Radiological and Pathological Findings in a Minor-mismatch Mouse Orthotopic Lung Transplant Model under Immunosuppression

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ABSTRACT

Background: Mouse orthotopic lung transplantation (MOLTx) models are extremely useful. However, there are only few studies on non-invasive monitoring methods for lung rejection in these models. Additionally, a model of chronic rejection has so far been difficult to reproduce consistently with MOLTx.

Objective: To determine if CT scan of the lung graft can be considered a useful noninvasive monitoring method of evaluation of graft rejection in an animal lung transplantation model.

Methods: Left MOLTx was performed from B10 donors into B6 recipients (from B6 donors for syngeneic controls). Following transplantation, 3 different doses of cyclosporine—5, 15, and 25 mg/kg daily—were administered in the first week to suppress acute rejection. Positive controls did not receive cyclosporine. 8 weeks after transplantation, CT and histological rejection grading were performed.

Results: The negative controls did not show any inflammation. Positive controls revealed moderate acute rejection (A3). A3 was also detected in the 5-mg/kg group (100%). The 15-mg/kg group (n=7) demonstrated A3 in 4 mice and mild acute rejection (A2) in the remaining 3 mice. In this group, 4 mice had bronchiolitis obliterans (BO; C1, 57%). The 25-mg/kg group (n=3) showed A3 changes in 1 and A2 in 2 mice. On CT scan, lungs without BO (C0) had radiodensities of -278.1±110.7 Hounsfield units (HU). C1 lungs had ground-glass opacity or atelectasis with -83.4±46.8 HU (C0 *vs*. C1, p<0.001). On grouping with A2 or A3 in C0, significant differences were detected: -375.3±41.2 *vs*. -185.0±38.4 HU (A2+C0 *vs*. A3+C0, respectively), p=0.01.

Conclusion: BO can be modeled using this MOLTx model by administration of subtherapeutic doses of cyclosporine. CT scans are a valid tool for monitoring of rejection following MOLTx.

KEYWORDS: Lung transplantation model; Acute rejection; Chronic rejection; Computed tomography; Mouse

INTRODUCTION

Lung transplantation is established as a treatment for severe end-stage lung failure. So far, the median survival af-

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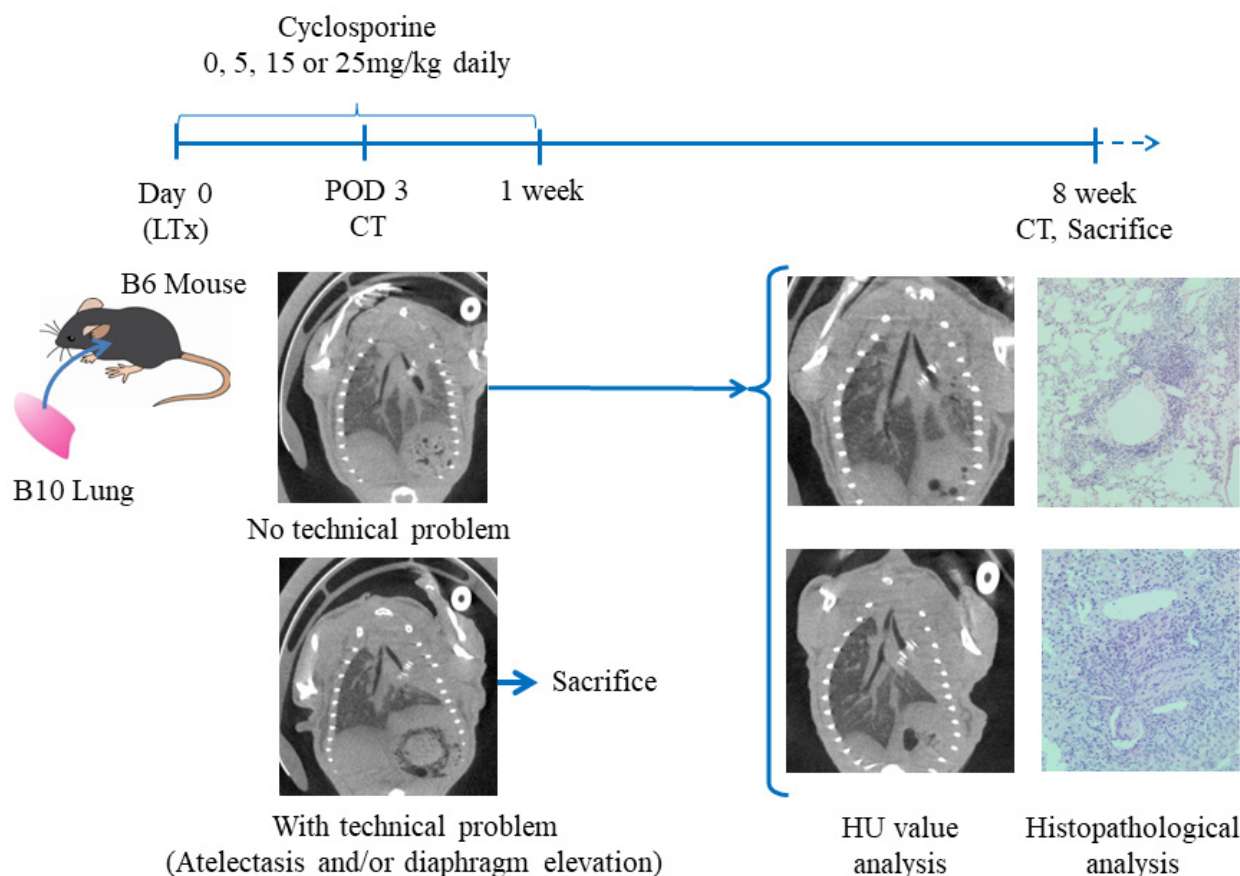Fax: +49-511-532-2852
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ter lung transplantation is only 5.8 years, according to the International Society for Heart and Lung Transplantation (ISHLT) [1]. The predominant cause of death is chronic lung allograft dysfunction (CLAD), which is histologically approved by the presence of bronchiolitis obliterans (BO) for which the largest risk factor is acute rejection [2, 3].

Since 2007, mouse orthotopic lung transplantation (OLTx) has successfully been performed by different research groups [4-6]. The mouse

Figure 1: Study protocol. The left lung was harvested from C57Bl/10J mice (B10: Jackson Laboratory, Sacramento, California) as the donor organ. The lung was orthotopically transplanted into a C57bl/6J (B6: bred in-house in the animal institute) mouse for allogenic transplantation. In the first week after transplantation, cyclosporine was injected subcutaneously daily at various doses (0 [positive controls], 5, 15 or 25 mg/kg). In negative controls, the donor lung was harvested from B6 mice. Thoracic computed tomography (CT) was performed three days after the transplantation. If CT findings indicated technical failure, the mice with failure were sacrificed. The remaining mice that had no operative complications were left alone until the 8th week after the transplantation. Thoracic CT was once again performed to measure the Hounsfield units (HU) value as a measure of lung parenchymal density. After the CT scan, the mice were sacrificed to histopathologically evaluate their lung for evidence of rejection.

OLTx model is challenging to create, yet is an extremely useful experimental model. Consistent development of BO using this model is likely to increase its utility as a research tool.

Recently, a minor histocompatibility complex mismatch model (the B10 to B6 strain combination) without immune suppression has been suggested as a murine chronic rejection OLTx model [7]. However, the combination is still controversial [8].

In the clinical monitoring of rejection after lung transplantation, strong acute lung rejection in recipients is accompanied by higher attenuation on computed tomography (CT). In addition, ground-glass opacity (GGO) is recognized in typical cases of acute and chronic rejection [9, 10]. However, in basic science fields, there is still only few studies related to the CT findings in mouse OLTx models and no statistical comparison of the CT findings has so far been made [11-13].

We performed B10 to B6 murine lung transplantation while administering subclinical doses of the calcineurin inhibitor, cyclosporine, to suppress acute rejection, extended the follow up to 8 weeks, and instituted CT scan monitoring of the lung graft in a quest to add a non-invasive rejection monitoring tool.

MATERIALS AND METHODS

Animals

This study protocol was approved by the German Animal Welfare Act (license number 16-2166). We used C57BL10/J substrain mice (B10: Jackson Laboratory, Sacramento, California) as the donors and C57BL6/J mice (B6: bred in house in the animal institute), weighing 25–30 g, as recipients for allogenic lung transplantation. Additionally, B6 mice were used as both the donor and recipient in negative controls. Animal handling and treatment were strictly maintained according to the German Animal Welfare Act.

Mouse Orthotopic Single Lung Transplantation

Our OLTx technique was modified from published techniques [4, 5]. Briefly, donor mice received analgesia with butorphanol (1 mg/kg) and were anesthetized with isoflurane under tracheal intubation. The intubation tube was connected to a ventilator (UNO microventilator, UNO Röestvaststaal, Zevenaar, the Netherlands). The lungs were flushed via the inferior vena cava with 5 mL heparinized Perfadex® (Vitrolife, Göteborg, Sweden), and the heart-lung block was excised and preserved on ice. Recipient mice received analgesia with metamizole (100 mg/kg) and were anesthetized with isoflurane administered through a breathing tube. The mouse was connected to the ventilator, and received a mixture of 1.2 L/min O_2 and 1.5% isoflurane. After left thoracotomy, the pulmonary artery and vein were separated from the bronchus. A 10-0 ligature was placed around the vein, and an 8-0 ligature was placed around the bronchus. Microvascular clamps were placed centrally at the hilus. First, the veins were anastomosed using the cuff-technic. Subsequently, the bronchus and arteries were anastomosed by the same method. The central side clamp was released, and the original left lung was removed. After confirming lung expansion, the thoracotomy was closed and the mouse was placed on a heating mat. After sufficient awakening, extubation was performed. The mouse was maintained on the heating mat for 5 days. During the period, butrophanol (1 mg/kg) and ciprofloxacin (7 mg/kg) were injected subcutaneously (every 12 hours and 24 hours, respectively, Fig 1).

Immunosuppressive Treatment

To prevent severe acute rejection and foster the development of BO/chronic rejection, we administered various doses of cyclosporine (Sandimmun®, Novartis Pharma, Nuremberg, Germany) subcutaneously in the first week after lung transplantation to create the following experimental groups: B6 to B6 transplantation, no cyclosporine; negative controls, B10 to B6, no cyclosporine; positive controls; and B10 to B6 transplantation with injection of 5 mg/kg, 15 mg/kg and 25 mg/kg cyclosporine every 24 hours as the treatment groups (n=6, 4, 4, 10, and 4 in the five groups, respectively). Cyclosporine dose titration aimed at achieving a sufficient, yet subclinical immunosuppressive effect of the drug.

CT Scanning

Mice were anesthetized with 1%–3% isoflurane in oxygen. µCT was performed using an Inveon µCT scanner (Siemens Medical Solutions USA, Inc, Knoxville, USA) equipped with Inveon Acquisition Workplace software 1.5 (Siemens). Projections were acquired over a total angle of 220° with an increment of 1° for technical control of the transplantation 3 days after the transplantation (POD 3-CT; 50 kV, 500 µA, 300 msec, binning 4, built-in 0.8 mm carbon filter). Before sacrificing the animals for histological analysis at the end of the 8th week, an additional respiratory-gated µCT was performed with 512 projections over a total angle of 360°. Data reconstruction was performed using bilinear interpolation, the Shepp-Logan reconstruction filter and without beam hardening correction. Image analysis was performed using Inveon Research Workplace software 2.3 (Siemens).

CT Image Analysis

In order to quantify tissue density, which is indicative of infiltration and inflammation, Hounsfield units (HU) of the images were calculated, with -1000 HU corresponding to air and 0 HU corresponding to water. Mean±SD HU values were then calculated. Density was measured within the left lung parenchyma,

Table 1: Therapy and results

Number	Combination	Therapy	Histological results			HU value
			A	B	C	
1	B6 to B6		0	0	0	-260.396
2	B6 to B6		0	0	0	-369.022
3	B6 to B6		0	0	0	-454.105
4	B6 to B6		0	0	0	-434.532
5	B6 to B6		0	0	0	-418.042
6	B10 to B6		3	2	0	-192.662
7	B10 to B6		3	2	0	-185.599
8	B10 to B6		3	1	0	-166.122
9	B10 to B6	CsA* 5 mg	3	2	0	-181.137
10	B10 to B6	CsA 5 mg	3	2	0	-187.285
11	B10 to B6	CsA 5 mg	3	2	0	-256.897
12	B10 to B6	CsA 15 mg	2	1	1	-53.7181
13	B10 to B6	CsA 15 mg	2	1	1	-22.4056
14	B10 to B6	CsA 15 mg	2	1	0	-409.235
15	B10 to B6	CsA 15 mg	3	1	0	-230.993
16	B10 to B6	CsA 15 mg	3	1	1	-124.449
17	B10 to B6	CsA 15 mg	3	1	1	-133.011
18	B10 to B6	CsA 15 mg	3	1	0	-128.707
19	B10 to B6	CsA 25 mg	3	1	0	-135.856
20	B10 to B6	CsA 25 mg	2	0	0	-317.305
21	B10 to B6	CsA 25 mg	2	2	0	-399.299

*CsA: Cyclosporine

which was defined visually as the left lung area after exclusion large airways and vessels.

Histopathological Evaluation
Next, the mice were sacrificed at 8th week post-transplantation for histopathological evaluation and classification of the lung changes in the five studied groups. Under full narcosis, the blood and spleen were removed; blood was flushed out of the vessels and organs with saline and 3.7% neutral-buffered formalin. Subsequently, the lungs and heart were removed en bloc, and the tissues were fixed with 3.7% neutral-buffered formalin overnight. The lungs were then cut transversely and embedded in paraffin, and were subsequently sectioned and stained with hematoxylin-eosin (HE), Elastica van Gieson (EVG) stain, or Periodic acid-Schiff (PAS) stain.

According to the consensus classification of clinical lung transplant rejection of the ISHLT,[14] the lung findings were diagnosed and classified pathologically (A: acute cellular rejection characterized by perivascular lymphocyte infiltration, B: small airway inflammation/lymphocytic bronchitis, and C: chronic airway rejection) in a blinded fashion by an experienced lung pathologist (DJ).

Statistical Analysis
GraphPad Prism 5 software (GraphPad Software, Inc, La Jolla, CA) was used for data analyses. Results were expressed as mean±SD. Student's t test was used to determine the difference between two groups. χ^2 test was used for comparison of frequencies. A p value <0.05 was considered statistically significant.
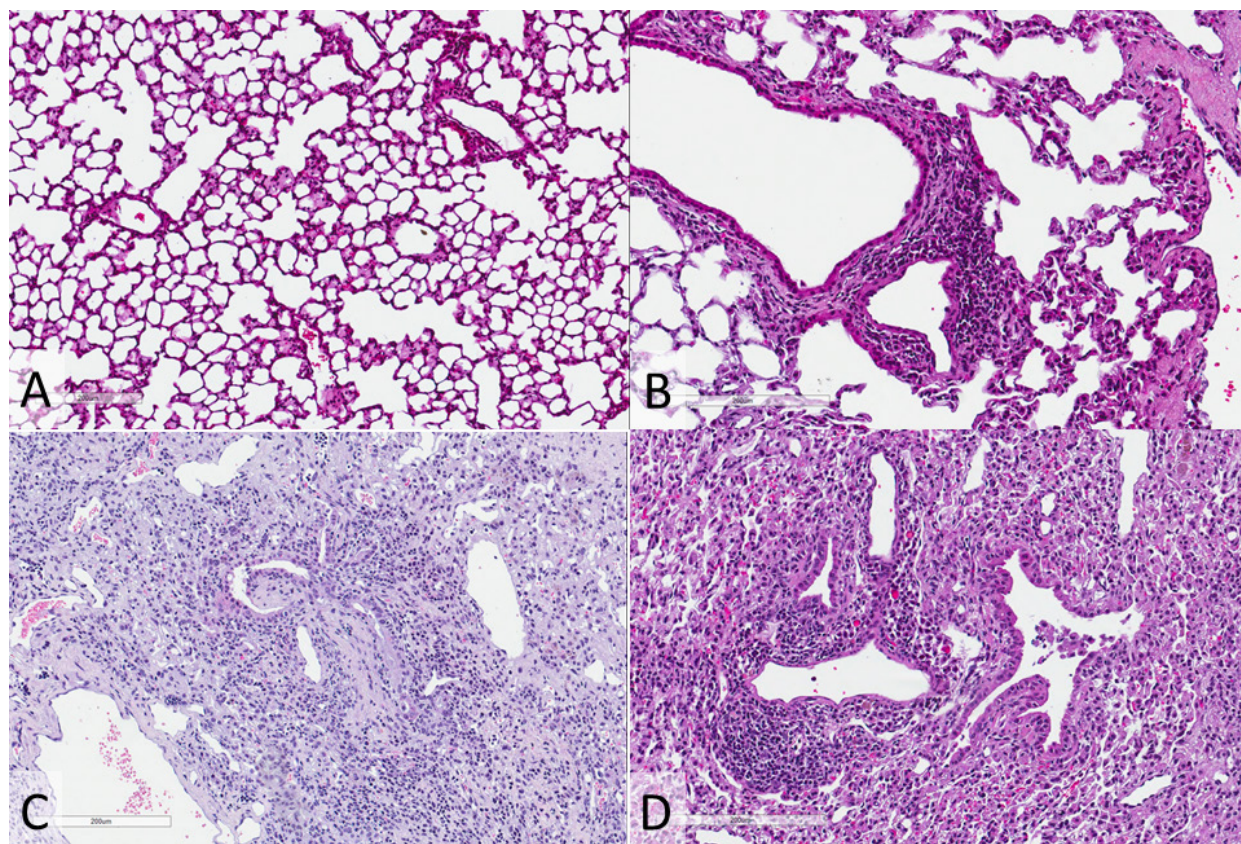
Figure 2: Histological findings of the lungs after lung transplantation (H&E stain). Transplanted lungs in the control group showed no rejection or small airway inflammation (A). In the positive controls, the transplanted lungs had a dense inflammatory cell population around peripheral arteries and the cells infiltrated into the lung parenchyma (grade A3). In addition, bronchioles in the transplanted lung also had a thick inflammatory cell layer around them (grade B2R, B). In the 15 mg/kg/day cyclosporine group, some bronchioles showed scarring of the submucosa and evidence of obliterative bronchiolitis (grade C1, C). The lungs of the 25 mg/kg/day cyclosporine group also showed evidence of mild cellular rejection (grade A2) and low-grade inflammation around the small airways (grade B1R), although no obliterative bronchiolitis was seen (grade C0, D).

RESULTS

According to the POD3-CT, mice with atelectasis and/or diaphragmatic paralysis (one negative control mouse, one positive control, one that was given 5 mg cyclosporine, three mice that were given 15 mg cyclosporine, and one mouse that was given 25 mg cyclosporine) were excluded. Finally, 21 mice were included in the analysis (21/28 mice, 75%, Table 1).

Histological Results

As seen on histological analysis at 8th week post-OLTx, the negative control group (B6 to B6) did not show acute rejection (A0), small airway inflammation (B0) or chronic rejection (C0; n=5, Fig 2A). Positive controls (B10 to B6 without immunosuppression) revealed moderate acute rejection (A3, 100%), low- to high-grade small airway inflammation (B1R or B2R, n=1 and 2, respectively), and no evidence of chronic rejection (C0) (n=3, Fig 2B). In B10 to B6 transplants given 5 mg/kg cyclosporine, A3 with B2R and C0 was seen (n=3). In the B10 to B6 transplants with 15 mg/kg cyclosporine group (n=7), A3 was seen in four mice (57%) and mild acute rejection (A2) was seen in three mice, corresponding to B1R. In this group, four mice also had obliterative bronchiolitis (C1, 57% Fig 2C). B10 to B6 mice treated with 25 mg/kg cyclosporine (n=3) showed A3 in one (33%) and A2 in two mice, and B1R and no chronic rejection (C0, Fig 2D).

Macroscopic Appearance of the Transplanted Lungs
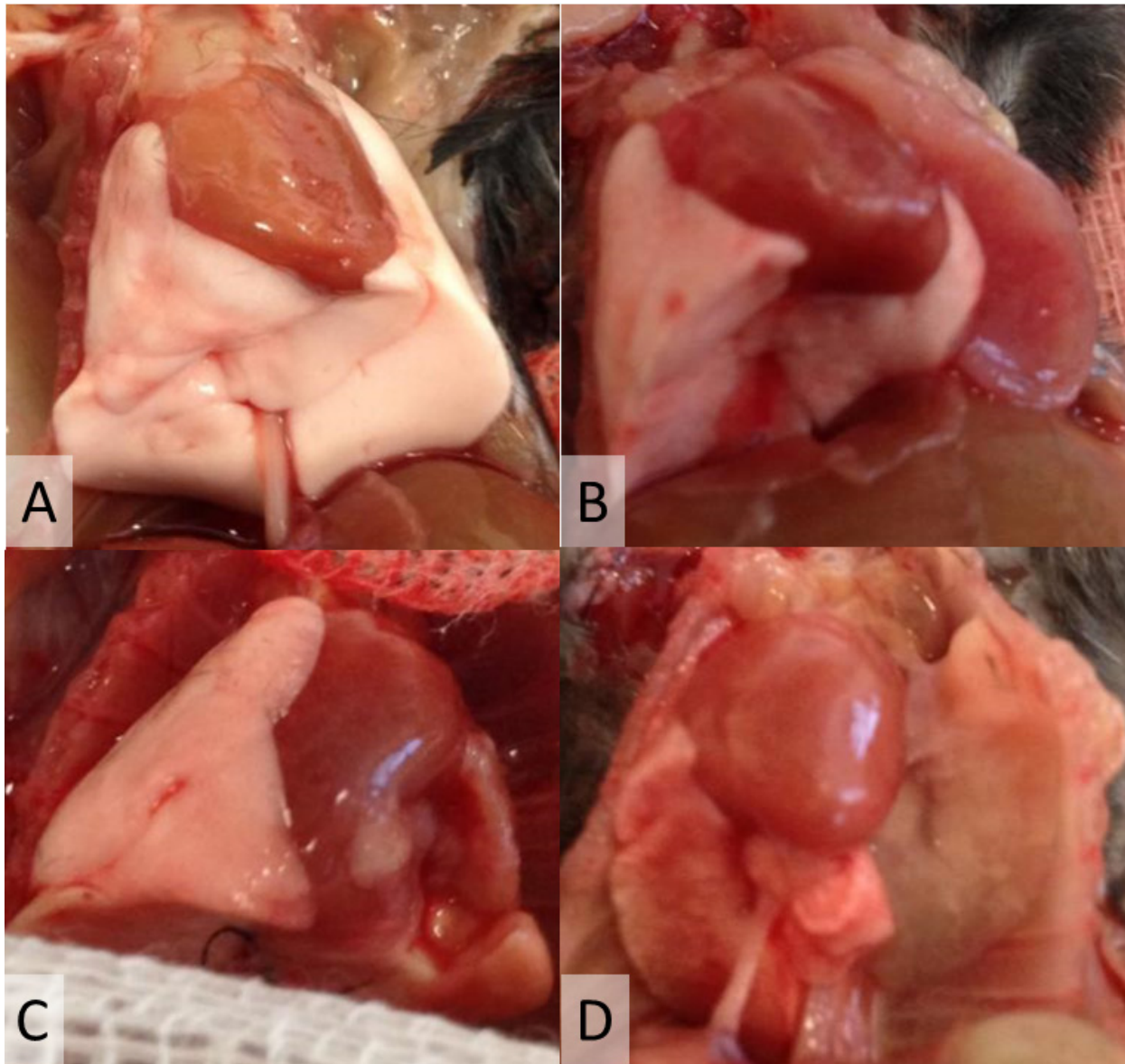
The negative control group had normal ap-

Figure 3: Macroscopic appearances of the transplanted lungs. The transplanted left lung of the control group had a similar appearance to the native right lung (A). The lungs of the allograft group showed swelling and reddening (B), and could not be inflated with positive pressure ventilation (compared to the inflated right lung (C). On the other hand, the lung with obliterative bronchiolitis did not shrink even in the expiration period compared to the shrunken right lung (D).

pearing lungs at 8th week post-OLTx (Fig 3A). Comparing the macroscopic findings with histological results, the acute rejection mice without chronic rejection had swollen and reddened lungs with histological A2 rejection (Fig 3B), or shrunken and edematous lungs with A3 rejection (Fig 3C), while the lungs with chronic rejection were pale and had low compliance (Fig 3D).

Comparison of BO Frequencies among Groups

BO was seen only in the 15 mg/kg cyclosporine group. The frequency of its appearance was also significantly (p=0.045) different from that observed in other groups.

HU Values of CT Images and Histological Results

Upon examination of the 8-week CT scans, those with A0 findings on histology had no infiltration (Fig 4A) and -387.2±69.4 HU values
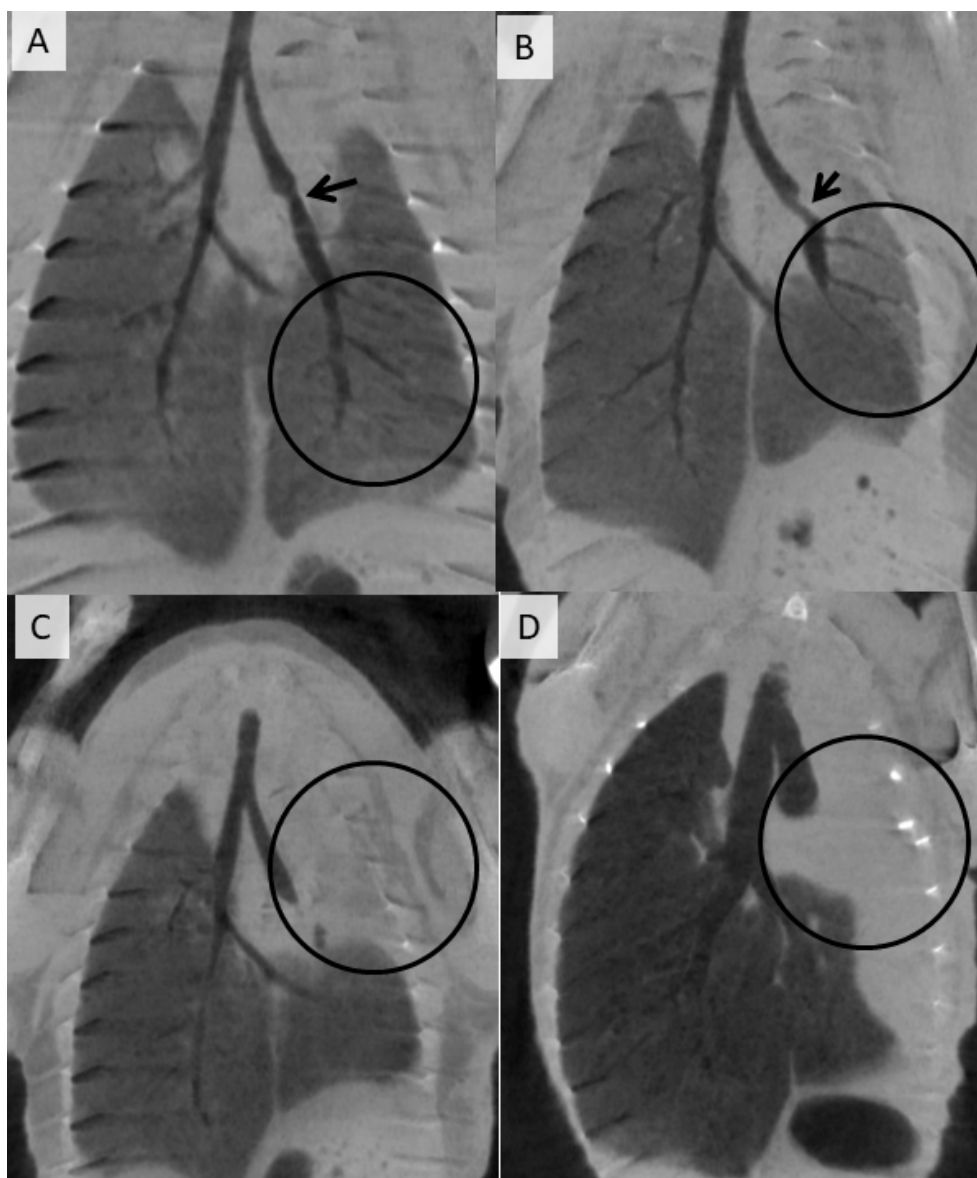
Figure 4: Macroscopic CT appearances of the transplanted lungs (A-D) and HU values in the entire cohort (E-G). The negative control group (B6 to B6) showed no infiltration in the lung (arrow: anastomosis (A). Acute rejection (A2-3) was associated with infiltration in the transplanted lung (B). In cases of chronic rejection, the transplanted lung still contained air, visible as ground-glass opacity, but the density was higher than in the acute rejection lung (C), or lungs showing atelectasis (D). There were significant differences between the no acute rejection group (grade A0) and the acute rejection group (grade A2+Grade A3, -387.2±77.6 *vs*. -196.6±116.1 HU, respectively: p=0.0013; E), and between the negative small airway inflammation group (grade B0) and positive small airway inflammation group (grade B1R+grade B2R, -375.6±75.0 *vs*. -187.3±115.3 HU, respectively: p=0.0010; F). All the lungs with obliterative bronchiolitis (grade C1) that had atelectasis were significantly different from the no obliterative bronchiolitis group (grade C0, -83.4±54.0 *vs*. -290.3±116.3 HU, respectively: p=0.0017; G).

in the left lung. Those with A2 or A3 histological findings had both GGO in the entire lung (Fig 4B) and -240.4±168.5 and 174.8±41.0 HU values, respectively. There were no significant differences between groups A0 and A2 (p=0.17), or A2 and A3 (p=0.48). However, upon grouping animals without (A0) or with acute rejection (A2+A3), significant differences in HU values were detected: -387.2±69.4 *vs*. -195.3±104.7 HU (p=0.002, Fig 4E).
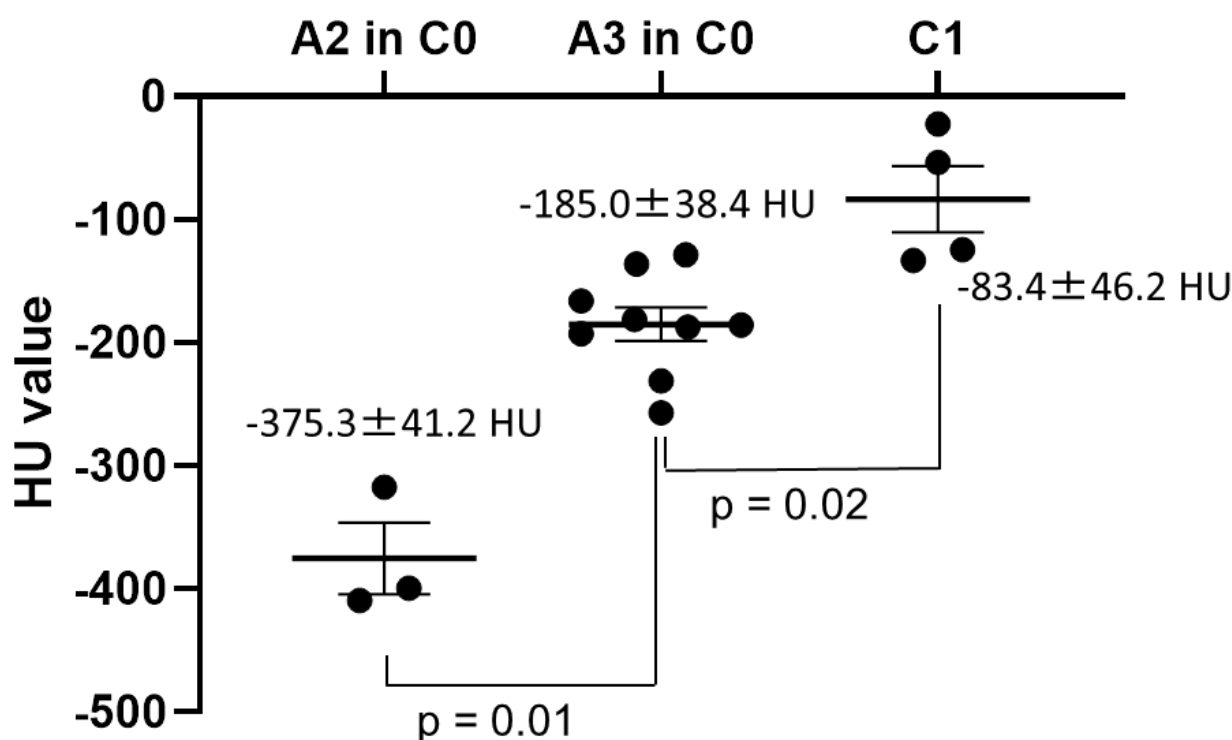
Figure 5: Comparison of Hounsfield unit (HU) values between C0 sub-classifications and C1 groups. Interestingly, when the C0 group was differentiated according to the acute rejection classification, significant differences in HUs were detected between A2 and A3 (-375.3±50.5 *vs*. -184.6±47.1, p<0.001). In addition, there was still a significant difference between A3 and C1 (-184.6±47.1 *vs*. -83.4±54.0, p=0.005).

Upon segregation into three groups according to the amount of inflammation, i.e., the B classification, there was a significant difference between groups B0 and B1R (-375.6±68.5 *vs*. -156.1±105.9 HU, p<0.001), but no significant difference between groups B1R and B2R (-156.1±105.9 *vs*. -233.8±78.4 HU, p=0.15). Significant differences were also detected between B0 and B1R+2R (-375.6±68.5 *vs*. -187.2±103.1 HU, p<0.001) (Fig 4F). Differentiation according to the presence of chronic rejection, i.e., according to the C classification, showed that the left lung showed dense GGO or atelectasis in C1 lungs (Figs 4C and D), with significant differences between C0 and C1 lungs (-278.1±110.7 *vs*. -83.4±46.8 HU, p<0.001, Fig 4G).

In addition, significant differences in HU were also detected when the C0 group was differentiated according to the presence of acute rejection (A classification) and on grouping of animals without (A0) or with acute rejection (A2+A3): -387.2±69.4 *vs*. -232.6±91.2 HU (p=0.006). In this cohort, there was also a sig-

nificant difference between A2 and A3 groups (-375.3±41.2 *vs*. -185.0±38.4, p=0.01; Fig 5). In addition, there was a significant difference between A3 and C1 (-185.0±38.4 *vs*. -83.4±46.8, p=0.02; Fig 5). Significant differences in densities were also detected when the C0 group was differentiated according to the B classification (presence of inflammation) and the no inflammation group (B0) was compared with the inflammation group (B1R+2R): B0 *vs*. B1R+2R (-375.6±75.0 *vs*. -233.4±105.1 HU, p<0.01). In addition, the HU value of the B1R+2R group without chronic rejection was significantly lower compared with the C1 group: B1R+2R with C0 *vs*. C1 (-224.9±91.5 *vs*. -83.4±46.8, p=0.005). However, there was still no significant difference between B1R and B2R without chronic rejection (-214.2±104.0 *vs*. -233.8±78.4 HU, p=0.76).

DISCUSSIONS

In this study, we aimed at development of an improved chronic rejection model of orthotopic

mouse lung transplantation, especially BO model, and evaluated the CT findings of the different degrees of rejection.

To induce BO, we first transplanted the left lungs orthotopically from B10 mice to B6 mice without immune suppression, according to the method described in a previous study.[6] However, we were only able to obtain lungs with grade A3 acute rejection without chronic rejection. The difference between our results and those of the previous study might be due to differences in the phenotypes of the mice at our institute, which could have been a phenocopy, i.e., characteristics caused by environmental conditions rather than genetically. A previous study used the combination of a crossed mouse donor to DBA/2J recipient to induce BO. Unfortunately, the combination, especially the donor, which is a crossed mouse between B6 and DBA, could become a large limitation in further studies because of its uniqueness.[15] Using B6 mice as the recipient, the percentage of BO development in previous studies was within a broad range of 0% and 44% [7, 8, 16, 17]. Recently, Yamada, et al, re-evaluated the combination and they also achieved a result of only moderate A3 acute rejection without BO, but with peribronchial fibrosis [8], which was also different from the results of the previous study [6]. They also concluded that the difference in the results could be due to strain variability and environmental factors.

Based on our results using this mouse strain combination, we gave several doses of immunosuppressive therapy with cyclosporine to the mice after transplantation, similar to the protocol after human lung transplantation, to induce several degrees of acute rejection with and without BO.

We previously reported in a clinical study that early regulatory T-cell frequencies are associated with less chronic rejection after lung transplantation [18], which suggests that the immune condition early after lung transplantation has a strong influence on the occurrence of BO. According to the study, we decided on administration of cyclosporine only in the first week after lung transplantation, instead of immunosuppressive treatment for the entire 8 weeks [19].

A previous study reported that the serum level of cyclosporine in mice after administration of 5 mg/kg/day of the drug ranged from approximately 50 to 380 ng/mL [20]. Generally, target trough levels of cyclosporine following human lung transplantation range from 100–450 ng/mL [21]. Therefore, we first administered 5 mg/kg/day cyclosporine to the mice. However, the rejection grade in the lungs of mice given 5 mg/kg/day cyclosporine was still A3 in our study. Hence, we next gave a dose of 15 mg/kg/day cyclosporine, which leads to a serum level of approximately 250–900 ng/mL [20]. During the 8-week rearing period of the 15 mg/kg/day cyclosporine-administered mice, we created the 25 mg group, by administering 25 mg/kg/day cyclosporine for 1 week. However, since we achieved several degrees of acute rejection with and without chronic rejection in the 15 mg cyclosporine group, we increased the sample number in this group.

Interestingly, there was no chronic rejection in the positive control, 5 mg or 25 mg cyclosporine groups. The aforementioned previous study reported that a minor mismatched strain model did not show any airway obliteration.[8] Another study reported that allografts with warm ischemia in a minor mismatch combination presented a higher ratio of occluded airways compared with allografts with only cold ischemia [22]. In clinical studies, a certain degree of bronchiolar damage, due to a certain degree of acute rejection, chronic aspiration of gastric juice, etc., exists along with BO formation [2, 3, 23]. In addition, when the damage is very severe, the lung tissue is likely to be destroyed before accumulation of fibroblasts. This indicates that for development of BO, the bronchial tissue needs to undergo only a certain degree of damage, with BO developing as a result of an abnormal repair process secondary to the damage. According to this phenomenon, we achieved BO not in the 5 mg or 25 mg group, but only the 15 mg cyclosporine mice in our study, indicating that our minor mismatch mouse model with immune suppression could be a pure immune-

induced chronic rejection model without the effects of other factors, and might also reflect a transition from acute rejection to chronic rejection.

In this study, we calculated the density of lung parenchyma in terms of HUs as a non-invasive method for monitoring rejection. The difference in HU values was affected largely by GGO lesions. GGO lesions are lesions of increased density, caused by partial filling of air spaces, partial collapse of alveoli, interstitial thickening, or increased capillary blood volume, as well as in cases of lung transplantation [9]. As part of the inflammatory changes due to rejection, the parenchyma developed histological changes of perivascular and peribronchial cellular infiltration and edema, which were reflected in the CT densities observed in our study; these were also revealed by macroscopic evaluation of acute rejection.

In addition, the densities of lungs with C1 were significantly higher than those with no chronic rejection (C0). In clinical evaluation, chronic rejection typically results in bronchial dilatation, bronchial wall thickening, and GGO mosaic attenuation [20]. However, in our results, the lungs with BO showed dense GGO in the entire lung or atelectasis, both of which increase HU values. In human transplantation, changes in bronchia do not usually affect the peripheral lung findings, because the diameter of the bronchium is large enough to avoid complete obliteration and the symptoms appear only through obliteration of the bronchioles. However, in mouse lung transplantation, changes in bronchia can affect the entire lung, because the diameter of the mouse trachea is as small as that of human bronchioles [24], indicating that the diameter of the mouse bronchus is much smaller than that of human bronchioles and that they can easily be obliterated. In our results, the obliterated peripheral bronchi were sufficient to affect the CT findings in the lungs with C1.

In this study, we performed a sub-analysis of HUs in the C0 group, because the effect of BO on the results of HU in the A and B classifications might be large. Interestingly, in this cohort, there was a significant difference between the A2 and A3 groups, but no significant difference between the B1R and B2R groups.

Acute rejection grades are classified according to the degree of inflammation around the arteries. In particular, A2 and A3 are differentiated based on whether there is expansion of infiltration into adjacent alveolar septa [14]. Differences in the findings of alveolar septa could also affect the difference in HU values. However, in B class changes, differentiation of B1R and B2R is not determined by alveolar infiltration, but is based on the condition of the bronchi and the surrounding tissue. This localized change could be the reason for the observed difference in HUs between B0 and B1+B2R, but no significant difference between B1R and B2R, as well as in the C0 group.

A limitation of this study was that we did not detect acute rejection of A1 severity, and did not investigate its immune mechanism, because the purpose of this study was not to detect the mechanism or immunological results, but to introduce a non-invasive rejection monitoring tool. Future studies should include A1 rejection as well. In addition, the dosage of immunosuppressives would need to be adjusted at each institution, because of the different phenotypes of the mice used at each institution.

In conclusion, our study indicated that BO can be modeled in a murine major histocompatibility mismatch OLTx model with subtherapeutic cyclosporine administration, which more closely approximates post-OLTx rejection in humans and is less cumbersome than previous models. CT scans are a valid tool for monitoring of rejection in this model, and can be used in future studies to assess the therapeutic effects in individual animals. Thus, we described an improved model of chronic rejection with BO in a more clinically relevant setting.

REFERENCES

1. Yusen RD, Edwards LB, Dipchand AI, *et al*. The Registry of the International Society for Heart and Lung Transplantation: Thirty-third Adult Lung and Heart-Lung Transplant Report-2016; Focus Theme: Primary Diagnostic Indications for Transplant. *J Heart Lung Transplant* 2016;**35**:1170-84.

2. Estenne M, Maurer JR, Boehler A, *et al*. Bronchiolitis obliterans syndrome 2001: an update of the diagnostic criteria. *J Heart Lung Transplant* 2002;**21**:297-310.

3. Aguilar PR, Michelson AP, Isakow W. Obliterative Bronchiolitis. *Transplantation* 2016;**100**:272-83.

4. Okazaki M, Krupnick AS, Kornfeld CG, *et al*. A mouse model of orthotopic vascularized aerated lung transplantation. *Am J Transplant* 2007;**7**:1672-9.

5. Jungraithmayr WM, Korom S, Hillinger S, Weder W. A mouse model of orthotopic, single-lung transplantation. *J Thorac Cardiovasc Surg* 2009;**137**:486-91.

6. Jungraithmayr W, Jang JH, Schrepfer S, *et al*. Small animal models of experimental obliterative bronchiolitis. *Am J Respir Cell Mol Biol* 2013;**48**:675-84.

7. Suzuki H, Fan L, Wilkes DS. Development of obliterative bronchiolitis in a murine model of orthotopic lung transplantation. *J Vis Exp* 2012;**10**:3947.

8. Yamada Y, Windirsch K, Dubs L, Kenkel D, *et al*. Chronic Airway Fibrosis in Orthotopic Mouse Lung Transplantation Models - An Experimental Reappraisal. *Transplantation* 2018;**102**:e49-e58. doi: 10.1097/TP.0000000000001917.

9. Park CH, Paik HC, Haam SJ, *et al*. HRCT features of acute rejection in patients with bilateral lung transplantation: the usefulness of lesion distribution. *Transplant Proc* 2014;**46**:1511-6.

10. Ng YL, Paul N, Patsios D, *et al*. Imaging of lung transplantation: review. *AJR Am J Roentgenol* 2009;**192**(3 Suppl):S1-13, quiz S14-9.

11. Oishi H, Martinu T, Sato M, *et al*. Halofuginone treatment reduces interleukin-17A and ameliorates features of chronic lung allograft dysfunction in a mouse orthotopic lung transplant model. *J Heart Lung Transplant* 2016;**35**:518-27.

12. Watanabe T, Martinu T, Chruscinski A, *et al*. A B cell-dependent pathway drives chronic lung allograft rejection after ischemia-reperfusion injury in mice. *Am J Transplant* 2019;**19**:3377-89.

13. Yoshida M, Oishi H, Martinu T, *et al*. Pentraxin 3 deficiency enhances features of chronic rejection in a mouse orthotopic lung transplantation model. *Oncotarget* 2018;**9**:8489-501.

14. Stewart S, Fishbein MC, Snell GI, *et al*. Revision of the 1996 working formulation for the standardization of nomenclature in the diagnosis of lung rejection. *J Heart Lung Transplant* 2007;**26**:1229-42.

15. Mimura T, Walker N, Aoki Y, *et al*. Local origin of mesenchymal cells in a murine orthotopic lung transplantation model of bronchiolitis obliterans. *Am J Pathol* 2015;**185**:1564-74.

16. Wu Q, Gupta PK, Suzuki H, *et al*. CD4 T Cells but Not Th17 Cells Are Required for Mouse Lung Transplant Obliterative Bronchiolitis. *Am J Transplant* 2015;**15**:1793-804.

17. Hirayama S, Sato M, Loisel-Meyer S, *et al*. Lentivirus IL-10 gene therapy down-regulates IL-17 and attenuates mouse orthotopic lung allograft rejection. *Am J Transplant* 2013;**13**:1586-93.

18. Salman J, Ius F, Knoefel AK, *et al*. Association of Higher CD4+ CD25high CD127low , FoxP3+ , and IL-2+ T Cell Frequencies Early After Lung Transplantation With Less Chronic Lung Allograft Dysfunction at Two Years. *Am J Transplant* 2017;**17**:1637-48. doi: 10.1111/ajt.14148. Epub 2017 Jan 24.

19. De Vleeschauwer S, Jungraithmayr W, Wauters S, *et al*. Chronic rejection pathology after orthotopic lung transplantation in mice: the development of a murine BOS model and its drawbacks. *PLoS One* 2012;**7**:e29802. doi: 10.1371/journal.pone.0029802. Epub 2012 Jan 6.

20. King MB, Jessurun J, Savik SK, *et al*. Cyclosporine reduces development of obliterative bronchiolitis in a murine heterotopic airway model. *Transplantation* 1997;**63**:528-32.

21. Scheffert JL, Raza K. Immunosuppression in lung transplantation. *J Thorac Dis* 2014;**6**:1039-53.

22. Chang JC, Leung JH, Tang T, *et al*. In the face of chronic aspiration, prolonged ischemic time exacerbates obliterative bronchiolitis in rat pulmonary allografts. *Am J Transplant* 2012;**12**:2930-7.

23. Hathorn KE, Chan WW, Lo WK. Role of gastroesophageal reflux disease in lung transplantation. *World J Transplant* 2017;**7**:103-116.

24. Hertz MI, Jessurun J, King MB, *et al*. Reproduction of the obliterative bronchiolitis lesion after heterotopic transplantation of mouse airways. *Am J Pathol* 1993;**142**:1945-51.