

# Netrin-1 reduces lung ischemia-reperfusion injury by increasing the proportion of regulatory T cells

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## Abstract

**Objective:** Inflammation is the primary mechanism of lung ischemia-reperfusion injury (LIRI) and neurologic factors can regulate inflammatory immune responses. Netrin-1 is an axonal guidance molecule, but whether Netrin-1 plays a role in LIRI remains unclear.

**Methods:** A mouse model of LIRI was established. Immunohistochemistry was used to detect expression of Netrin-1 and to enumerate macrophages and T cells in lung tissue. The proportion of regulatory T cells (Tregs) was assessed by flow cytometry. Levels of apoptosis were assessed by terminal deoxynucleotidyl transferase dUTP nick end staining.

**Results:** Numbers of macrophages and T cells in the lung tissues of mice with LIRI were elevated, while expression of netrin-1 was significantly decreased. Flow cytometry showed that the proportion of Tregs in mice with LIRI was significantly decreased. The proportion of Tregs among lymphocytes was positively correlated with netrin-1 expression. *In vitro* experiments showed that netrin-1 promoted an increase in Treg proportion through the A2b receptor. Animal experiments showed that netrin-1 could inhibit apoptosis and reduce T cell and macrophage infiltration by increasing the proportion of Tregs, ultimately reducing LIRI. Treg depletion using an anti-CD25 monoclonal antibody blocked the effects of netrin-1.

**Conclusion:** Netrin-1 reduced LIRI by increasing the proportion of Tregs.

## Keywords

Netrin-1, ischemia-reperfusion injury, regulatory T cell, inflammation, lung, A2b receptor

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## Introduction

Multiple factors can lead to lung ischemia-reperfusion injury (LIRI) including pulmonary embolism, lung transplantation, cardiac arrest and severe trauma. LIRI has a high mortality rate, so prevention and treatment are both clinically important.<sup>1,2</sup> Inflammation is the primary mechanism of LIRI. During reperfusion of ischemic lung tissues, inflammatory cell infiltrates release pro-inflammatory molecules such as tumor necrosis factor- $\alpha$  and interleukin (IL)-6, causing further damage to lung tissues.<sup>3,4</sup> In addition, inflammatory molecules released from ischemic sites can enter the peripheral blood, causing systemic organ and tissue damage.<sup>5</sup> Therefore, regulation of inflammatory responses is important for the prevention and treatment of LIRI.

Regulatory T cells (Tregs) are immune regulatory cells that play an important role in maintaining immune homeostasis.<sup>6</sup> Tregs inhibit the proliferation and activation of effector T cells by directly contacting them or secreting immunosuppressive molecules.<sup>7</sup> Human leukocyte antigen (HLA)-G and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) play important roles in immunosuppression by Tregs. Several studies have found that Tregs can protect the brain, heart, liver and kidney against ischemia-reperfusion injuries.<sup>8–10</sup> Tregs reduced the infiltration of macrophages and lymphocytes into renal tissue following ischemia-reperfusion by secreting IL-10 and transforming growth factor (TGF)- $\beta$ . The number of Tregs was negatively correlated with the degree of ischemia-reperfusion injury.<sup>11</sup> Therefore, increasing the number of Tregs may have a therapeutic effect on LIRI.

In recent years, many neurologic factors have been found to regulate immune responses. Netrin-1 is an axonal guidance molecule that plays an important role in nerve growth and axon formation.<sup>12</sup> Many

studies have found that netrin-1 can reduce myocardial ischemia-reperfusion injury and inhibit cardiomyocyte apoptosis, although the specific mechanisms remain unclear.<sup>13,14</sup> Regulation of inflammatory responses may be one mechanism through which netrin-1 exerts these effects. Netrin-1 can inhibit the migration and aggregation of white blood cells and reduce the release of pro-inflammatory cytokines.<sup>15–17</sup> The goals of this study were to investigate whether netrin-1 can be used to treat LIRI and to explore the role of Tregs in its mechanism of action.

## Materials and methods

### *Establishment of a mouse model of LIRI*

The mouse model of LIRI was established as previously reported.<sup>18</sup> Briefly, following anesthesia, the mouse trachea was incised and mechanical ventilation was performed using a small animal ventilator. The second and third ribs were cut along the left sternum, the thoracic cavity was opened, and the left lung was exposed. The left hilum was clamped for half an hour, and then the arterial clamp was loosened to allow reperfusion of lung tissue. The study was approved by the Ethics Committee of Wenzhou Central Hospital (No: 20170361) and was performed according to the Guide for the Care and Use of Laboratory Animals of Wenzhou Central Hospital.

### *Histological staining*

Mice were divided into a control group (sham operation) and a LIRI group (LIRI model). After 3 hours of reperfusion, lung tissues of mice in both groups were collected, fixed with 10% formalin, and then paraffin sections were prepared. Some sections were stained with hematoxylin and eosin (H&E) to observe the overall morphology of lung tissue. Some sections were used for

immunohistochemistry to assess expression of netrin-1 as well as numbers of CD68+ macrophages and CD3+ T cells in lung tissue. All antibodies were purchased from Abcam (Cambridge, UK). Netrin-1 expression was quantified using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA). The numbers of macrophages and T cells in each high power field were counted using a light microscope.

### Flow cytometry

After 3 hours of reperfusion, peripheral blood was collected and lymphocytes were separated using lymphocyte separation medium (TBD, Tianjin, China). Anti-CD4 and -CD25 antibodies were added for 15 minutes, then the lymphocytes were washed three times with phosphate-buffered saline (PBS). One hour following membrane rupture, an anti-Foxp3 antibody was added and incubated for 15 minutes. After three washes with PBS, the proportion of CD4+CD25+Foxp3+ Tregs was assessed by flow cytometry. Tregs were sorted by fluorescence-activated cell sorting and cultured in RPMI-1640 containing 10% fetal bovine serum (Hyclone, Logan, UT, USA). After 48 hours, levels of IL-10 and TGF- $\beta$  in the supernatant were assessed by ELISA (Huijia, Xiamen, China) and expression of HLA-G and CTLA-4 on Tregs was assessed by flow cytometry. All antibodies were purchased from BD Biosciences (San Diego, CA, USA).

### In vitro experiments

Peripheral blood of mice in the LIRI group was collected. Lymphocytes were separated using lymphocyte separation medium and cultured in RPMI-1640 containing 10% fetal bovine serum. The cells were divided into four groups: (i) a control group (an equivalent volume of PBS was added to the culture medium), (ii) a netrin-1 group

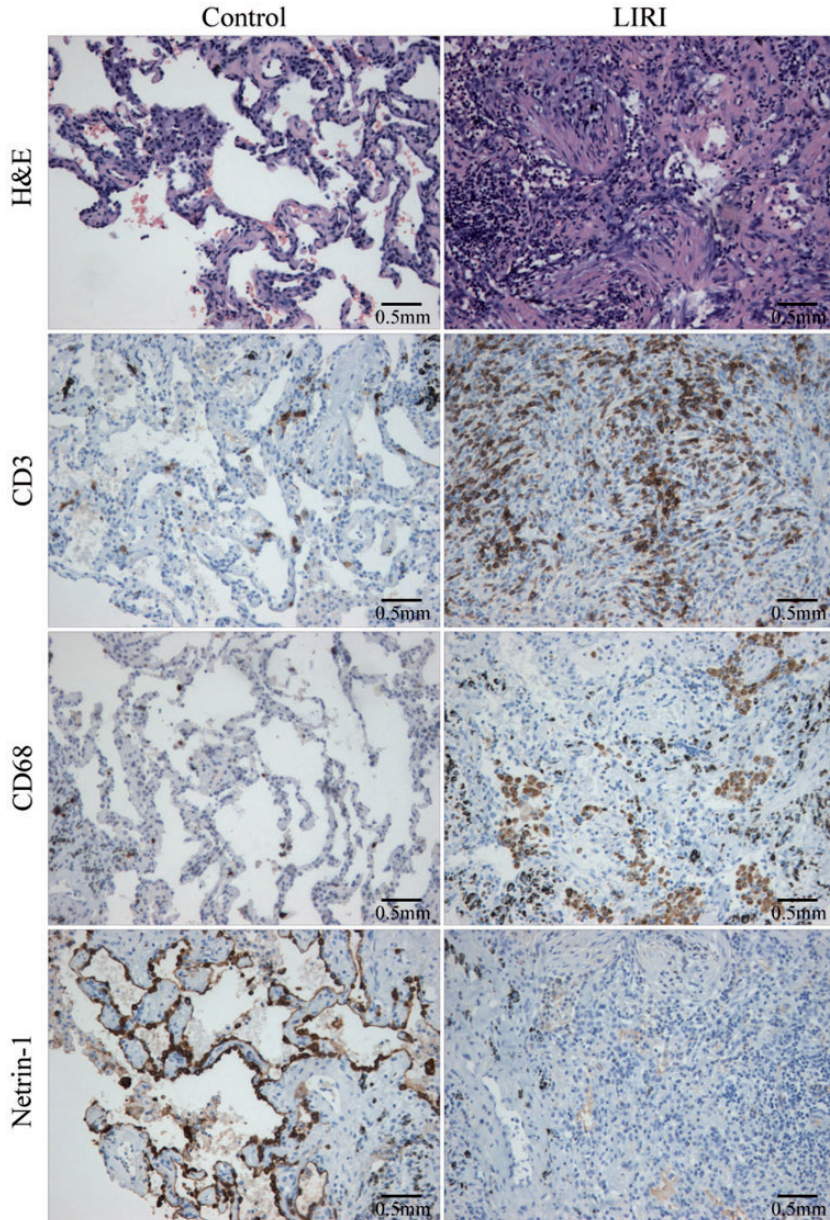
(10 ng/mL of netrin-1 was added to the culture medium), (iii) an A2b blockade group (10 ng/mL of netrin-1 and an A2b neutralizing antibody were added to the culture medium), and a deleted in colorectal cancer (DCC) blockade group (10 ng/mL of netrin-1 and a DCC neutralizing antibody were added to the culture medium). After 48 hours of continuous culture, the proportion of Tregs among lymphocytes was assessed by flow cytometry.

### Animal experiments

Mice were divided into three groups: (i) a LIRI group (LIRI model mice), (ii) a netrin-1 group (receiving intraperitoneal injections of netrin-1 4 hours prior to LIRI model establishment), and (iii) a PC61 group (receiving intraperitoneal injections of netrin-1 and the anti-CD25 monoclonal antibody PC61 4 hours prior to LIRI model establishment).<sup>19,20</sup> After 24 hours of reperfusion, peripheral blood was collected and the number of Tregs in peripheral blood was assessed by flow cytometry. Lung tissues were collected and overall pathological changes were assessed by H&E staining. The numbers of CD3+ T cells and CD68+ macrophages were assessed by immunohistochemistry. Levels of apoptosis were assessed using terminal deoxynucleotidyl transferase dUTP nick end (TUNEL) staining.

### Statistical analysis

Statistical analyses were carried out using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Data were presented as mean  $\pm$  standard deviation. Differences between multiple groups were assessed using one-way analysis of variance and the Bonferroni *post hoc* test. Values of  $P < 0.05$  were considered statistically significant.



**Figure 1.** Histological staining. Compared with the control group, the numbers of macrophages and T cells in lung tissues of LIRI mice were significantly increased, while expression of netrin-1 was significantly decreased.

## Results

### *Expression of netrin-1 was significantly decreased in lung tissues of LIRI mice*

H&E staining showed that lung tissue structures in the control group were basically normal with a small number of infiltrating inflammatory cells detected. In the LIRI group, lung tissue was necrotic and infiltrated with many inflammatory cells. Immunohistochemistry showed that the numbers of macrophages and T cells in lung tissues of LIRI mice were significantly increased compared with the control group. By contrast, the expression of netrin-1 was significantly decreased in LIRI mice (Figure 1).

### *The proportion of Tregs was significantly decreased in LIRI mice*

Compared with the control group, the proportion of Tregs among lymphocytes in the LIRI group was significantly decreased. Treg proportion was positively correlated with netrin-1 expression (Figure 2).

We further studied the functions of Tregs in LIRI. We found that there was no

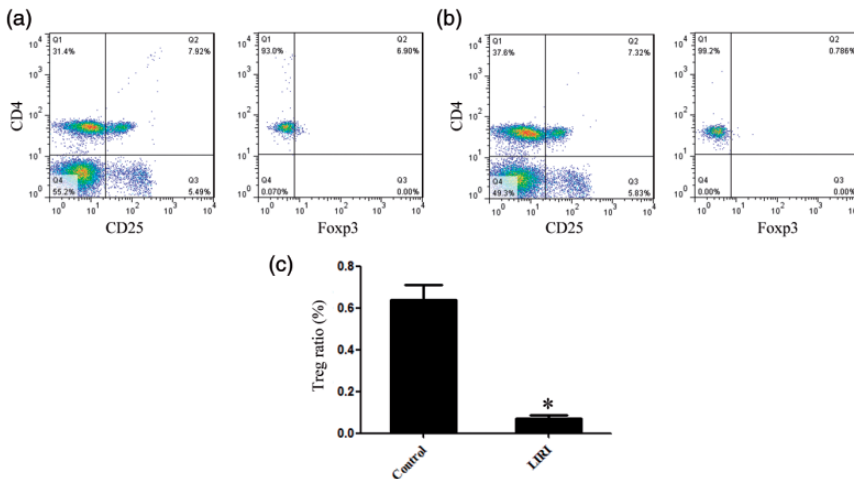
significant difference in IL-10 and TGF- $\beta$  concentrations in the supernatants of Tregs purified from control and LIRI mice. There was also no significant difference in the expression of HLA-G and CTLA-4 on the Tregs of control and LIRI mice. These results suggested that there was no significant difference in Treg function in these two groups of mice (Figure 3).

### *Netrin-1 promoted an increase in Treg proportion in vitro*

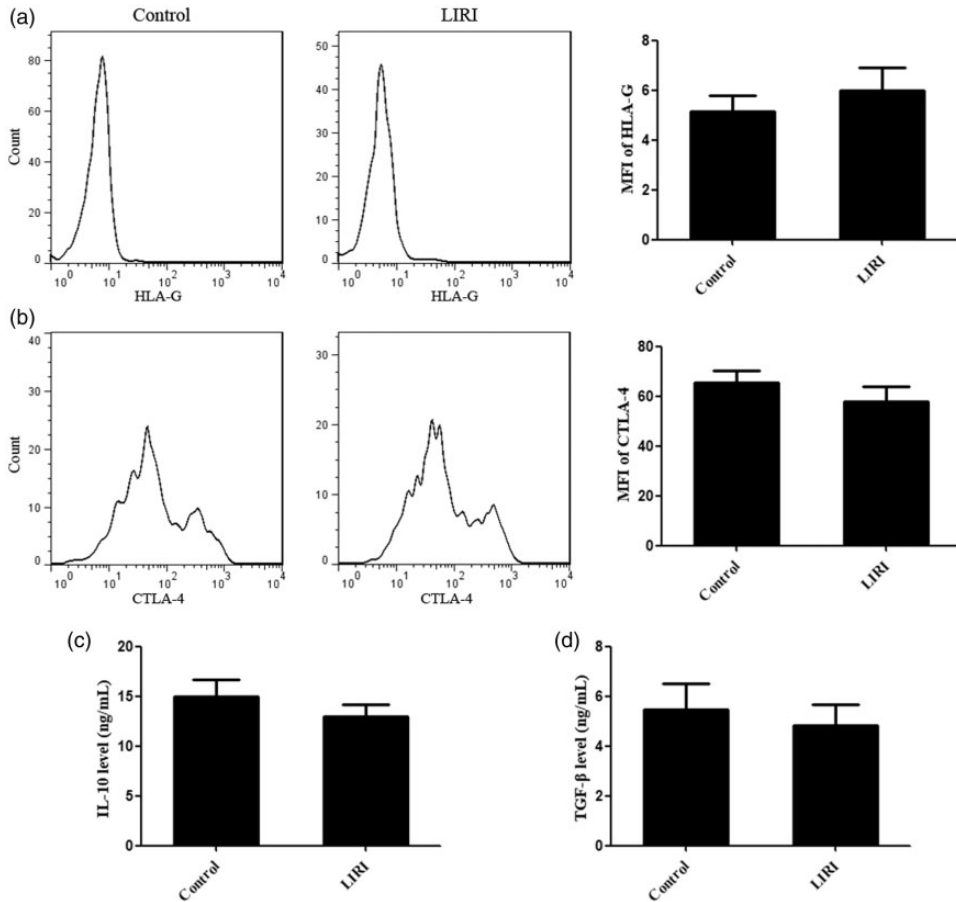
Compared with the control group, the proportion of Tregs in netrin-1 treated lymphocytes from LIRI mice was significantly increased. A2b receptor blockade significantly inhibited the function of netrin-1, while DCC blockade had no significant effect on its function (Figure 4).

### *Netrin-1 protected the lung against ischemia-reperfusion injury*

H&E staining demonstrated partial necrosis of lung tissue and infiltration by a large



**Figure 2.** Changes in Treg proportion associated with LIRI. Flow cytometry was used to assess the proportion of Tregs in control (a) and LIRI mice (b). The proportion of Tregs in LIRI mice was significantly decreased (c).



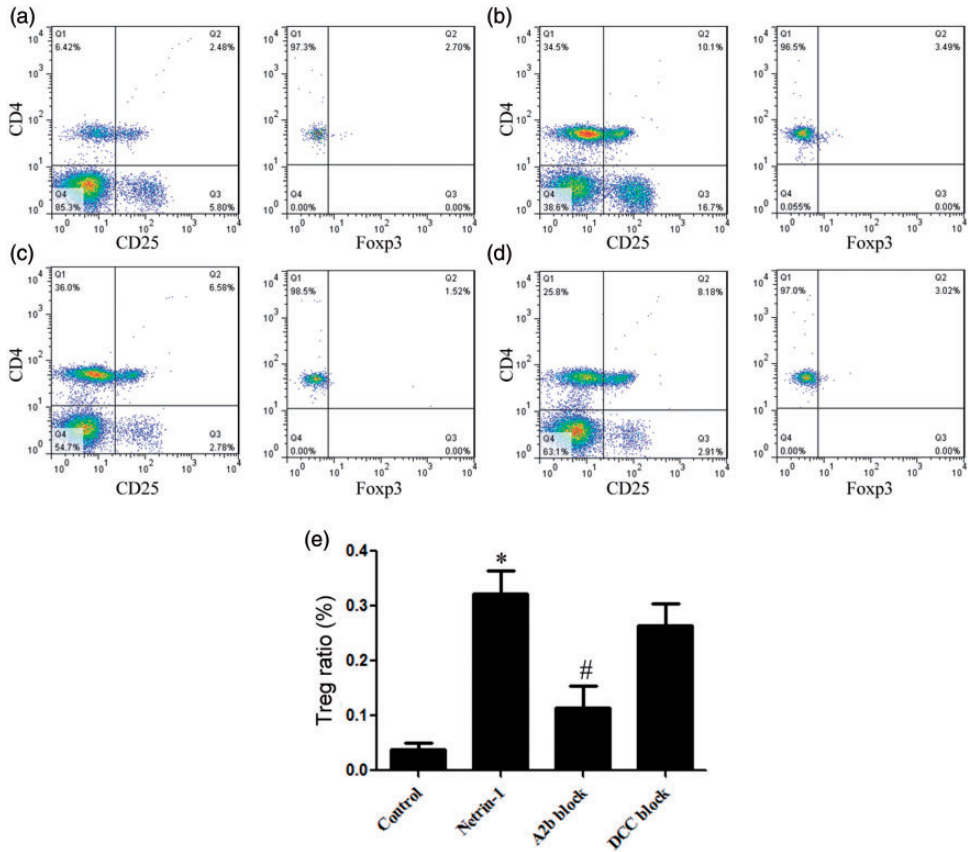
**Figure 3.** Treg function in LIRI and control mice. (a, b) There was no significant difference in the expression of HLA-G and CTLA-4 on Tregs derived from control and LIRI mice. (c, d) There was no significant difference in levels of IL-10 and TGF- $\beta$  in the supernatants of Tregs purified from control and LIRI mice. MFI, mean fluorescence intensity.

number of inflammatory cells in LIRI mice. Compared with the LIRI group, netrin-1 treatment resulted in decreased lung tissue necrosis and inflammatory cell infiltration. Depletion of Tregs using the anti-CD25 antibody PC61 completely blocked the effects of netrin-1. TUNEL staining showed that 16.4% of cells were apoptotic in LIRI mice, while treatment with netrin-1 decreased the percentage of apoptotic cells by 76.3%. PC61

completely blocked the effects of netrin-1 on apoptosis (Figure 5).

#### *Netrin-1 increased the Treg proportion in vivo*

Compared with the LIRI group, the proportion of Tregs in netrin-1-treated mice was 6.07-fold higher. Compared with netrin-1-treated mice, the proportion of



**Figure 4.** Netrin-1 treatment promoted an increase in Treg proportion. Flow cytometry was used to assess the proportion of Tregs in control (a), netrin-1-treated (b), A2b blockade (c) and DCC blockade (d) mice. (e) Compared with the control group, the Treg proportion in netrin-1-treated mice was significantly increased. A2b receptor blockade significantly inhibited the function of netrin-1, while DCC blockade had no significant effect on its function. \*vs. control,  $P < 0.05$ ; # vs. netrin-1,  $P < 0.05$ .

Tregs in PC61-treated mice decreased by 84.64% (Figure 6).

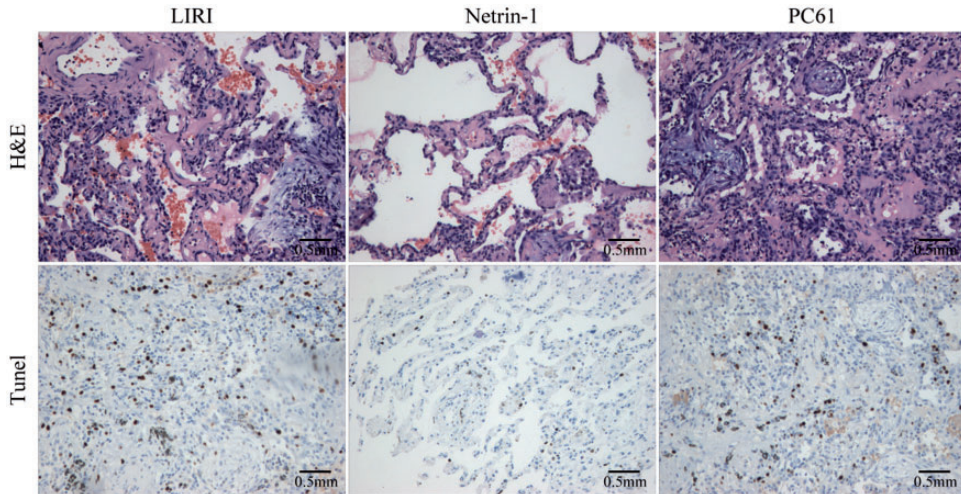
### *Netrin-1 inhibited inflammatory responses in lung tissue following ischemia-reperfusion*

Immunohistochemistry showed that netrin-1 inhibited the infiltration of macrophages and T cells into lung tissue. The numbers of macrophages and T cells present in the lung tissues of PC61-treated mice were

significantly higher than those of mice treated with netrin-1 alone (Figure 7).

## **Discussion**

In recent years, the increasing number of patients undergoing lung transplantation and cardiac arrest has significantly increased the incidence of LIRI. The mortality rate of patients with LIRI is very high, but there is no effective treatment at present.<sup>21</sup> Thus, it is very important to identify new prevention and treatment methods.



**Figure 5.** Netrin-1 protected the lung against ischemia-reperfusion injury. Netrin-1 inhibited apoptosis induced by ischemia-reperfusion. Compared with the LIRI group, the percentage of apoptotic cells decreased by 76.3%. Treatment with the anti-CD25 antibody, PC61 completely blocked the effect of netrin-1. (100 $\times$ ).

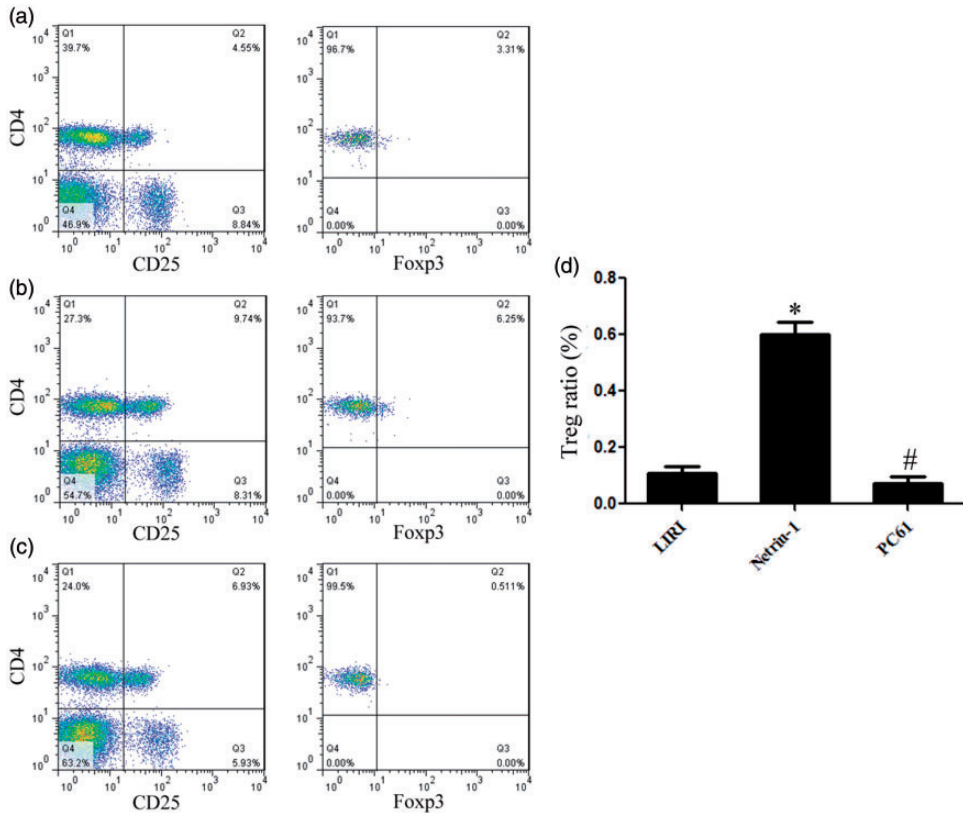
Inflammation is the primary mechanism of ischemia-reperfusion injury in lung tissue and other organs. Regulating inflammation can effectively alleviate LIRI.<sup>22</sup> Tregs are the most important immune regulatory cells in peripheral blood, and suppress immune responses through two mechanisms: (i) Tregs express immunosuppressive molecules (HLA-G and CTLA-4) that can inhibit inflammatory cells through direct cell contact, and (ii) Tregs can secrete large amounts of IL-10 and TGF- $\beta$  to inhibit inflammation and promote the proliferation the activities of other types of regulatory immune cells.<sup>23,24</sup> Our study found that the immunoregulatory functions of Tregs in control and LIRI mice were similar, suggesting that LIRI had no significant effect on Treg function.

Many clinical experiments have shown that Tregs can have therapeutic effects on organ transplant rejection and autoimmune diseases.<sup>25,26</sup> For ischemia-reperfusion

injury, Treg transplantation can significantly reduce infiltration of macrophages and lymphocytes into ischemic tissue and can inhibit apoptosis.<sup>27,28</sup> Our results showed that the proportion of Tregs was significantly decreased in LIRI mice, suggesting that the inflammatory response was activated in these mice. Excessive inflammation can cause further damage to lung tissues. Many inflammatory factors can also enter the peripheral blood and damage other important tissues and organs. Therefore, increasing the proportion of Tregs and maintaining immune homeostasis may be an effective strategy to treat LIRI.

There is a strong connection between the nervous system and inflammatory responses. In recent years, many studies have found that neurological factors can regulate inflammation. Brain-derived neurotrophic factor can regulate the inflammatory microenvironment, promote IL-10 expression, and induce macrophage polarization from M1 to M2.<sup>29</sup>



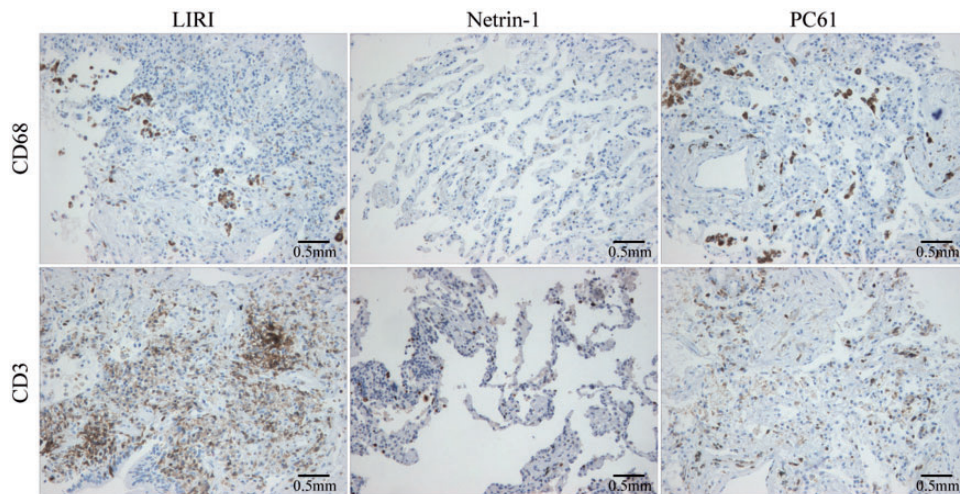


**Figure 6.** Netrin-1 increased the Treg proportion *in vivo*. Flow cytometry was used to assess the proportion of Tregs in the LIRI (a), netrin-1 (b) and netrin-1 + PC61 (c) groups. (d) Compared with the LIRI group, the proportion of Tregs in the netrin-1 group was 6.07-fold higher. Compared with the netrin-1 group, the proportion of Tregs in the PC61 group decreased by 84.64%. \*vs. LIRI,  $P < 0.05$ ; # vs. netrin-1,  $P < 0.05$ .

Netrin-1 can also regulate inflammatory responses. Some studies have found that netrin-1 can inhibit the proliferation of macrophages and reduce the damage caused by inflammatory cells in ischemic tissue.<sup>30,31</sup> Our study points to a new mechanism through which netrin-1 can regulate inflammation. Netrin-1 increases the proportion of Tregs and inhibits inflammation induced during ischemia-reperfusion injury. Immunohistochemistry showed that netrin-1 treatment could significantly inhibit LIRI-

associated apoptosis and reduce lymphocyte and macrophage infiltration into ischemic lung tissue. A2b receptors may play a key role in this process. A2b receptor is the primary adenosine receptor expressed in endothelial cells, dendritic cells and lymphocytes, and plays a key role in pulmonary inflammation.<sup>32,33</sup>

In conclusion, netrin-1 reduced LIRI by increasing the proportion of Tregs. Our data suggest a new strategy for the treatment and prevention of LIRI.



**Figure 7.** Netrin-1 inhibited inflammatory responses in lung tissue following ischemia-reperfusion. Netrin-1 also inhibited infiltration of macrophages and T cells into lung tissue. The numbers of macrophages and T cells in lung tissue of PC61-treated mice were significantly higher compared with mice treated with netrin-1 alone. (100×).

### Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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