Therapeutic Effect of Intravenous Infusion of Perfluorocarbon Emulsion on LPS-Induced Acute Lung Injury in Rats

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Abstract

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS) are the leading causes of death in critical care. Despite extensive efforts in research and clinical medicine, mortality remains high in these diseases. Perfluorocarbon (PFC), a chemical compound known as liquid ventilation medium, is capable of dissolving large amounts of physiologically important gases (mainly oxygen and carbon dioxide). In this study we aimed to investigate the effect of intravenous infusion of PFC emulsion on lipopolysaccharide (LPS) induced ALI in rats and elucidate its mechanism of action. Forty two Wistar rats were randomly divided into three groups: 6 rats were treated with saline solution by intratracheal instillation (control group), 18 rats were treated with LPS by intratracheal instillation (LPS group) and the other 18 rats received PFC through femoral vein prior to LPS instillation (LPS+PFC group). The rats in the control group were sacrificed 6 hours later after saline instillation. At 2, 4 and 6 hours of exposure to LPS, 6 rats in the LPS group and 6 rats in LPS+PFC group were sacrificed at each time point. By analyzing pulmonary pathology, partial pressure of oxygen in the blood (PaO2) and lung wet-dry weight ratio (W/D) of each rat, we found that intravenous infusion of PFC significantly alleviated acute lung injury induced by LPS. Moreover, we showed that the expression of pulmonary myeloperoxidase (MPO), intercellular adhesion molecule-1 (ICAM-1) of endothelial cells and CD11b of polymorphonuclear neutrophils (PMN) induced by LPS were significantly decreased by PFC treatment *in vivo*. Our results indicate that intravenous infusion of PFC inhibits the infiltration of PMNs into lung tissue, which has been shown as the core pathogenesis of ALI/ARDS. Thus, our study provides a theoretical foundation for using intravenous infusion of PFC to prevent and treat ALI/ARDS in clinical practice.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Acute lung injury/acute respiratory distress syndrome (ALI/ARDS), which were firstly described in 1967, clinically manifest as respiratory distress and refractory hypoxemia [1]. The condition is defined by a series of pathological changes, including pneumone-dema and micro-atelectasis, which are caused by diffuse injury of alveolar-capillary membrane due to severe infection, trauma, shock, acidosis or toxic inhalation [2,3]. As a clinical complication of severe ALI, ARDS is a leading cause of morbidity and mortality in critically ill patients [4,5]. It is initiated by injury to the lung, often in the setting of pneumonia or sepsis, which can result in pulmonary edema and significant hypoxia [3]. Although positive end-expiratory pressure (PEEP) [6], high frequency oscillatory ventilation (HFOV) [7,8] and prone positioning [9,10] have been used to relieve the severe hypoxia due to ALI/ARDS, none of these interventions is able to decrease mortality in randomized clinical trials [3,7–9]. Despite extensive efforts have been made in experimental and clinical medicine, there are no effective pharmacological treatments for ALI/ARDS existing to date. Multiple therapeutic attempts, including inhaled

nitric oxide [11,12], corticosteroids [13], β2-agonists [14,15], surfactants [16], and immunomodulating agents such as IL-10 [17] have failed.

Perfluorocarbon (PFC), a molecule consisting wholly of fluorine and carbon, is characterized by high gas solubility, fast release, low surface tension, high volume-quality, average volatility, good histocompatibility and the absence of absorption and metabolism *in vivo* [18,19]. Because its capability of dissolving large amounts of physiologically important gases (mainly oxygen and carbon dioxide), PFC has been used for liquid breathing medium [20,21]. Liquid breathing has been proposed as a means of improving gas exchange in infants with acute respiratory failure since the 1970s. Greenspan *et al.* reported that application of liquid perfluorochemical ventilation in human preterm neonates with ARDS resulted in marked improvement in lung distensibility and oxygenation [22]. Partial liquid ventilation (PLV) using PFC and vaporized PFC inhalation have also been proven to improve gas exchange and survival in infants with severe respiratory distress syndrome [23–25]. However, these interventions need establish-

ment of artificial airway to facilitate PFC into the lungs, which is not suitable for treating patients with early ALI.

PFC emulsions with exceptionally small particles, which could be infused intravenously, have been clinically evaluated as artificial oxygen carriers to reduce allogeneic blood transfusions or to improve tissue oxygenation [26]. Fluosol DA-20%, a 20% w/v PFC emulsion comprising 14% w/v perfluorodecalin and 6% w/v perfluorotripropylamine emulsified primarily with a synthetic poloxamer, was the first successful commercial development of an injectable PFC emulsion. It was approved for intravenous use by the United States Food and Drug Administration as "oxygen therapeutic" for treatment of myocardial ischemia at the time of balloon angioplasty in 1989 [27]. However, it was withdrawn in 1992 because of cumbersome preparation (stored frozen) and the application of autoperfusion catheters, which make it no need to use PFC during balloon angioplasty [28,29]. However, due to the multiple potential use of PFC, on-going efforts continue to try to get a safe PFC for use in humans. Recently, Oxygent, an improved second-generation concentrated PFC emulsion based on perflu-bron (perfluorooctyl bromide; C8F17Br), was developed, which has a shelf-life of up to 2 years at 2°C to 8°C. Oxygent is initially designed for use as a temporary intravenous oxygen carrier, but there are many attractive potential use for Oxygent, such as to treat tissue ischemia, augment tumor PO_2 levels to enhance sensitivity to radiation and chemotherapy, preserve tissues and prolong storage time of an organ (e.g., kidney) prior to transplantation [29]. Previous studies have shown that PFC can be incorporated in breathing medium during liquid ventilation in patients or experimental animals with ALI/ARDS [23–25]. However, it is not known whether intravenous infusion of PFC emulsions can protect from ALI when administered at the early stage of disease process.

In this study, we investigated the effect of intravenous infusion of PFC emulsion on LPS induced ALI in rats and explored the potential molecular mechanisms of its action. Our results demonstrated that PFC infusion could significantly relieve LPS induced acute lung injury, improve gas exchange, and attenuate inflammatory reactions.

Materials and Methods

Ethics statement

All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health. The study protocol was approved by the Laboratory Animal Ethics Committee of Affiliated Hospital of Logistical College of Chinese People's Armed Police Forces. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

PFC emulsion

PFC emulsion is purchased from Double Crane Pharmaceuticals, Beijing, China, which is an improved second-generation concentrated PFC emulsion based on perflubron (perfluorooctyl bromide; C8F17Br). The current 60% w/v perflubron-based formulation (AF0144) is emulsified with phospholipid as the only surfactant, has an initial median particle diameter of 0.16 to 0.18 μm.

Animal model

Forty two male Wistar rats (6 weeks old, 200±51 g) were randomly divided into three groups: control group (n = 6), LPS group (n = 18) and LPS+PFC group (n = 18). Rats in the LPS group were anesthetized by intraperitoneal injection with 2% pentobarbital at a dose of 40 mg/kg. The tracheas were gradually freed from surrounding tissues and instilled with 2 mg/mL of LPS (from *E. coli* O111:B4, Sigma, St. Louis, MO) at a dose of 1 mg/kg. The rats were placed upright and their bodies were rotated by hand to ensure good distribution of LPS in lung. Rats in LPS+PFC group received PFC emulsion through the femoral vein at 6 mL/kg, 30 min prior to LPS instillation as described above. At 2, 4 and 6 hours of exposure to LPS, 6 rats in the LPS group and 6 rats in LPS+PFC group were anesthetized by pentobarbital again and then blood was collected from abdominal aorta. After blood collection, the rats were sarrificed right away and lung tissues were collected. In control group, the rats were treated the same as in LPS group, except LPS is substituted with an equal volume of normal saline (NS) and the rats were sacrificed 6 hours later.

Blood gas analysis

One mL Blood from abdominal aorta was collected after 2, 4 and 6 hours of LPS treatment or 6 hours of NS treatment. PaO2 was measured by using a Radiometer ABL 625 Blood Gas Analyzer (Copenhagen, Denmark).

Analysis of wet-to-dry weight ratio of lung tissue

After NS or LPS treatment, the rats were killed and the left lung was isolated. After blotting off blood and other contaminants, the wet weight of lung tissue was measured. Then the lung was dried in a 70°C oven for 72 h and the dry weight was measured. The wet/dry weight ratio of lung was calculated.

Determination of myeloperoxidase expression in lung tissue

After the rats were sacrificed, the right lower lobe of lung was isolated and snap-frozen. The expression of myeloperoxidase (MPO) in lung was detected using enzyme-linked immunosorbent assay (ELISA, Bluegene, China) according to the manufacturer's instructions. Briefly, the lung tissue were homogenized and centrifuged at 12,000 g for 15 min at 4°C. The supernatants were added into a microtiter plate (100 μl/well) precoated with a murine anti-MPO mAb, then added 10 μl balance buffer and 50 μl enzyme conjugate to each well. After incubation for 1 h at 37°C, the plate was washed for 5 times followed by addition of the substrate and stop solution. Optical density (OD) at 450 nm was measured using a microplate reader. All the samples were assayed in triplicate.

Detection of CD11b expression on polymorphonuclear neutrophils by flow Cytometry

Fifty μL of anticoagulated blood from abdominal aorta of rats was incubated with 10 μL of mouse anti-rat monoclonal CD11b-FITC antibody or mouse IgG$_1$-FITC (Santa Cruz Biotechnology, Santa Cruz, USA) for 15 min in the dark at room temperature. 450 μL hemolysin was added to the mixture, followed by 10 min incubation. After centrifugation at 1200 rpm for 5 min, supernatant was discarded and the pellet was washed with phosphate buffered solution (PBS) buffer once and suspended in 450 μL of PBS buffer for flow Cytometry analysis. Data were analyzed using Cell Quest software (BD Biosciences). CD11b expression levels were presented as mean fluorescent intensity (MFI) of positive cells.

Histology and ICAM-1 immunostaining

The upper and middle lobes of right lung were fixed in 10% formalin for 24 h. The tissues were dehydrated, embedded in
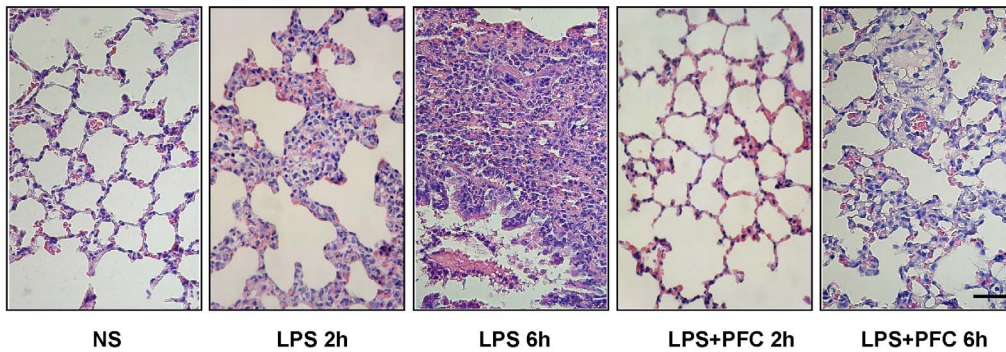
Figure 1. Histological examination of the lung sections from the rats treated by NS, LPS or LPS+PFC. The rats were treated with NS (n = 6) or LPS (n = 36) by intratracheal instillation. 18 rats received PFC through femoral vein prior to LPS instillation (LPS+PFC). Lung tissues were collected at the indicated time points after exposure to LPS and stained with HE. Scale bar, 25 μm.
doi:10.1371/journal.pone.0087826.g001

paraffin and cut into 5 mm sections. The tissues were stained with hematoxylin and eosin (HE staining) after deparaffinization, and evaluated under an optical microscopy (Olympus BX51, Japan).

The expression of ICAM-1 in lung was determined by immunostaining. After deparaffinization and rehydrating, paraffin sections were placed into a pressure cooker containing antigen retrieval buffer (0.01 M citrate buffer, pH 6.0), cooked with full pressure for 2 minutes to unmask antigens. Immunostaining was performed by incubating the sections with mouse anti-rat ICAM-1 monoclonal antibody (1:200, Abcam, MA) overnight at 4°C, biotin-conjugated secondary antibody (ZSGB-bio, China) at 37°C for 1 h, and streptavidin-HRP (ZSGB-bio, China) at 37°C for 30 min. 3,3-Diaminobenzidine (DAB, ZSGB-bio, China) was then used to visualize immunohistochemical staining. Cell nuclei were counterstained with hematoxylin. Images were obtained with an Olympus BX51 microscope and the proportion of positive staining cells was analyzed with Image-Pro plus 5.1 software. The expression of ICAM-1 in lung tissue was presented as mean photodensity.

For histology and immunostaining analysis, the slides were renamed by arabic number followed by a double-blinded examination by two pathologists.

Statistical analysis

Data were analyzed using Statistical Product and Service Solutions (SPSS) statistical software version 13.0 (SPSS Inc., Chicago, IL) and expressed as mean ± standard deviations (SD). Within-group comparisons were analyzed using single factor analysis of variance and between–group comparisons were analyzed using one-sample t-test. Within group comparisons of results at different time intervals were analyzed using F test. P values of less than 0.05 were considered to be statistically significant.

Results

General observations

After 6 hours of treatment with normal saline or LPS, rats in the control group developed tachypnea that disappeared rapidly without any abnormal secretion, whereas the rats in LPS group exhibited tachypnea, cyanotic limbs and lips, mouth and nasal hemorrhage. The rats treated with LPS also had matted hair, slow reactions, listlessness and they rejected water. In addition, three of the rats in LPS group developed diarrhea. Rats in LPS+PFC group were more alert than those in LPS group, and they were

able to escape capture as well as had no evidence of oral or nasal hemorrhage.

Histopathological observation of lung tissue

We examined the histology of lung tissues of all the rats in this study by hematoxylin and eosin staining (Figure 1). There was no obvious difference in the lung tissue of rats treated with normal saline (NS) compared with normal untreated rats (data not shown). However, in LPS group the rats developed expansion and congestion of pulmonary minute vessels and alveolar septum capillaries. The alveolar walls burst, and the alveolar space was narrowed. In addition, partial alveoli were damaged and the alveolar septum was broadened. There was significant infiltration of PMN cells in the alveoli and pulmonary interstitial tissue, accompanied by hemorrhage and pulmonary interstitial edema. The injury got more and more serious with increased time of exposure to LPS. Importantly, we found that pretreatment of PFC emulsion markedly reduced the severity of pulmonary histopathological injury induced by LPS. In the lung of rats in LPS+PFC groups, there is wider alveolar space, less PMN cells infiltration, thinner alveolar septae and reduced hemorrhage comparison with the lung of rats only treated with LPS, as shown in Figure 1.

Arterial blood gas analysis

PaO2 is a measurement of the partial pressure of oxygen dissolved in plasma, which indicates how much oxygen was available in alveoli to dissolve in blood. We collected blood from abdominal aorta after NS or LPS treatment and analyzed PaO2. We found that PaO2 in the LPS group was significantly reduced compared to that in the control group (P<0.01) and it further decreased with longer exposure to LPS (Figure 2A). However, PFC treatment significantly increased PaO2 values in LPS+PFC group compared with LPS group after 2, 4 and 6 h of exposure to LPS (P<0.01) (Figure 2A), which may be due to improved O2 transport by PFC as well as the reduced lung injury as described above.

Determination of lung wet-to-dry weight ratio

The lung wet-to-dry (W/D) weight ratio was used as an index of lung water accumulation, which is an indicator of the degree of lung edema. As shown in Fig 2B, the W/D ratio was significantly higher in LPS group compared with control group, and it further increased with longer time of exposure to LPS (P<0.05 or 0.01). After 4 and 6 hours of exposure to LPS, the lung W/D ratio in LPS+PFC group was significantly (P<0.05 or 0.01) lower than that
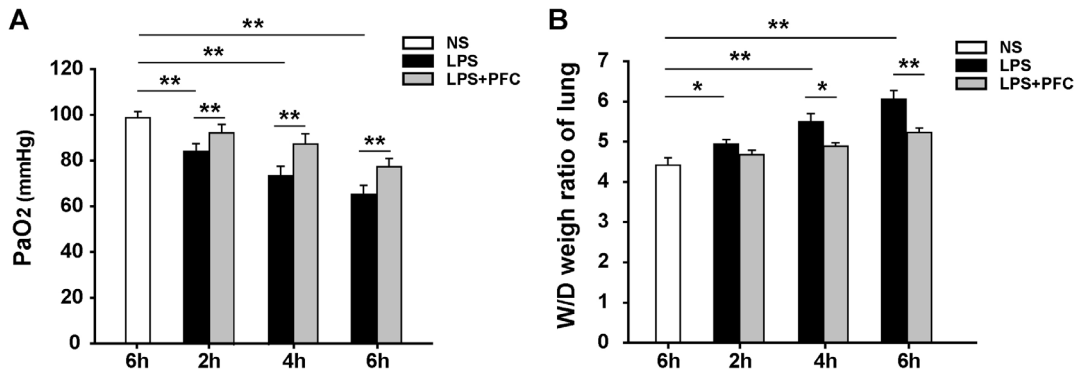
Figure 2. Pretreatment of PFC improves PaO$_2$ and wet/dry weight ratio of lung in LPS induced acute lung injury. After the indicated time of NS or LPS exposure, PaO$_2$ in the blood from abdominal aorta were measured (A); the wet/dry weight ratio of left lung in each rat was evaluated (B). Results are given as mean ± SD (n = 6). * $P<0.05$, ** $P<0.01$ between the indicated groups.
doi:10.1371/journal.pone.0087826.g002

in LPS group (Figure 2B), indicating that pretreatment of PFC could decrease the degress of lung edema induced by LPS. Together with lung histology and arterial blood gas analysis, these data for the first time demonstrated the therapeutic effect of intravenous infusion of PFC emulsion on LPS-induced acute lung injury in rats.

Analysis of myeloperoxidase expression in lung tissue

The expression of myeloperoxidase (MPO), which is an enzyme mainly presenting in azurophil granules of PMNs, was used as an index of the number of PMNs in the lung. It also reflected the degree of pulmonary retention and accumulation of PMN [30]. By using ELISA, we showed that the expression level of MPO in the lung was increased with LPS exposure time and its expression in LPS group was significantly ($P<0.01$) higher than that in the control group at all three exposure time points (Figure 3). However, the expression of MPO in the PFC group was significantly ($P<0.05$ or 0.01) lower than that in the LPS group. This result suggests that PFC treatment reduces infiltration of PMNs into lung tissue, which may explain the reduced inflammation and lung injury observed previously.
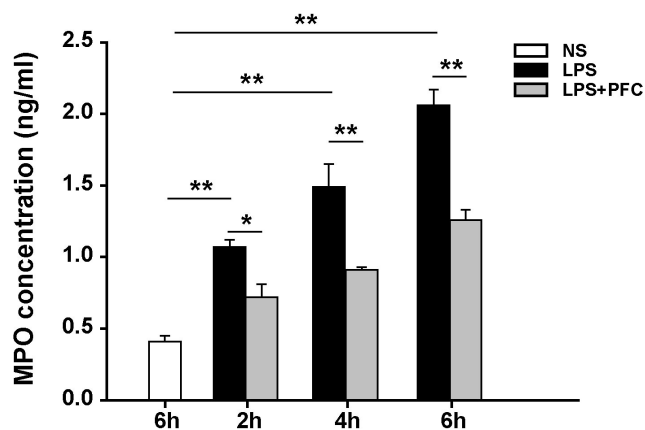

Figure 3 PFC reduces the expression of MPO in lung treated with LPS. After the indicated time of NS or LPS exposure, the right lower lobe of lung was isolated and the expression of myeloperoxidase (MPO) in lung was detected using enzyme-linked immunosorbent assay. Results are given as mean ± SD (n = 6). * $P<0.05$, ** $P<0.01$ between the indicated groups.
doi:10.1371/journal.pone.0087826.g003

Analysis of CD11b expression on polymorphonuclear neutrophils in circulation

The first step of recruitment of PMNs to the inflamed tissue is the attachment of circulating PMNs to vascular endothelial cells, which is mediated by the interaction between integrins expressed on PMNs, such as αMβ2, and cell adhesion molecules expressed on endothelial cells, such as intercellular adhesion molecule-1 (ICAM-1) [31]. CD11b is one protein subunit forming the heterodimeric integrin αMβ2 molecule, which mediates inflammation by regulating leukocyte adhesion and migration and has been implicated in several immune processes such as phagocytosis, cell-mediated cytotoxicity, chemotaxis and cellular activation [32].

To reveal of the molecular mechanism of PFC's anti-inflammation effect, we analyzed the expression of CD11b on PMNs in abdominal aorta blood by flow Cytometry (Figure 4). We showed that the expression of CD11b was increased with the longer exposure to LPS. At 6 hours of exposure to LPS, the expression of CD11b in LPS group was 6-fold ($P<0.01$) higher compared with control group (Figure 4B). Interestingly, intravenous infusion of PFC emulsion significantly ($P<0.01$) decreased the expression of CD11b on circulating PMNs, in comparison with LPS group, at all three time points (Figure 4B).

Analysis of ICAM-1 expression in lung tissue

Next, we characterized ICAM-1 expression in lung tissue by immunostaining and brown granules indicated ICAM-1 expression (Figure 5). In control group, low expression of ICAM-1 was observed on the surface of vascular endothelia, bronchiolar and alveolar epithelium (Figure 5A). In LPS group, claybank or dark brown granules were presented in the vascular, bronchiolar and alveolar epithelium after 2 h exposure to LPS, and this ICAM-1 signal markedly increased at 6 h of exposure to LPS (Figure 5A). However, in the rats treated with PFC, the lung exhibited MUCH weaker staining of ICAM-1 compared with the rats in LPS group (Figure 5A). Furthermore, we quantified these immunostaining with Image-Pro software and presented the expression of ICAM-1 in lung with mean photo densities, which were significantly ($P<0.05$ or 0.01) higher in LPS group than that in the control group (Figure 5B). The expression of ICAM-1 progressively increased from 2 hours to 6 hours of exposure to LPS (Figure 5B). PFC significantly inhibited the increase of ICAM-1 during the process of ALI and the mean photodensities of ICAM-1 in PFC group were markedly lower than that in LPS group ($P<0.05$ or 0.01) (Figure 5B).
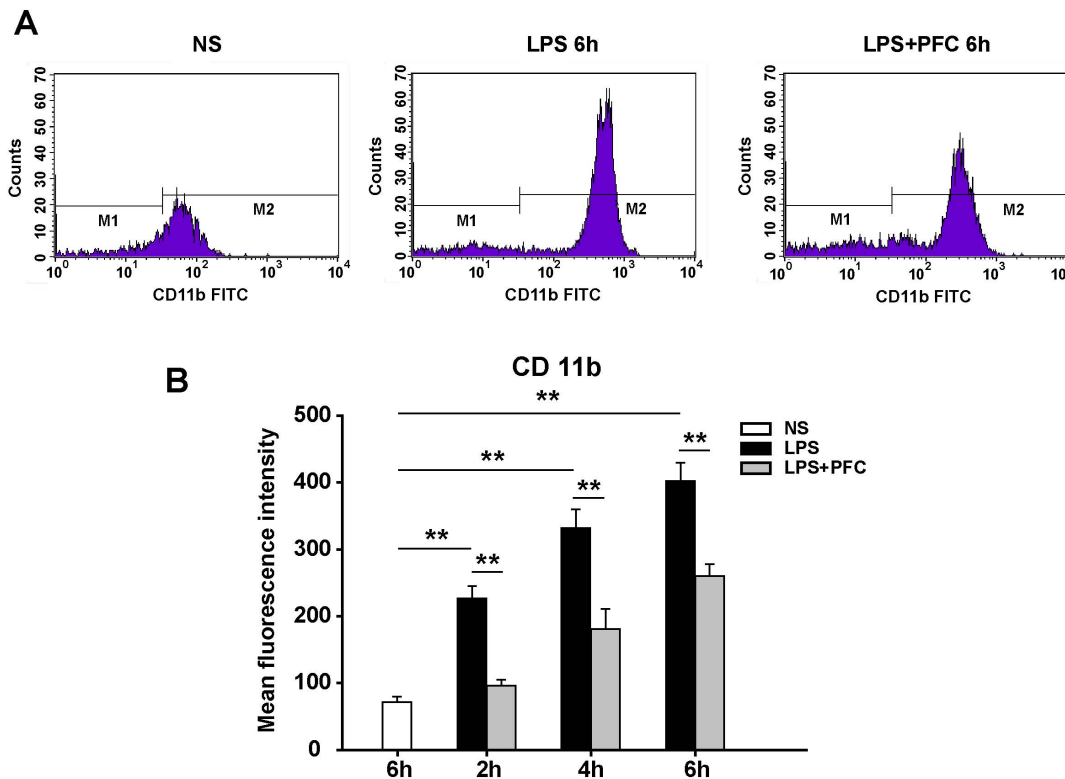
A

B

Figure 4. PFC reduces the expression of CD11b on circulating PMNs from rats treated with LPS. (A) Representative flow cytometry plots detecting CD11b on PMN cells at 6 h after NS or LPS exposure are shown. (B) Mean fluorescence intensity was measured to evaluate the expression of CD11b on PMN cells in different groups. Results are given as mean ± SD (n = 6). * $P<0.05$, ** $P<0.01$ between the indicated groups.
doi:10.1371/journal.pone.0087826.g004

Together, our data indicate that intravenous infusion of PFC emulsion reduces PMNs infiltrating into lung tissue by decreasing the expression of CD11b on circulating PMNs and ICAM-1 on vascular endothelia, bronchiolar and alveolar epithelium, which may explain its therapeutic effect on LPS induced ALI.

Discussion

In this study, we for the first time demonstrated that intravenous infusion of PFC emulsion can alleviate lung injury using a LPS-induced ALI rat model. ALI/ARDS is a common, clinically intensive condition affecting the respiratory system. It is associated with high mortality and there is no effective treatment available to date [33]. Recently, PFC has been used as a novel way to prevent and treat ALI in the form of liquid ventilation (LV), partial liquid ventilation (PLV) or vaporized perfluorocarbon inhalation [23–25,34], which can improve respiratory function and alleviates pulmonary injury and there was no significant adverse effect of PFC on lung, liver or kidney was observed in these studies [35,36]. However, these interventions need establishment of artificial airway to facilitate PFC into the lungs, which is not suitable for treating patients with early ALI. Thus, in this study, we chose intravenous infusion of PFC emulsion and evaluated its therapeutic effect on ALI.

Here we showed that in a LPS-induced ALI rat model, intravenous infusion of PFC emulsion i) significantly improved lung function, indicated by increased arterial blood PaO_2 and decreased lung wet-dry weight ratio (Figure 2); ii) reduced pulmonary injury, indicated by preserved lung structure (Figure 1) and decreased neutrophilic granulocyte infiltration into alveoli and pulmonary interstitial (Figure 3). These data indicate

that intravenous infusion of PFC emulsion may be used to treat ALI, especially its early stage, in clinic.

There is good agreement that ALI/ARDS is an inflammatory injury of lung that mainly characterized by PMN infiltration. Enzymes, oxygen radicals and inflammatory mediators released from inflammatory cells were found to be the main factors associated with ALI [37]. The same study demonstrated that during ALI PMN cells adhered to vascular endothelium, crossed endothelial barrier and migrated to the region of inflammation. Based on these findings, it has been proposed that the interaction between inflammatory cells and adhesion molecules on the surface of vascular endothelia may be an important molecular mechanism responsible for ALI [38].

The mechanism of PFC protecting against ALI/ARDS remains controversial. It has been previously shown that PFC prevents PMN activation and decreases the synthesis and release of inflammatory mediators by macrophages [39,40]. It may also serve as a physical barrier blocking the entrance of inflammatory cells and factors from alveoli into pulmonary parenchyma or coating the activated inflammatory cells and inhibiting their interaction with other activated cells [41]. Here we showed that PFC decreased the infiltration of PMN in lung by down-regulating the expression of CD11b on circulating PMNs (Figure 4) and ICAM-1 on endothelial cells (Figure 5), which is the core pathogenesis of ALI. In previous studies, both ICAM-1 and CD11b/CD18 have been shown to play a crucial role in the conjugation of PMN cells to the endothelium [42,43]. CD11b and CD18 expressed by PMNs adhere to ICAM-1 on the surface of activated endothelia with high affinity [44]. This interaction mediates the migration and effusion of PMN cells, resulting in
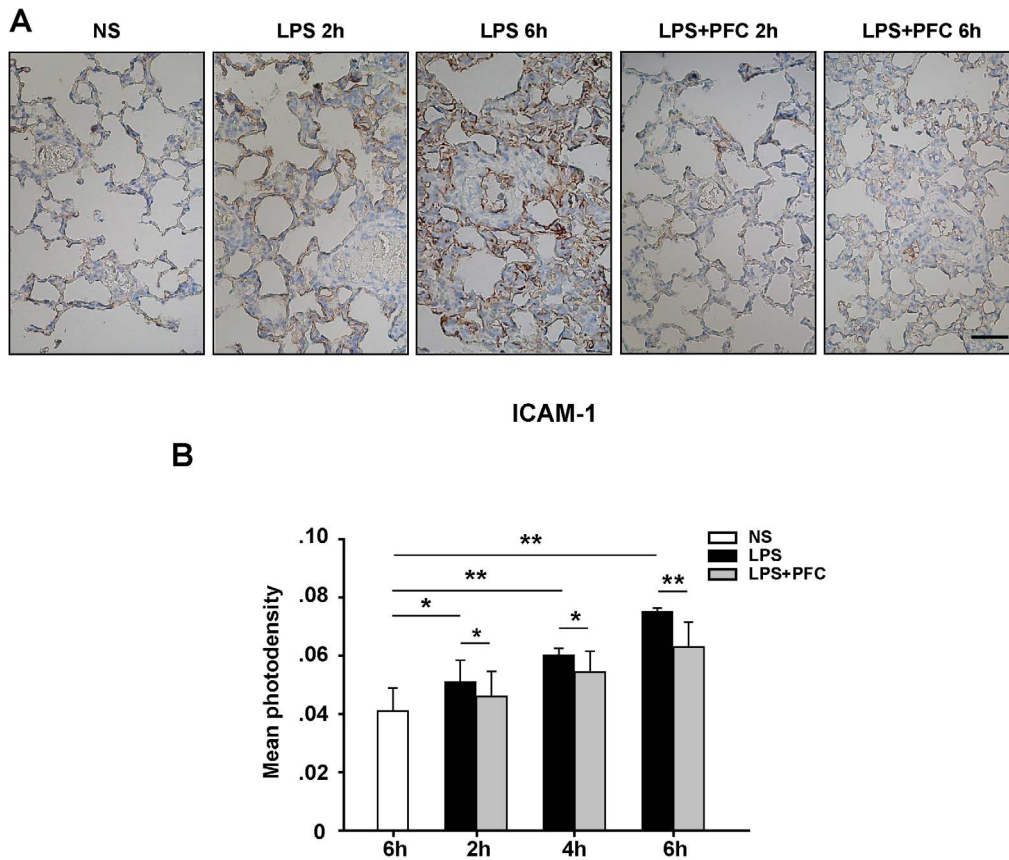
A

ICAM-1

B

Figure 5. PFC attenuates the expression of ICAM-1 induced by LPS in lung tissue. (A) Representative immunohistochemistrical staining of ICAM-1 in lung tissue after NS or LPS exposure. Scale bar, 25 µm. (B) Mean photodenisity was measured to evaluate the expression of ICAM-1 in lung tissue. Results are given as mean $\pm$ SD (n = 6). * $P<0.05$, ** $P<0.01$ between the indicated groups.
doi:10.1371/journal.pone.0087826.g005

their accumulation at inflammatory region, which in turn strengthens and exaggerates inflammatory reaction, finally causing lung injury [45]. Wyman *et al.* found that ICAM-1 was not only needed to promote the pulmonary accumulation of PMNs, but also trigger the release of elastinase and other hydrolases from PMNs [46]. In line with these findings, it has been reported that ALI can be alleviated using ICAM-1 antibodies or genetic deletion of ICAM-1 in mice model [47].

Together, our study demonstrates the therapeutic effect of intravenous infusion of PFC emulsion on ALI. Importantly, this strategy avoids the establishment of artificial airway to facilitate PFC into the lungs as used in previous studies, thus it is more suitable for treating patients with early ALI. Moreover, we

explored the molecular mechanism which may be responsible for this therapeutic effect of PFC. We showed that PFC decreases the infiltration of PMNs in lung by down-regulating the expression of CD11b on circulating PMNs and ICAM-1 on endothelial cells, which provides a theoretical foundation for using intravenous infusion of PFC to prevent and treat ALI/ARDS in clinical practice.

Author Contributions

Conceived and designed the experiments: SKH HJF. Performed the experiments: HD XFY JQS. Analyzed the data: HD QL. Wrote the paper: HD QL NXL.

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