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Original Article

Inducible costimulatory molecule deficiency induced imbalance of Treg and Th17/Th2 delays rejection reaction in mice undergoing allogeneic tracheal transplantation

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Abstract: Objective: This study aimed to investigate the role of inducible costimulatory molecule (ICOS) pathway in the rejection reaction of mice undergoing allogeneic tracheal transplantation. Methods: The bronchus was separated from wide-type (WT) BalB/c mice and transplanted into WT BalB/c mice, C57 mice and *icos*$^{-/-}$ mice to prepare the obliterative bronchiolitis (OB) animal model. The transplanted bronchus was pathologically examined; flow cytometry was done to detect the T cell subsets and activity of the bronchus and spleen of recipient mice. Results: 21 d after transplantation, evident rejection reaction was observed and the proportion of Th2 and Th17 cells increased significantly in the bronchus and spleen in C57 mice receiving allogeneic tracheal transplantation when compared with mice with autologous transplantation, but the proportion of Treg cells was comparable between them. When compared with WT BalB/c mice, the proportion of Th2, Th17 and Treg cells reduced markedly and rejection reaction was attenuated in *icos*$^{-/-}$ mice receiving tracheal transplantation, although rejection reaction was still noted. Conclusion: *icos* knockout may delay the rejection reaction after tracheal transplantation, which might be ascribed to the imbalance among Th2, Th17 and Treg cells.

Keywords: Lung transplantation, inducible costimulatory molecule, chronic rejection, bronchiolitis obliterans, Th2, Th17

Introduction

Lung transplantation is an exclusive and effective strategy for the therapy of end-stage lung disease, but patients undergoing lung transplantation usually have a low survival rate when compared with patients undergoing transplantation of other solid organs [1]. Chronic rejection is a major factor influencing the long term survival of patients receiving lung transplantation. Bronchiolitis obliterans syndrome (BOS) is a major cause of death in patients after lung transplantation and pathologically characterized by obliterative bronchiolitis (OB) and fibrosis of terminal bronchioles [1, 2]. Mice with allogeneic tracheal transplantation are preferred animals used in studies on OB. These mice have been applied in several studies and promising results have been achieved [3, 4]. Inducible costimulatory molecule (ICOS) is one of members of costimulatory CD28 family and is mainly expressed on T cell subsets including Th1 cells, Th2 cells, Th17 cells, Treg cells and Tfh cells. ICOS play important roles in the acute and chronic rejection reaction after transplantation of organs or cells [5-8]. Studies have shown that to block ICOS pathway may prolong the survival time of animals receiving transplantation of the kidney, liver and heart [9-11]. However, the role of ICOS/ICOSL pathway in lung transplantation, especially the chronic rejection reaction, and the specific mechanisms are still

poorly understood. In the present study, icos knockout mice were used to establish the chronic rejection model in mice with allogeneic tracheal transplantation, aiming to investigate the influence of ICOS/ICOSL pathway on the rejection reaction and the potential mechanisms.

Materials and methods

Animals

Male inbred mice aged 8-10 weeks were used and included 40 BalB/C mice and 10 C57BL/6 mice weighing 20-22 g. Animals were purchased from the Shanghai Laboratory Animal Research Center. ICOS knock-out male mice aged 8-10 weeks (B6.129P2-Icos^{tm1Mak}/J; n = 10) were prepared with C57 mice and purchased from JACKSON lab and reproduced in the Animal Center of Tongji University. All these animals were housed in the Animal Center of Tongji University.

Lung transplantation

Experiment was done twice. BalB/C mice served as donors and recipient mice were divided into 3 groups: (1) Homogenic transplantation (n = 5): recipients were BalB/C mice; (2) wide-type (WT) allogeneic transplantation (n = 5): recipients were WT C57BL/6 mice; (3) *icos*$^{-/-}$ allogeneic transplantation (n = 5): recipients were *icos*$^{-/-}$ B6 mice. Donor mice were sacrificed by cervical dislocation and the neck was sterilized with 75% ethanol. A middle incision was made at the neck and the subcutaneous tissues were separated to expose the trachea. The main bronchus was collected as long as possible (about 8-10 mm) and divided into 2 segments which were placed in PBS. The trachea was collected under an aseptic condition and not contaminated by blood. Recipient mice were intraperitoneally anesthetized with chloral hydrate and the hair on the skin of the back was removed and the skin was sterilized. An incision (about 3-5 mm) was made in the back, and the subcutaneous tissues were separated to form a “skin bag” in which there was no blood. The collected trachea was placed into the “skin bag” which was then sutured with 0 suture. The wound was treated with chlortetracycline eye ointment to prevent infection. One trachea was embedded in the back at both sides, and the operation from trachea collection to tracheal transplantation was done within 5 min.

Main reagents

RPMI-1640, fetal bovine serum (FBS; Gibco, USA), Flow Cytometry Staining Buffer, Fixation / Permeabilization Concentrate, Fixation/Permeabilization Diluent, CD4-FITC, CD25-APC, Foxp3-PE, IL-4-PE-Cy7, IL-17-PE (eBioscience, USA), Phorbol 12-myristate 13-acetate (PMA), ionomycin, Brefeldin A Solution (BFA) (Sigma), CD3 and CD28 expansion kit for T cell activation (Miltenyi Biotec, USA) were used in the present study.

Sample collection

At 21 d after tracheal transplantation, the trachea was harvested from the skin bag. In brief, mice were sacrificed by cervical dislocation and the skin bag was carefully separated to avoid pressing the trachea. The transplanted trachea and spleen were collected and used in following experiments: (1) pathological examination: the trachea was fixed in 10% neutral formaldehyde, embedded in paraffin and cut into sections followed by HE staining and pathological analysis; (2) flow cytometry: the trachea was cut into blocks (1 mm^3) and digested with 1.0 mg/ml type IV collagenase (Sigma) at 37°C for 30 min, and single cell suspension was prepared.

Collection of spleen mononuclear cells

Mice were sacrificed by cervical dislocation and the spleen was collected under an aseptic condition. Cell suspension of the spleen was prepared with 3 ml of RPMI-1640 by using a 200-mesh filter, centrifugation was done at 1000 r/min (centrifugation diameter: 17.5 cm) for 5 min and the supernatant was removed. Red blood cells were lysed with Tris-NH4Cl buffer, followed by centrifugation. Then, the resultant cells were re-suspended in 2 ml of PBS. After centrifugation, the supernatant was removed, and cells were re-suspended in RPMI-1640 at a density of 1×10^6/mL.

Detection of intracellular cytokines

The trachea and spleen were used to prepare single cell suspension and then these cells were seeded into 96-well plates. Subsequently, 26 ng/mL PMA, 1 μg/mL ionomycin and 10 μg/

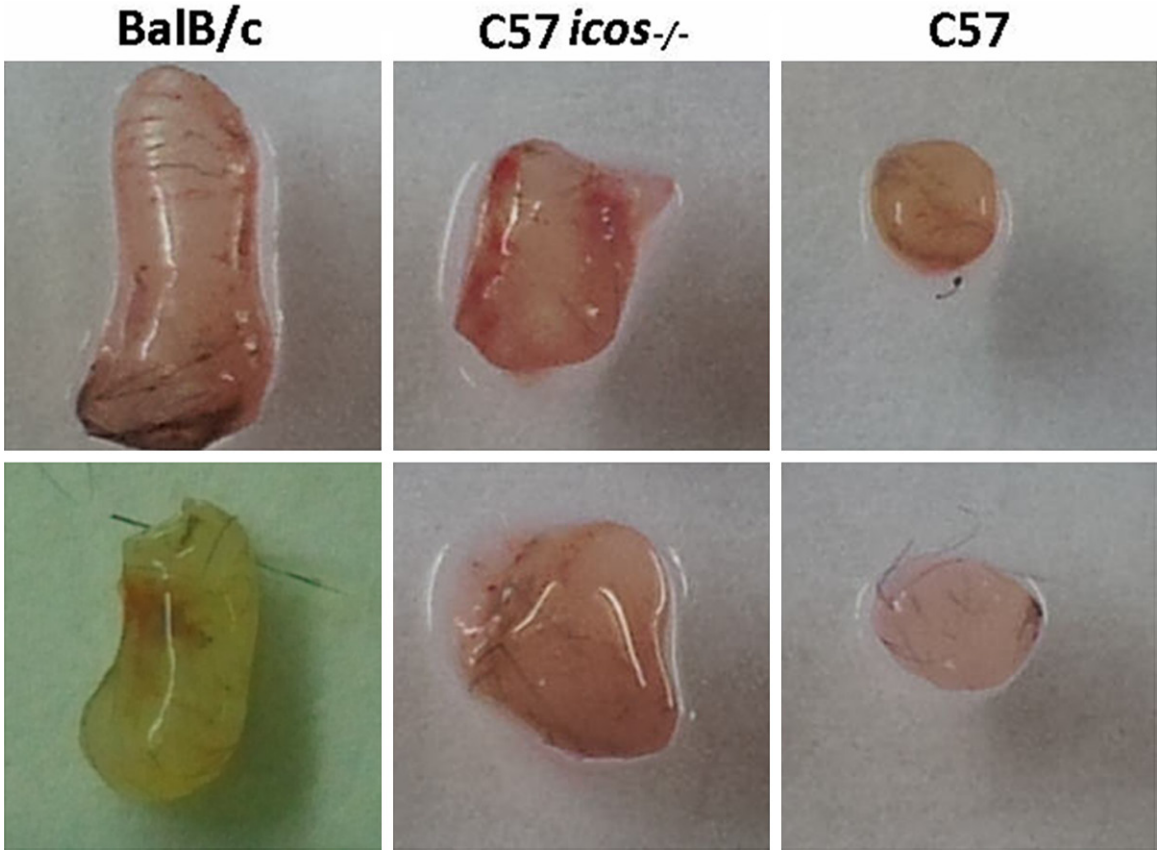

Figure 1. Trachea in different groups was collected at 21 d after transplantation. The Tracheal size in *icos*$^{-/-}$ mice was larger than that in WT mice at 21 d after transplantation.

mL BFA were added to each well, followed by incubation at 37°C in an environment with 5% CO_2 for 4 h. Cells were harvested and washed with PBS. Then, cells were incubated with CD4-FITC at room temperature in dark for 20 min. After washing in PBS, the supernatant was removed, and cell fixation solution (500 μl) was added, followed by incubation at 4°C for 30 min. After addition of 1 ml of penetrating solution, centrifugation was done and the supernatant was removed. Following addition of 50 μl of penetrating solution, fluorescence conjugated IL-17, IL-4 or FoxP3 antibody was added, followed by incubation at 4°C over night. On the second day, 500 μL of penetrating solution was added, followed by centrifugation. The supernatant was removed, and 200 μL of PBS was used to re-suspend these cells. Flow cytometry was done to detect above cytokines.

Statistical analysis

The expression of different cytokines after different treatments was compared with oneway analysis of variance. A value of $P < 0.05$ was considered statistically significant.

Results

Rejection reaction is delayed in icos$^{-/-}$ *mice receiving tracheal transplantation*

In different groups, all the mice survived after transplantation, and there were no wound infection and edema. At 21 d after tracheal transplantation, mice were sacrificed by cervical dislocation, and the skin of the back was incised. 1) In autologous transplantation group, there was no adhesion between trachea and skin, and the trachea was collected smoothly. The trachea was intact in shape and transparent, the cartilage rings were clear and macroscopically visible, and the openings at both ends were evident. 2) In WT C57 group, the trachea shrunk significantly, became soft and opaque and adherent to the skin requiring sharp separation. There were no visible cartilage rings and openings at both ends. In several

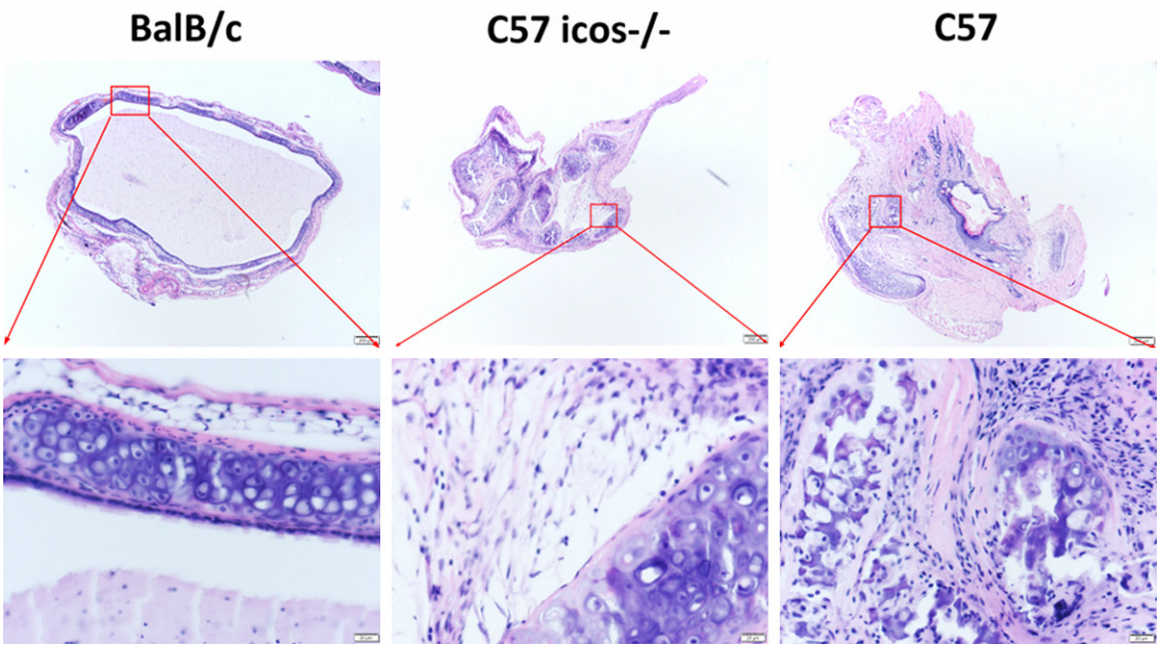

Figure 2. HE staining of the trachea in different groups. The rejection reaction was attenuated in *icos*$^{-/-}$ mice at 21 d after transplantation (upper: 40×; lower: 400×).

animals, yellow or brown necrotic tissues were expelled from the trachea during the collection of the trachea. 3) In *icos*$^{-/-}$ mice, the cartilage rings were evident and intact in several mice. Luminal occlusion was observed in several mice, and the trachea became soft and it was easy to separate the trachea from the skin. The size of the trachea was larger than that in WT C57 mice (**Figure 1**).

HE staining of the trachea showed 1) in autologous transplantation group, there were clear layers and lumen in the trachea, and mucus was observed in the lumen which had infiltration of some inflammatory cells. There was no fibrous hyperplasia, epithelial cells were intact in the lumen, and the tracheal cartilage was complete and had no edema and calcification; 2) In WT C57 mice, the transplanted trachea collapsed, the number of tracheal cartilage reduced and calcification was observed in these tracheal cartilage, the lumen was absent or even closed, and infiltration of a large amount of granulocytes and lymphocytes was observed in the basement membrane and submucosa. The tracheal epithelial cells were absent, fibrous granulation tissues increased, and angiogenesis was also observed. In a fraction of tissues, skin tissues were noted. The skin tissues were not removed aiming to assure the integrity of the trachea because there was adhesion between trachea and skin. 3) In *icos*$^{-/-}$ mice, the tracheal pathology was milder than that in WT C57 group. The tracheal cartilages collapsed, but the chondrocytes were not significantly destructed. The tracheal calcification was mild, the lumen shrunk and became irregular, and infiltration of some inflammatory cells and hyperplasia of fibroblasts were observed in the trachea (**Figure 2**).

ICOS deficiency causes imbalance among Treg, Th2 and Th17 cells and attenuates GVHD

The trachea and spleen were collected from mice of different groups and digested in collagenase. Flow cytometry was done to detect the Th2 cells, Th17 cells and Treg cells after activation with CD3 and CD28. **Figure 3** showed, when compared with autologous transplantation group, the proportion of CD4+ cells remained unchanged in the leukocytes of the spleen, the proportion of Treg cells in CD4+ cells increased slightly, but the proportion of Th17 cells and Th2 cells in CD4+ cells increased markedly. In *icos*$^{-/-}$ mice, the proportion of CD4+ cells in the leukocytes of the spleen reduced markedly when compared with WT mice and autologous transplantation mice. The proportion of Th17 cells and Th2 cells in CD4+ cells

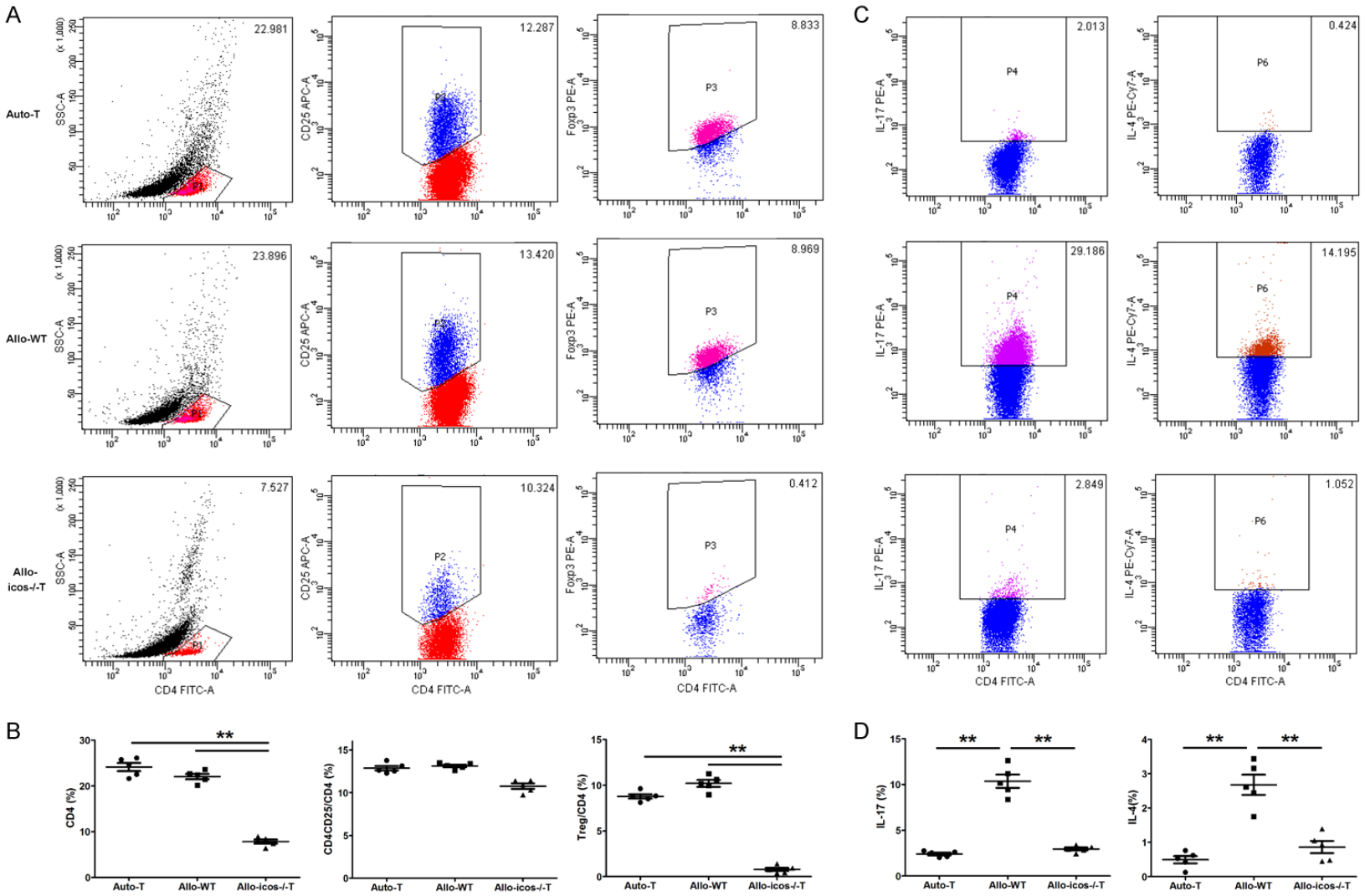

Figure 3. Imbalance among Treg cells, Th2 cells and Th17 cells in the spleen of mice in different groups. At 21 d after tracheal transplantation, the spleen was collected from mice in different groups. After stimulation with CD3 and CD28, the proportion of CD4+ cells in leukocytes, and that of Treg cells (CD4+CD25+Foxp+), Th2 cells (CD4+IL-4+) and Th17 cells (CD4+IL-17+) in CD4+ cells were determined by flow cytometry. A. Proportion of CD4+ cells and Treg cells in different group (flow cytometry); B. Statistical analysis of data from representative 2 experiments in A; C. Proportion of Th2 cells and Th17 cells in different groups (Flow cytometry); D. Statistical analysis of data from representative 2 experiments in C.

also reduced dramatically when compared with WT mouse group, but was slightly higher than that in autologous transplantation group. However, the proportion of Treg cells in CD4+ cells was significantly higher than that in WT mouse group and autologous transplantation group.

Furthermore, the trachea was collected and digested with collagenase, and single cell suspension was prepared. Cells were stimulated with CD3 and CD28, and the proportion of Th2 cells, Th17 cells and Treg cells was determined by flow cytometry. In autologous transplantation, there were no obvious lymphocyte subsets and CD4+ cells (data not shown). **Figure 4** showed Th2 cells, Th17 cells and Treg cells in the trachea. When compared with WT C57 mice, the proportion of Th17 and Th2 cells in the trachea reduced significantly in *icos*$^{-/-}$ mice, and more obvious reduction was observed in the proportion of Treg cells. The changes in the proportion of these cells in the trachea were similar to those in the spleen.

Discussion

Although the quality of life is improved and the survival time prolonged in a majority of patients receiving lung transplantation with the progression of modern medicine, the therapeutic outcome varies among individuals. More than 80% of patients undergoing lung transplantation may survive for 1 year, but the 5-year and 10-year survival rate is lower than 50% and 20%, respectively, which is significantly lower than that in patients receiving the transplantation of the liver or kidney. The main reason for low survival rate of patients undergoing lung transplantation is ascribed to OB, the clinical manifestation of which is BOS [12]. The pathophysiological processes underlying the occurrence and development of OB is still poorly understood. Some investigators propose that the autoimmunity against autologous antigens also plays an important in the pathogenesis of OB besides immune response to antigens from the donor [13, 14]. The chronic rejection after lung transplantation is mainly attributed to the CD4+ T cell mediated immune airway injury. Currently, which subset of CD4+ T cells plays a key role in the post-transplantation chronic rejection is still controversial and there is no effective strategy for the prevention and therapy of this chronic rejection. There is evidence showing that Th2 cells and Th17 cells play an important role in the occurrence and development of BOS and may serve as crucial factors promoting the occurrence and progression of OB [15-17], but Treg cells may inhibit the progression of OB. Thus, there is an antagonism between Th2/Th17 cells and Treg cells, and the balance between Th17 cells and Treg cells may be used to predict the risk for BOS after lung transplantation [14]. The occurrence of OB after lung transplantation is closely associated with the biological balance among Th17 cells, Th2 cells and Treg cells.

ICOS is one of costimulatory molecules essential for the complete activation and functions of T lymphocytes. Generally, ICOS may activate the immunity and enhance the immune function. The constitutive expression of ICOS ligand is found in B cells and dendritic cells which are professional antigen presenting cells. ICOS/ICOSL pathway functions mainly to enhance the recalling proliferative reaction, activate T cells to generate cytokines and mediate the functions of T cells. To block ICOS/ICOSL pathway may promote the differentiation of Th1 cells into Th2 cells. ICOS is expressed on both Th2 cells and Th17 cells and important for the functions of both cell types [18]. In addition, Treg cells also express ICOS. Whether ICOS deficiency influences the rejection after transplantation is not clear. There is evidence showing that to block the ICOS pathway may prolong the survival time of transplanted heart, liver and kidney [9-11]. Two single nucleotide polymorphisms of ICOS gene (rs10183087 and rs4404254) influences the expression of ICOS and is closely associated with the delayed kidney function and non-functioning kidney after kidney transplantation. Another SNP of ICOS gene (rs10932037) is related to the survival of grafts. However, the SNP of other costimulatory molecules (such as CD28, CTLA-4 and PD-1) has no relationship with the function of transplanted kidney. These findings indicate the importance of ICOS/ICOSL pathway in the prognosis of grafts [19]. The gene polymorphism of ICOS of both donors and recipients is closely related to the outcome after organ transplantation [20]. Thus, ICOS/ICOSL may play important roles in the acute and chronic rejection after transplantation of organs and cells, and ICOS/ICOSL may influence the survival and function of grafts or even affect the survival time of patients receiving transplantation. However,

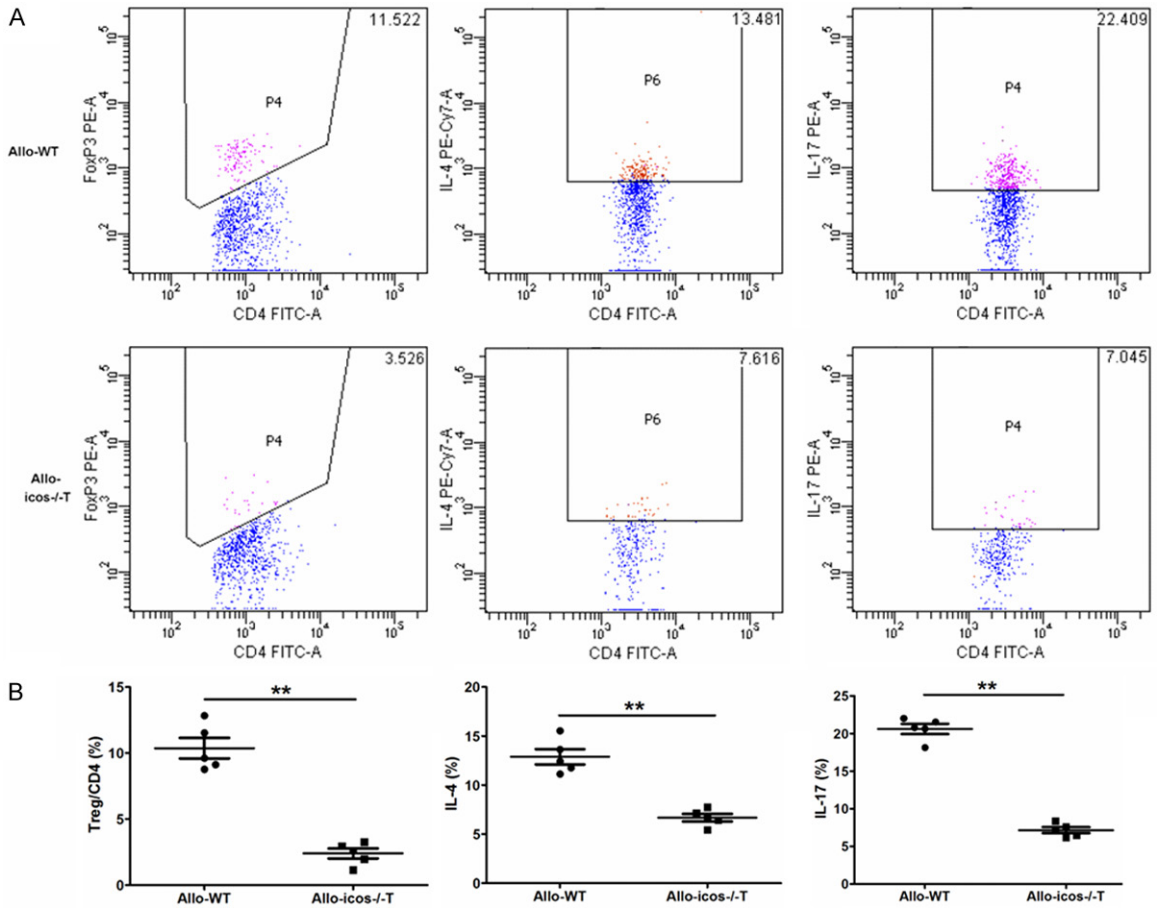

Figure 4. Imbalance among Treg cells, Th2 cells and Th17 cells in *icos*$^{-/-}$ mice after trachea transplantation. At 21 d after tracheal transplantation, the trachea was collected, and digested with collagenase. Single cell suspension was prepared, and cells were then treated with CD3 and CD28. The proportion of Treg cells, Th2 cells and Th17 cells was determined in CD4+ cells. A. Proportion of Treg cells, Th2 cells and Th17 cells in WT mice and *icos*$^{-/-}$ mice (flow cytometry); B. Statistical analysis of data from 3 representative experiments in A.

the influence of ICOS pathway on the immune rejection after lung transplantation and whether to block ICOS pathway may attenuate or abolish the occurrence and development of OB are still unclear.

To date, few studies have been conducted to investigate the role of ICOS/ICOSL pathway in the rejection reaction after lung transplantation, and only one study published in 2008 was found in the available studies [21]. In this study, mouse trachea was transplanted, and results showed tracheal occlusion was evident at 4 weeks after transplantation. In addition, infiltration of a large amount of DC, CD4+ T cells and CD8+ T cells were observed in the transplanted trachea. When compared with isogeneic transplantation, the mRNA expression of ICOS increased by 10 times in the trachea of allogeneic transplantation group. However, whether ICOS is directly involved in the rejection after lung transplantation and which type of cells and mechanisms are involved in the occurrence of rejection have never been reported.

In present study, *icos*$^{-/-}$ mice were recruited as recipients, aiming to investigate the rejection and its mechanisms after tracheal transplantation. Our results showed the trachea shrunk and became soft, the tracheal lumen was closed, the cartilage rings were invisible and the trachea was adherent to the skin at 21 d after allogeneic transplantation in WT mice. Pathological examination also showed typical features of chronic rejection. However, in *icos*$^{-/-}$ mice, the trachea size was larger than that in WT C57 mice, cartilage rings were visible and

intact in several mice, and congestion was observed in the trachea of several mice. In remaining *icos*$^{-/-}$ mice, the lumen was closed, the trachea became soft and mildly adherent to the skin, but it was easy to separate the trachea. Pathological examination showed the infiltration of inflammatory cells and fibrosis in the trachea in *icos*$^{-/-}$ mice were milder than those in WT mice. These findings suggest that ICOS deficiency inhibit and attenuate the progression of rejection after tracheal transplantation, but fails to completely abolish the rejection. Further investigation of T cell subsets in the mouse spleen and trachea revealed that the proportion of Th17 cells and Th2 cells in the trachea and spleen increased significantly in WT mice, but the proportion of CD4 cells in the spleen and that of Th17 and Th2 cells in the spleen and trachea reduced markedly in *icos*$^{-/-}$ mice. Of interest, although the proportion of Treg cells in the spleen of WT cells was slightly higher than that in mice with autologous transplantation, the proportion of Treg cells in the spleen of *icos*$^{-/-}$ mice reduced significantly when compared with autologous transplantation mice and WT C57 mice. In addition, the proportion of Treg cells in the trachea of *icos*$^{-/-}$ mice was also markedly lower than that in WT C57 mice. Our findings were consistent with those in previous studies [15-17]. Available findings indicate that both Th17 cells and Th2 cells are involved in the occurrence and progression of rejection after allogeneic transplantation. Although the proportion of inhibitor Treg cells increases slightly, the functions of Th17 cells and Th2 cells are not completely inhibited in the whole body and the grafts, resulting in the presence of rejection. In this process, ICOS/ICOSL plays an important role in the functions of Th17 cells and Th2 cells. ICOS deficiency firstly inhibit the generation of CD4+ cells. In the rejection, although ICOS reduce the production of Th17 cells and Th2 cells and compromise their functions to delay and attenuate the rejection after transplantation, ICOS deficiency also reduce the proportion of Treg cells, and attenuate the inhibitory effect of Treg cells on Th17 cells and Th2 cells. Thus, Th17 cells and Th2 cells may partially exert pro-inflamamtory effect, and pathological features of mild rejection were also observed in the trachea. We speculate that ICOS deficiency may regulate the balance between Th17/Th2 cells and Treg cells to attenuate the rejection reaction after allogeneic tracheal transplantation.

Taken together, our study for the first time reported that ICOS deficiency may significantly delay and inhibit the rejection reaction after tracheal transplantation, which may be mainly ascribed to the reduced differentiation of CD4+ T cells, reduced proportion of Th2 cells and Th17 cells and decreased production of functional cytokines. After lung transplantation, blocking the ICOS/ICOSL pathway to inhibit the functions of Th2 cells and Th17 cells and taking measures to preserve Treg cells may become promising strategies for the prevention and therapy of complications of chronic rejection after lung transplantation. The specific mechanisms are required to be further studied.

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Disclosure of conflict of interest

None.

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