR mimic negative control or inhibitor negative control. The effects of transfection on miRNA expression levels were assessed by RT-qPCR. Following 48 h of transfection, miR-17-3p levels were markedly increased in A549 cells transfected with the miR-17-3p mimic compared with the negative control, and were reduced by the transfection with the miR-17-3p inhibitor ($P<0.01$; Fig. 4). Following transfection, cells were divided into 4 groups as detailed in the experimental protocol and were harvested at 2, 4, 6 and 8 h after treatment for protein expression analysis.

Western blotting was used to assess ICAM-1 protein expression in the A549 cells following transfection (Fig. 5). In the cells transfected with miR-17-3p mimics or the mimic negative control, the anti-inflammatory effects of PFC were greater in the mimic group compared with the negative control group at each time point (Fig. 5A-D). In the cells transfected with the miR-17-3p inhibitor or the inhibitor negative control, the anti-inflammatory effects of PFC were greater in the inhibitor negative control group compared with the inhibitor group at 2 and 4 h (Fig. 5E and F), however, were not at 6 and 8 h (Fig. 5G and H). In summary, transfection of the miR-17-3p mimic in A549 cells enhanced the anti-inflammatory effects of PFC, whereas the miR-17-3p inhibitor weakened the anti-inflammatory effects of PFC at early time points.

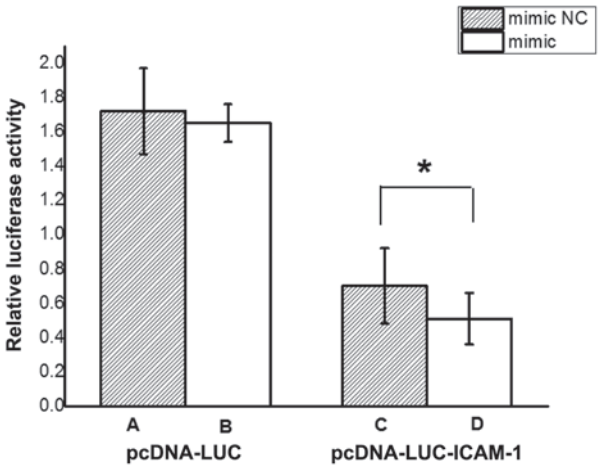

Figure 6. Interaction between miR-17-3p and ICAM-1 3'UTR assessed by luciferase reporter gene assays. The 293T cells were transfected for 6 h. At 48 h post-transfection, the relative luciferase activity analysis of each group was obtained by normalizing the *Renilla* luciferase activity to the firefly luciferase activity. Group A was co-transfected with pcDNA-LUC, miR-17-3p, and the *Renilla* vector; Group B was co-transfected with pcDNA-LUC, miR negative control and the *Renilla* vector; Group C was co-transfected with pcDNA-LUC-ICAM-1, miR negative control and the *Renilla* vector; and Group D was co-transfected with pcDNA-LUC-ICAM-1, miR-17-3p, and the *Renilla* vector. The relative luciferase activity of group D was significantly lower than group C, whereas there was no difference between group A and B. Values are presented as the mean ± standard deviation, n=3. $^{*}P<0.05$. miT, microRNA; ICAM-1, intercellular adhesion molecule-1; NC, negative control.

Interaction of miR-17-3p with the ICAM-1 3'UTR. Luciferase reporter gene assays were used to assess the interaction between miR-17-3p and the ICAM-1 3'UTR. miR-17-3p mimics or the mimic negative control, and pcDNA-LUC-ICAM-1 or pcDNA-LUC, and *Renilla* vector were co-transfected into 293T cells. Following 48 h of transfection, the relative luciferase activity analysis of group A was 1.65±0.25; group B was 1.72±0.22; group C was 0.7±0.15; and group D was 0.51±0.11. The relative luciferase activity of group D was significantly lower than group C ($P<0.05$), whereas there was no difference between groups A and B ($P>0.05$). These data suggest an interaction between miR-17-3p and the ICAM-1 3'UTR, and that ICAM-1 is a target gene of miR-17-3p.

Discussion

In the present study, the effects of PFC on the expression of ICAM in LPS-induced A549 cells were observed, with the aim of determining the potential anti-inflammatory mechanism. The significant observations include the following: i) As PFC is insoluble in water, cell-culture experiments with A549 cells should be performed using a PFC-DMEM suspension; ii) LPS induces ICAM-1 production in A549 cells, and PFC attenuates ICAM-1 mRNA and protein expression levels in LPS-induced A549 cells, with no significant effect on untreated cells; iii) PFC increased the expression of miR-17-3p in LPS-induced A549 cells; iv) miR-17-3p mimics enhanced the anti-inflammatory effects of PFC, whereas miR-17-3p inhibitors weakened the anti-inflammatory effects of PFC at early time points; and v) ICAM-1 is a target of miR-17-3p. Thus, it may be concluded that PFC attenuates ICAM-1 expression in LPS-induced A549 cells, and increased miR-17-3p expression may be an associated mechanism.

ALI/ARDS is a critical clinical syndrome with progressive dyspnea, refractory hypoxemia and high mortality. The pathological features include inflammatory reaction and alveolar-capillary membrane injury resulting from severe infection, trauma and shock. Alveolar epithelial cells are an important cell group associated with lung injury, and in addition to being the target of inflammatory cells and mediators, are active inflammatory cells and effector cells. They serve important roles in the occurrence and progression of ALI/ARDS (35,36). Previous studies have shown that alveolar epithelial cells are stimulated to produce a number of inflammatory mediators such as TNF-α, ICAM-1, monocyte chemotactic protein-1, interleukin (IL)-8 and IL-6 (35,36). Certain reports have indicated that ICAM-1 serves an important role during the entire development and progression of ALI/ARDS (37-40). ICAM-1 is a cell surface glycoprotein that belongs to the immunoglobulin superfamily of adhesion molecules. ICAM-1 is the counter-receptor for the β_2-integrins, lymphocyte function-associated antigen and macrophage-1 antigen, and is involved in leukocyte trafficking and lymphocyte activation (41). Under physiological conditions, ICAM-1 is expressed at low levels in epithelial cells (42,43) and is induced by cytokines (TNF-α, IL-1) and bacterial LPS (44). The increased expression of ICAM-1 on epithelial cells is a prerequisite for leukocyte trafficking through the endothelial and epithelial barrier in ALI/ARDS, and facilitates the adhesion and activation of leukocytes (mainly polymorphonuclear leukocytes) and pulmonary vascular endothelial cells in addition to inducing the "cascade effect" of inflammatory mediators in the lung (37-40). Therefore, the measurement of soluble ICAM-1

levels may be useful for identifying the patients with ALI at the highest risk of poor outcomes (43,44). As ICAM-1 serves an important role in ALI/ARDS, the inhibition of ICAM-1 expression may contribute to the prevention and treatment of ALI.

In the current study, the aim was to observe the effects of PFC on the expression of ICAM-1 in LPS-induced alveolar epithelial cell and to determine the potential mechanism. The A549 cells (a human pulmonary alveolar cell carcinoma cell line with epithelial type II cell properties) were stimulated with LPS as an *in vitro* model of ARDS. From this model, PFC was observed to have a protective effect on alveolar epithelial cells. Additionally, PFC attenuates ICAM-1 mRNA and protein expression in LPS-induced A549 cells. Further experiments indicated that PFC increased the production of miR-17-3p in LPS-induced A549 cells, and that transfection with a miR-17-3p mimic enhanced the anti-inflammatory effects of PFC, whereas transfection with a miR-17-3p inhibitor weakened the anti-inflammatory effects of PFC at early time points. To conclude, miR-17-3p may be involved in the anti-inflammatory effects of PFC by targeting ICAM-1. However, the underlying mechanisms of how PFC affects miR-17-3p remain unknown.

miR-17-3p has 22 nucleotides, and there are several studies about its functions (45). A study by Suárez *et al* (30) has shown that ICAM-1 is a target of TNF-induced miR-17-3p in endothelial cells. Jiang and Li (46) confirmed that miR-17-3p expression is regulated by TNF-α and LPS in HeLa cells. In addition, miR-17-3p induces carcinoma by targeting vimentin (47), mediates stress responses by targeting MDM2 (48), and inhibits angiogenesis by downregulating fetal liver kinase-1 (49). In certain clinical studies, circulating miR-17-3p in serum has been indicated to be a potential non-invasive biomarker for colorectal cancer screening (50,51). Other studies have shown that the miR-17-92 cluster, which encodes miR-17-3p (and 6 other miRNAs), is expressed in numerous mammalian tissues, and this cluster contributes to the development of the heart, lungs, blood vessels and the immune system (52,53). In addition, miR-17-3p is able to induce tumorigenesis (54,55) and alters the expression of cell-cycle-related genes (56). As ARDS is a complex inflammatory disease, and miRNAs serve critical roles in the inflammatory response, it was suggested that influencing miR-17-3p may be an anti-inflammatory mechanism of PFC.

Despite considerable investigation, new treatments have not been discovered, and respiratory support remains the predominant method of treating ALI/ARDS. Liquid ventilation with PFC has been used clinically for over 50 years, and shows potential for the treatment of ARDS. In recent years, the major studies regarding liquid ventilation have focused on animal experiments or *in vitro* experiments with few clinical trials. Based on these animal studies and cell experiments, liquid ventilation holds promise to improve low pulmonary compliance and gas exchange in addition to reducing pulmonary inflammatory responses, and ultimately improving the prognosis of animals with ARDS (6-14). However, the results of clinical randomized controlled trials (RCTs) and basic research have produced conflicting results, and the outlook of liquid ventilation has been disappointing. Results from clinical trials did not demonstrate an improved outcome of partial liquid ventilation in patients with ARDS compared with conventional mechanical ventilation (57-59). In addition, no significant differences in the number of days free from the ventilator, incidence of mortality or any pulmonary-related parameter were observed (57-59). Although these results may seem discouraging, it is too early to conclude that there is no effect of PFCs in patients with ARDS. Further multi-center clinical RCTs are required to provide the most convincing evidence. A phase II clinical trial of perfluorocarbon inhalation treatment of ALI/ARDS is underway at the Chinese People's Liberation Army General Hospital (Beijing, China) (ClinicalTrials.gov identifier: NCT 01391481), and the results are awaited. As a novel treatment, the mechanism of action of liquid ventilation is not fully understood and further basic and clinical research is required.

In summary, the present study observed that PFC attenuates ICAM-1 mRNA and protein expression in LPS-induced A549 cells. Notably, for the first time, to the best of our knowledge, miR-17-3p was reported to be involved in the anti-inflammatory effects of PFC. Transfection with a miR-17-3p mimic enhanced the anti-inflammatory effects of PFC, whereas transfection with a miR-17-3p inhibitor weakened the anti-inflammatory effects of PFC at early time points. In addition, miR-17-3p administration was demonstrated to inhibit the expression of ICAM-1, and miR-17-3p was shown to target ICAM-1. In the present study, evidence of the biological anti-inflammatory effects are reported, which improve the understanding of the protective effects of PFC. These findings are likely to have important implications in the application of PFC in the treatment of ALI/ARDS, and further investigation of miRNAs and PFC will pave the way to developing a novel therapeutic approach to the treatment of ALI/ARDS.

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