Original Article

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Dysfunction of peripheral regulatory T cells predicts lung injury after cardiopulmonary bypass

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SUMMARY Lung injury caused by cardiopulmonary bypass (CPB) increases the mortality after cardiac surgery. Previous studies have shown that regulatory T cells (Tregs) play a protective role during CPB, but the correlation between Tregs and CPB-induced lung injury remains unclear. Here, we conducted a prospective study about Treg cells in patient receiving CPB. Treg cells were collected from patients before the CPB operation (pre-CPB Tregs), and the effect of pre-CPB Tregs on the occurrence of CPB-induced lung injury was evaluated. Data showed that the baseline level of Treg cells in peripheral blood were lower in patients who developed lung injury after CPB, compared to those who did not develop lung injury after CPB. Function analyses revealed that pre-CPB Tregs from CPB-induced lung injury patients presented decreased ability in suppressing the proliferation and IFN-γ production of CD4 and CD8 T cell. Also, pre-surgery levels of TGF- β and IL-10 were markedly lower in lung injury patients than in non-lung injury patients. In addition, PD-1 and Tim-3 expression on pre-CPB Tregs were significantly lower in CPB-induced lung injury patients than the CPB patients without lung injury. Above all, we found impaired peripheral Treg responses in CPB-induced lung injury patients, indicating a potential role of Treg cells in the early diagnosis of CPB-induced lung injury.

Keywords Regulatory T cell, lung injury, cardiopulmonary bypass

1. Introduction

Cardiopulmonary bypass (CPB) procedure is a basilic component of conventional cardiac surgeries (*1*). Pulmonary dysfunction after CPB was described almost 40 years ago (*2*), and lung injury is one of the most common complications with non-cardiogenic refractory hypoxemia as the main clinical manifestation. About 2% patient with lung injury could develop into acute respiratory distress syndrome (ARDS) with about 15.5% mortality. Therefore, identifying specific warning biomarkers is critical for preventing morbidity and mortality of lung injury after CPB.

Lung injury caused by CPB might be attributed to the disorder and unbalance of immune response and different $CD4^+$ T cells are involved in the development of lung injury (*3,4*). Regulatory T cell (Treg) is a subset of $CD4^+$ T cells, which is significant for immune homeostasis and maintaining self-tolerance. These cells were termed suppressor cells originally (*5*), expressing CD25 as well as Foxp3. It has been reported that Treg cells play a critical role in resolution of acute lung injury (ALI) in mice. Data showed that Rag-1 mice exhibited a profound impairment in resolution of lung injury, which could be reversed by administration of Treg cells, while depletion of Tregs in wild type mice delayed recovery (*6*). Besides, the recovery of ALI was also attributed by upregulation of Tim-3 on Tregs. However, the effects of Tregs on lung injury patients after CPB are still not clear.

Here, we showed that the dysfunction of Treg cells in patients with CPB-induced lung injury appeared before the surgery, which suggests a new biomarker of CPB-induced lung injury and a therapeutic approach through increasing Treg population and function.

2. Materials and Methods

2.1. Ethics statement

Ethical approval (YXLL-KY-2020-56) was obtained from the Research Ethics Committee of Qianfoshan Hospital. Informed consent was obtained from every patient and the study protocols were approved by the Research Ethic committee of the Shandong Province Qianfoshan Hospital. The ethical consideration for

this study followed the principles of the Declaration of Helsinki.

2.2. Patients

Patients scheduled for cardiac surgery under cardiopulmonary bypass between October 2020 and January 2021 at our hospital were enrolled in the study. Exclusion criteria included heart failure, severe pulmonary hypertension, preoperative presence of respiratory distress syndrome, age > 75, and without providing consent form. They were grouped into non-lung injury and lung injury groups based on the presence or absence of lung injury after CPB. The oxygenation index was calculated according to the results of blood gas analysis and ventilator parameters within 24 hours after operation. Lung injury group was defined if the oxygenation index was less than 200, otherwise it belonged to non-lung injury group.

2.3. Isolation of peripheral blood mononuclear cells (PBMCs) and cell culture

PBMC were obtained by Ficoll-Hypaque (Thermo Fisher, Waltham, MA, USA) centrifugation (Eppendorf, Hamburg, Germany) of heparinized blood and resuspended in RPMI (GIBCO, Grand Island, NY, USA) tissue culture medium containing 10% fetal calf serum (GIBCO, Grand Island, USA), streptomycin (GIBCO, Grand Island, USA), and penicillin (GIBCO, Grand Island, USA).

2.4. Serum

Ten mL of whole-blood samples were collected from patients. Serum samples were obtained after centrifugation at 800× g for 10 min, aliquoted and stored at –80°C until assayed.

2.5. Enzyme-linked immunosorbent assay

Cytokines in the sera were (IL-10, TGF-β, IFN-γ and IL-17A) measured by Bio-Plex Pro™ Human Cytokine Assays® (Bio-Rad Laboratories, Inc., Hercules, CA, USA). In brief, the serum samples were diluted 4-fold with the diluting solution, and centrifuged at 10,000× g for 5 minutes. Fifty μL of the supernatant was used for the cytokine assay in accordance with the manufacturer's instruction.

2.6. Flow cytometry

PBMCs were stained with different antibodies according to the manufacturer's instruction, in which antibodies used for the experiments included APC/CY7-CD8, BV605-CD4, PE/CY7-CD25, FITC-PD-1, PE-Tim-3 or isotype-matched control IgG. All the antibodies and isotype controls were purchased from BD PharMingen San Diego, CA, USA. After staining, cells were washed twice with PBS and were subjected to flow cytometry analysis using a FACS Foterassa (BD, San Diego, CA, USA). Analyses of flow cytometry were performed by FlowJo software.

2.7. Intracellular cytokine staining

PBMCs were stimulated with 20 ng/mL phorbol myristate acetate and 1 μg/mL ionomycin (Sigma-Aldrich, St. Louis, MO, USA) for 6 h to detect IFN-γ- or IL-17A-producing T cell frequencies in patients with lung injury or non-lung injury. Brefeldin A (10 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) was added to cultured PBMCs for 4 h. Stimulated PBMCs were washed in phosphate-buffered saline (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate, 2 mM potassium dihydrogen phosphate, pH 7.4, Sigma-Aldrich, St. Louis, MO, USA) and incubated with PC/CY7-CD4, APC/CY7-CD8a or matched isotype (Thermo Fisher, Waltham, MA, USA) for 30 min in dark at 4°C. PBMCs were then fixed in 4% formaldehyde, permeabilized with 0.1% saponin (Sigma-Aldrich, St. Louis, MO, USA) and stained v450-INF-γ or FITC-IL-17A (Thermo Fisher, Waltham, MA, USA) or matched isotype control monoclonal antibody (Thermo Fisher, Waltham, MA, USA). Cells were analyzed using a FACS Foterassa (BD, San Diego, CA, USA).Analyses of flow cytometry were performed by FlowJo software.

2.8. Foxp3 staining

For intracellular staining of Foxp3, cells were fixed and permeabilized with Foxp3 staining buffer (Thermo Fisher, Waltham, MA, USA), then stained with allophycocyanin-conjugatedanti-humanFoxp3 mAbs (0.5 μg per 10^6 cells; Thermo Fisher, Waltham, MA, USA). Lymphocytes were gated with characteristic low forward scatter/side scatter, using a FACS Foterassa instrument. Analyses of flow cytometry were performed by FlowJo software.

2.9. Isolation of $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells

$CD4^+$ T cells were isolated from PBMC using magnetic bead separation. To isolate $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells, purified $CD4^+$ T cell population were incubated with PE-labeled anti-CD25 Ab (BD PharMingen San Diego, CA, USA) and were isolated by BD Arial (BD, San Diego, CA, USA).

2.10. *In vitro* proliferation assays

$CD4^+CD25^-$ cells (5×10^4) from healthy donor were

Table 1. Staining Intensity of ALM and SSM lesions

Items	Non-lung injury	Lung injury	*p* value
Number	26	14	
Age	62.46 ± 2.03	62.35 ± 2.33	0.97
Gender (male/female)	16/10	5/9	0.19
BMI	24.71 ± 0.58	24.74 ± 0.90	0.98
NYHA (I/II/III/V)	0/0/22/4	0/0/13/1	0.64
During operation			
Cardiopulmonary bypass time (min)	148.31 ± 6.81	151.64 ± 8.41	0.77
Auxiliary circulation time (min)	76.19 ± 6.65	67.21 ± 7.55	0.40
Lowest Hct	28.53 ± 0.75	31.21 ± 1.23	0.06
Oxygenation index (before surgery)			
PaO_2/FiO_2 (mmHg)	363.88 ± 7.26	362.31 ± 12.56	0.91

BMI, body mass index; NYHA, New York heart association. Continuous variables with a normal distribution were represented in mean ± SEM, continuous variable with a non-normal distribution were presented as the median. Categorical variables were presented as number (%).

cultured in 96-well plates (0.2 mL) with 5×10^4 $CD4^+CD25^+$ (from patients) for 3 days. Proliferation was measured in triplicates by the expression of CFSE (Sigma-Aldrich, St. Louis, MO, USA).

2.11. Statistical analysis

All data were analyzed using SPSS 22.0 software. Data were presented as mean ± standard error of the mean (SEM). Percent detectable were compared by Pearson's chi-squared test. The differences among the two different groups were evaluated with student *t* test. Graphs were prepared with Prism version 8 (GraphPad Software Inc., La Jolla, CA). For all tests, p values less than 0.05 were considered significantly different. n.s. not significant, * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$.

3. Results

3.1. Patient characteristics

According to the inclusion/exclusion protocol, 40 patients (21 male and 19 female) who underwent cardiovascular surgery between October 2020 and January 2021 were enrolled in this study. Among those patients, 26 did not develop lung injury after CPB, and 14 developed injury after the surgery (Table 1). The average age, gender, BMI, and NYHA class between these two groups before surgery were similar. In addition, CPB time, auxiliary circulation time, and lowest Hct during surgery were similar between those two groups.

3.2. Low level of peripheral Treg cells in lung injury patients before surgery

Considering a certain correlation between Treg cells and progression of lung injury after cardiopulmonary bypass, it would be interesting to clarify the difference of Tregs between non-lung injury and lung injury patients before surgery. We examined Treg cells in PBMCs from 40 patients before the CPB operation (pre-CPB Tregs), in which 14 developed lung injury after the surgery. As shown in Figure 1, the proportion (Figures 1b and 1c) of pre-CPB Tregs was lower in lung injury patients compared with non-lung injury patients, while little difference was found between lung injury and non-lung injury patients in Th1 (Figure 1d, e and Th17 (Figures 1f and 1g) cells, indicating there was already difference in Treg cells but not in other T helper subsets between lung injury and non-lung injury patient even before cardiopulmonary bypass.

3.3. Suppressive role of Tregs in the proliferation of effector T lymphocytes

Treg cells were originally described as suppressing proliferation of other lymphocyte subsets. Thus, we speculated that the dysfunction of peripheral pre-CPB Treg cells may actively participate in the onset of lung injury after CPB. We isolated pre-CPB Treg cells from patients and co-cultured with $CD4^+CD25^-$ (Figures 2a and 2b) or with $CD8^+CD25^-$ (Figures 2c and 2d) cells from healthy donors. Both effector CD4 and CD8 T cells cocultured with pre-CPB Treg cells from lung injury patients showed higher proliferation ability compared to those with pre-CPB Tregs from non-lung injury patients. These results suggested that pre-CPB Tregs from lung injury patients presented reduced ability to suppress the proliferation of effector CD4 and CD8 T cells.

3.4. Inhibition of Tregs on function of effector T lymphocytes

As Treg cells were reported to inhibit the function of other effector T lymphocytes, next we examined the cytokine produced by effector T lymphocytes that cocultured with the pre-CPB Treg cells from patients. Results showed that more IFN-γ^+ CD4 T cells were found in $CD4^+CD25^-$ T cells co-cultured with pre-CPB Tregs from lung injury patients than with those from non-lung injury patients (Figure 3b). Similarly, IFN-γ-

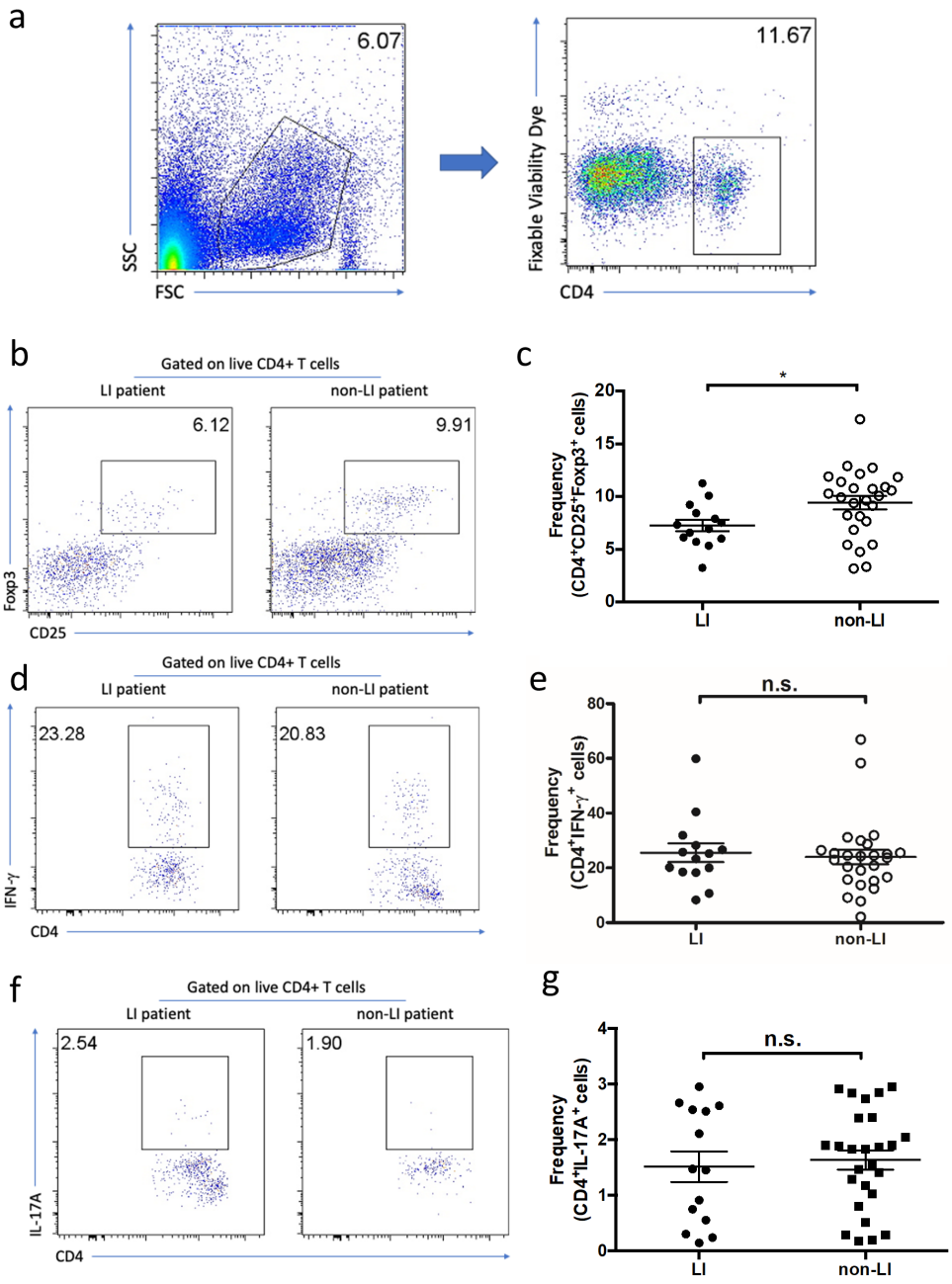

Figure 1. Treg cells in lung injury and non-lung injury patients. PBMC from lung injury and non-lung injury patients before CPB respectively were analyzed for Treg, Th1 and Th17 cells by flow cytometry. **a**. The flow cytometry gating strategy for live $CD4^+$ T cells (applied for all gating strategy in this study). **b**. Representative plots for flow cytometry data of Treg cells ($CD4^+CD25^+Foxp3^+$) in PBMC. **c**. The frequency of Treg cells in **(a)**. **d**. Representative plots for flow cytometry data of Th1 cells ($CD4^+IFN\text{-}\gamma^+$) in PBMC. **e**. The frequency of Th1 cells in **(d)**. **f**. Representative plots for flow cytometry data of Th17 cells ($CD4^+IL\text{-}17A^+$) in PBMC. **g**. The frequency of Th1 cells in **(f)**. Error bars denote mean ± SEM. Each dot in **c**, **e** and **g** represented an individual.Statistical analysis was performed using t test analysis, * $p < 0.05$.

producing CD8 T cells increased when cocultured with pre-CPB Tregs from lung injury patients compared to those with pre-CPB Tregs from non-lung injury patients (Figure 3d). These data suggested that pre-CPB Tregs from lung injury patients presented defect in inhibiting cytokine secreting by effector T lymphocytes.

3.5. Altered TGF-β and IL-10 levels in serum

Treg cells can play immunosuppressive roles by excreting TGF-β and IL-10. Considering lower amount of pre-CPB Treg cells in LI patients, we examined levels of TGF-β, IL-10, IFN-γ, and IL-17A in serum of the patients before surgery. Data showed that the pre-surgery levels of TGF-β (Figure 4a) and IL-10 (Figure 4b) were markedly lower in lung injury patients than in non-lung injury patients meanwhile IFN-γ (Figure 4c) and IL-17A (Figure 4d) had similar levels between serum from Lung injury and non-lung injury patients, indicating the defect of Treg cells in cytokine

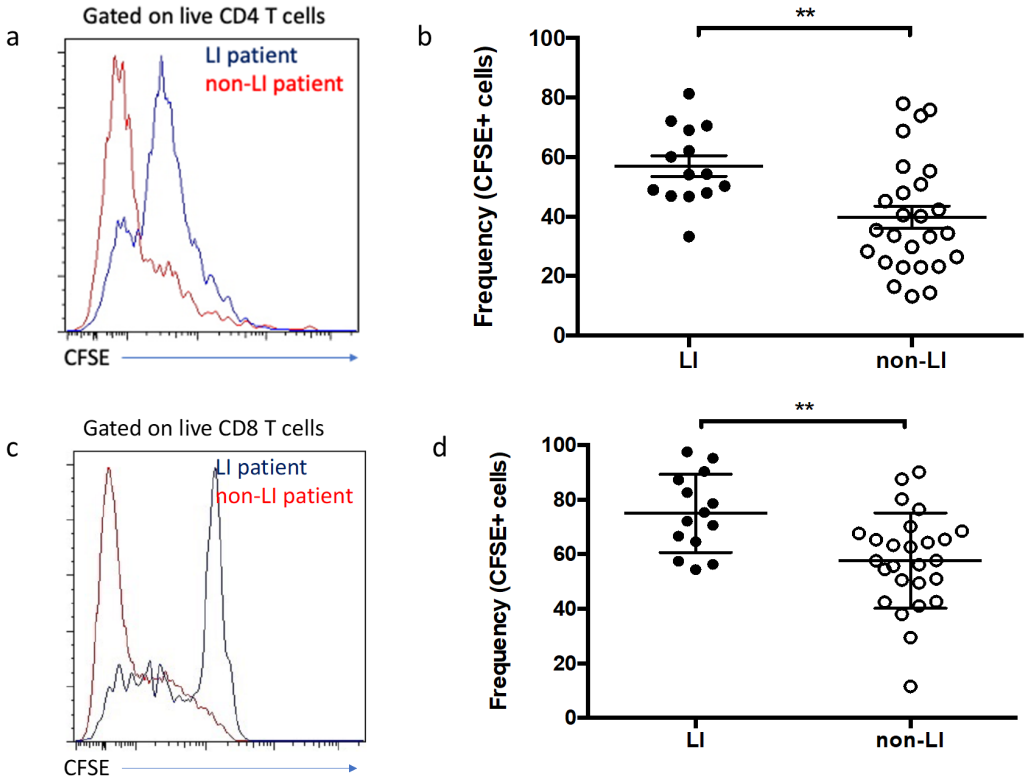

Figure 2. Inhibition of Treg cells on CD4 and CD8 T cells. Treg cells ($CD4^+CD25^+$) were isolated from periphery blood of lung injury or non-lung injury patients before CPB, then co-cultured with $CD4^+CD25^-$ or $CD8^+CD25^-$ from healthy donor. **a**. Representative histogram for flow data of CFSE expression in $CD4^+CD25^-$ T lymphocytes post co-cultured with Treg cells. **b**. Frequency of $CFSE^+$ cells in **a**. **c**. Representative histogram for flow data of CFSE expression in $CD8^+CD25^-$ T lymphocytes post co-cultured with Treg cells. **d**. Frequency of $CFSE^+$ cells in **c**. Each dot in **b** and **d** represented an individual. Statistical analysis was performed using *t* test analysis, * $p < 0.05$.

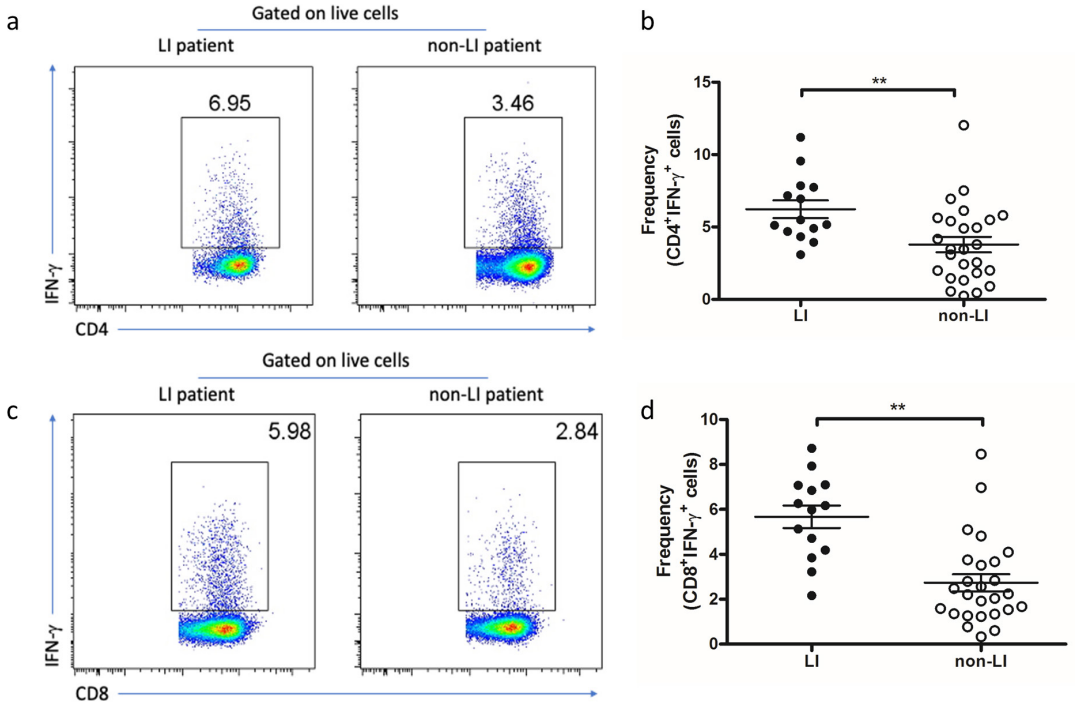

Figure 3. IFN-γ-producing T cells in lung injury and non-lung injury patients. Treg cells ($CD4^+CD25^+$) were isolated from periphery blood of lung injury or non-lung injury patients before CPB, then co-cultured with PBMC from healthy donor. **a**. Representative plotsfor flow data of $CD4^+IFN\text{-}\gamma^+$ cells post co-cultured with Treg cells. **b**. The frequency of $CD4^+IFN\text{-}\gamma^+$ cells in **(a)**. **c**. Representative plots for flow data of $CD8^+IFN\text{-}\gamma^+$ cells post co-cultured with Treg cells. **d**. The frequency of $CD8^+IFN\text{-}\gamma^+$ cells in **(c)**. Each dot in **b** and **d** represented an individual. Statistical analysis was performed using *t* test analysis, * $p < 0.05$.

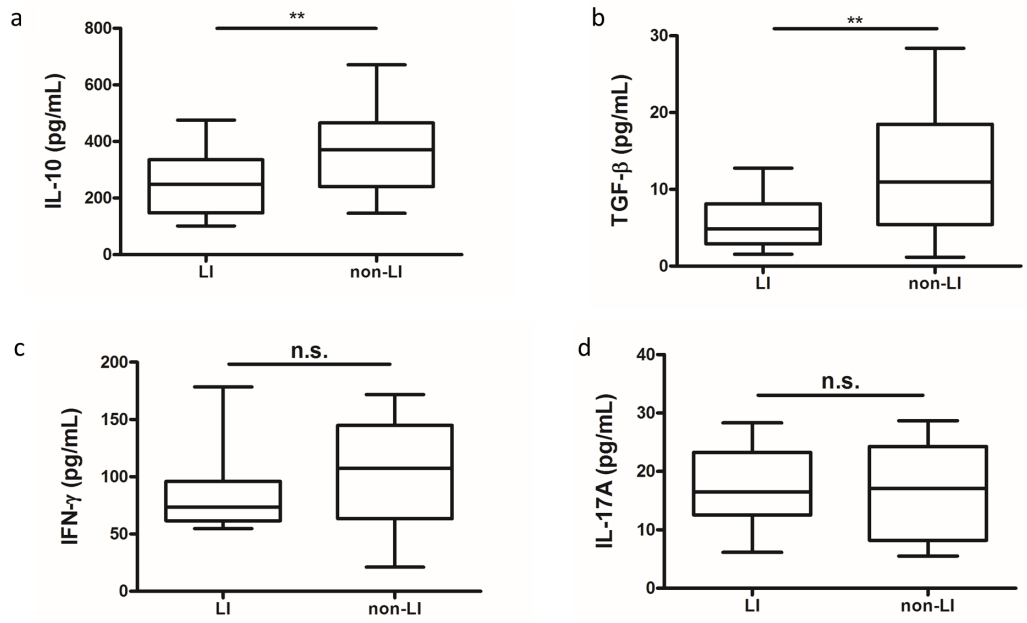

Figure 4. Levels of cytokines from serum in lung injury and non-lung injury patients. The levels of TGF-β **(a)**, IL-10 **(b)**, IFN-γ **(c)** and IL-17A **(d)** in serum from lung injury and non-lung injury patients were detected by ELISA. Each dot represented an individual. Statistical analysis was performed using *t* test analysis, * $p < 0.05$.

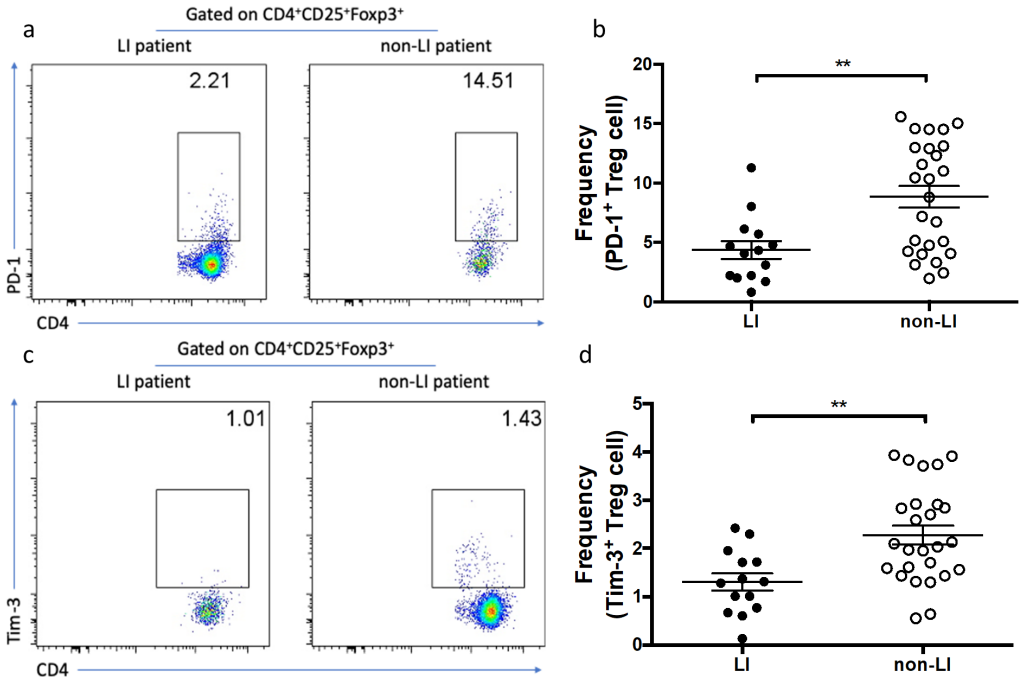

Figure 5. Expression of PD-1 and Tim-3 on Treg cells. Inhibitory immune checkpoint expression on Tregs in PBMC from lung injury and non-lung injury patients were analyzed by flow cytometry. **a**. Representative plots for flow data of $CD4^+CD25^+Foxp3^+PD\text{-}1^+$ cells in peripheral blood. **b**. The frequency of $CD4^+CD25^+Foxp3^+PD\text{-}1^+$ cells in **(a)**. **c**. Representative plots for flow data of $CD4^+CD25^+Foxp3^+Tim\text{-}3^+$ cells in peripheral blood. **d**. The frequency of $CD4^+CD25^+Foxp3^+Tim\text{-}3^+$ cells in **(c)**. Each dot in **b** and **d** represented an individual. Statistical analysis was performed using *t* test analysis, * $p < 0.05$.

production in lung injury patients.

3.6. Expression of PD-1 and Tim-3 on Tregs

PD-1 and Tim-3 could be expressed on Tregs and modulate function of these cells. Also, previous studies demonstrated that PD-1-PD/L1 pathway plays a central role in lung protection during acute lung injury. In addition, higher expression of Tim-3 on Tregs was associated with better clinical outcome in acute lung injury patients. Thus, we further examined the expression of PD-1 and Tim-3 on peripheral pre-CPB Tregs from lung injury and non-lung injury patients. We observed less $PD\text{-}1^+$ Treg cells among PBMC from

lung injury patients than from non-lung injury patients (Figure 5b). Similarly, lower level of Tim-3$^+$ Tregs was also found in pre-CPB Tregs from lung injury patients compared with non-lung injury patients (Figure 5d).

4. Discussion

In this study, we showed a decrease in peripheral Treg cells both in population and in function from lung injury patients. Less pre-CPB Tregs were detected in lung injury patients than in non-lung injury patients, and these cells were with lower suppressive ability to the proliferation and cytokine secretion of effector T cells. Also, declined serum levels of TGF-β and IL-10 indicated impaired Treg function in cytokine production in lung injury patients. In addition, expression of PD-1 and Tim-3 on pre-CPB Tregs were lower in lung injury patients compared with those in non-lung injury patients. All these data indicate there is a difference in Treg cells between lung injury and non-lung injury patients before cardiac surgery, and propose Tregs as a potential biomarker for lung injury diagnosis during CPB.

Previous studies have suggested that circulating humoral and inflammatory factors mediate the pulmonary injury associated with CPB due to exposure to foreign material (*7*). Other factors such as complement activation, ischemia-reperfusion injury, proteases, arachidonic acid metabolites, endotoxin, and bacterial translocation also contribute to this process (*8*). Apoptosis, also known as programmed cell death, plays important roles in disease states (*9*). Some supporting treatment can decrease the occurrence of cardiomyocyte apoptosis in the CPB process (*10,11*) and protect cardiomyocyte (*12,13*). But the exact mechanism underlying the process is unclear. Treg cells, either natural or induced, suppress a variety of physiologic and pathological immune responses (*14,15*). These cells have been considered as a potential target for treating several lung injuries. Previous research mainly focused on Tregs in mice model. However, research about Tregs in human lung injury after CPB remains unclear. Interestingly, in our study, lower level with impaired function of Treg cells was found in lung injury patients before surgery than in non-lung injury patients, suggesting patients with better immunosuppressive function before surgery may have less lung injury after CPB. This could explain why patients with similar pre-operation conditions underwent same surgical process could develop into different degree of lung injury post-operation.

Mechanism of Treg regulation in lung injury remains unclear (*16,17*). Programmed cell death receptor 1 (PD-1) pathway is critical to maintain the intricate balance between positive and negative signals to ensure adequate immune protection against pathogens and yet prevent over activation of lymphocytes. Similar to PD-1, compelling evidence is emerging for the role of Tim-3 in peripheral immune tolerance, autoimmune response, and antitumor and antiviral immune evasion (*18*). Studies have shown that Tim-3 is constitutively expressed on natural Tregs and has been identified as a regulatory molecule of alloimmunity through its ability to modulate CD4$^+$ T cell differentiation in mice. In humans, Tim-3 expression on Treg cells identifies a population highly effective in inhibiting pathogenic Th1- and Th17-cell responses. Here, we observed decreased expression of PD-1 and Tim-3 on Treg cells in lung injury patients before surgery, indicating the involvement of these molecules through Treg cells and may play an important role in the development of CPB-induced lung injury.

This study reported the difference of Treg cells between lung injury and non-lung injury patients before cardiac surgery, and proposed Tregs as a potential biomarker for lung injury diagnosis during CPB. However, the sample size of this current study is relatively small, and population-based studies with large sample size are required to further verify the findings in this present work. Additionally, we only compared the population and function of pre-CPB Tregs between lung injury and non-lung injury patient. Further studies to identify specific molecules of Treg cells in CPB-induced lung injury and investigate the mechanism for Treg cells on lung injury development after CPB are required.

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