Maintenance of Airway Epithelium in Acutely Rejected Orthotopic Vascularized Mouse Lung Transplants

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Lung transplantation remains the only therapeutic option for many patients suffering from end-stage pulmonary disease. Long-term success after lung transplantation is severely limited by the development of bronchiolitis obliterans. The murine heterotopic tracheal transplantation model has been widely used for studies investigating pathogenesis of obliterative airway disease and immunosuppressive strategies to prevent its development. Despite its utility, this model employs proximal airway that lacks airflow and is not vascularized. We have developed a novel model of orthotopic vascularized lung transplantation in the mouse, which leads to severe vascular rejection in allogeneic strain combinations. Here we characterize differences in the fate of airway epithelial cells in nonimmunosuppressed heterotopic tracheal and vascularized lung allograft models over 28 days. Up-regulation of growth factors that are thought to be critical for the development of airway fibrosis and interstitial collagen deposition were similar in both models. However, while loss of airway epithelial cells occurred in the tracheal model, airway epithelium remained intact and fully differentiated in lung allografts, despite profound vascular rejection. Moreover, we demonstrate expression of the anti-apoptotic protein Bcl-2 in airway epithelial cells of acutely rejected lung allografts. These findings suggest that in addition to alloimmune responses, other stimuli may be required for the destruction of airway epithelial cells. Thus, the model of vascularized mouse lung transplantation may provide a new and more physiologic experimental tool to study the interaction between immune and nonimmune mechanisms affecting airway pathology in lung allografts.

Keywords: lung transplantation; mouse; airway epithelium; Bcl-2

Lung transplantation is the treatment of choice for many patients suffering from end-stage pulmonary disease. Despite improvements in 1-year survival rates, the long-term outcome after lung transplantation remains far worse than that after the transplantation of other solid organs. Survival rates of lung transplant recipients are only 49% at 5 years and 25% at 10 years (1). The main cause for late death after lung transplantation is bronchio-

CLINICAL RELEVANCE

Our study describes the fate of airway epithelial cells in a novel model of vascularized aerated orthotopic mouse lung transplants. This provides a new experimental tool to study airway pathology in lung transplantation.

litis obliterans syndrome, which is thought to be a manifestation of chronic allograft rejection (2, 3). The pathologic changes of bronchiolitis obliterans syndrome are referred to as bronchiolitis obliterans. The histologic hallmark of the syndrome is bronchiolitis obliterans characterized by loss of airway epithelial cells and partial or complete occlusion of the lumina of terminal and respiratory bronchioles by inflammatory and fibrous tissue.

While the mechanisms leading to the development of bronchiolitis obliterans are poorly understood, airway epithelial cells are thought to represent an important cellular target during lung allograft rejection. It has been hypothesized that injury and apoptosis of airway epithelial cells with subsequent aberrant tissue repair leads to excessive fibroproliferation (4). Risk factors for the development of bronchiolitis obliterans after lung transplantation have been categorized into alloimmune-dependent and alloimmune-independent factors. There is evidence for a relationship between the frequency and severity of acute rejection episodes and the subsequent development of chronic rejection (5). Alloimmune-independent risk factors include ischemia-reperfusion injury, bacterial and viral respiratory infections, and gastroesophageal reflux (6–8). These alloimmune-independent risk factors are inflammatory stimuli that may damage the airway epithelium directly or alter the milieu in the lung allograft to increase antigenicity and thereby indirectly influence the alloimmune response. Once established, therapeutic options are limited, as bronchiolitis obliterans is generally not reversible. To date, augmentation of immunosuppression has failed to prevent the progression of fibrotic airway obliteration in the majority of these patients, and retransplantation remains the only option (9–11).

The vast majority of studies investigating the pathogenesis of bronchiolitis obliterans have used the mouse model of heterotopic tracheal transplantation. This model employs the transplantation of trachea into a subcutaneous pouch of the recipient mouse (12). While airway epithelial cells remain normally differentiated in syngeneic transplants and the airway lumen remains patent, allogeneic tracheal grafts develop progressive infiltration with mononuclear cells, epithelial damage, fibroproliferation, and ultimately luminal obliteration by 4 weeks after

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the transplant. This model has several advantages, such as relative technical simplicity, reproducibility and, more importantly, the ability to use multiple knockout and transgenic mouse strains for analysis of injury. It is therefore not surprising that in the absence of more physiologic models of lung transplantation this model has been used to gain mechanistic insights into the pathogenesis of fibrous obliteration of the airway (13, 14). However, the heterotopic tracheal transplant model has several shortcomings, which may limit its relevance with regard to the ability to draw conclusions relative to the pathogenesis of bronchiolitis obliterans in lung allografts. For example, changes in the trachea may not reflect the bronchiolitis obliterans observed in small airways after lung transplantation. Furthermore, unlike the airways in lung allografts, heterotopic tracheas are not exposed to the environment and, perhaps more importantly, heterotopic tracheal grafts are not vascularized.

Our laboratory has recently developed a model of aerated vascularized single-lung transplantation in the mouse (15). We have used this as a model of acute rejection, as it successfully reproduces the histopathologic findings of acute human lung allograft rejection. The goal of the present study was to characterize temporal histopathologic changes after allogeneic orthotopic lung transplantation over an extended period with a focus on the fate of airway epithelial cells. We demonstrate that contrary to the progressive de-differentiation and loss of airway epithelial cells in the heterotopic tracheal transplant model, the airway epithelial cells remain intact in allogeneic orthotopic allografts despite acute vascular rejection and progressive necrosis.

MATERIALS AND METHODS

Orthotopic Lung Transplantation and Heterotopic Tracheal Transplantation

Balb/cJ (H-2Kd) and C57BL/6J (H-2Kb) (referred to as Balb and B6, respectively) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Orthotopic lung transplants and heterotopic tracheal transplants were performed in the syngeneic B6 → B6 and allogeneic Balb/c → B6 strain combinations. Donor and recipient mice were anesthetized with ketamine (0.1 mg/g; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (0.01 mg/g; VEDCO Inc., St. Joseph, MO) administered by intraperitoneal injection. Left orthotopic vascularized lung transplants were performed using cuff techniques as previously described (15). Tracheas were transplanted into a subcutaneous pocket behind the neck of the recipient. Animals did not receive any immunosuppression. The animals were housed in a barrier facility, within air-filtered cages, after surgery and before evaluation. Some lung transplant recipients were immunosuppressed with MR1 (anti-CD40Ligand, 250 µg, intraperitoneally) on the day of transplant and CTLA4 Ig (200 µg, intraperitoneally) two days after the transplant.

Magnetic Resonance Imaging

Respiratory-gated, spin-echo magnetic resonance (MR) images of anesthetized mice were collected as previously described with a small-animal MR scanner based on an Oxford Instruments (Oxford, UK) 4.7 Tesla, 40-cm bore magnet (16). The magnet is equipped with Magnex Scientific (Oxford) actively shielded, high-performance (10 cm ID, ~50 G/cm, ~200 µs rise-time) gradient coils and Techon gradient power supplies and is interfaced with a Varian NMR Systems (Palo Alto, CA) INOVA console. All data were collected using a Stark Contrast (Erlangen, Germany) 2.5-cm birdcage-style rf coil. Synchronization of MR data collection with animal respiration was achieved with a respiratory-gating unit and images were collected during post-expiratory periods. Imaging parameters were TR ~ 3 s, TE = 20 ms, FOV 2.5 cm × 2.5 cm², slice thickness = 0.5 mm.

Histology and Immunohistochemistry

Animals were analyzed at 7, 14, 21, or 28 days after transplantation. Lungs were inflation-fixed with 10% buffered formalin using 25 cm of

pressure. Transplanted tracheas were dissected from subcutaneous tissues and fixed in 10% buffered formalin. Tissue sections were analyzed with hematoxylin plus eosin, trichrome, and Alcian blue stains. Immunostaining was performed using antigen retrieval methods as previously described (17). Primary antibodies and dilutions used were as follows: mouse anti-β-tubulin IV, 1:250 (BioGenex, San Ramon, CA); goat anti-human Clara cell secretory protein (CCSP), 1:10,000 (kindly provided by Barry Stripp and Susan Reynolds, University of Pittsburgh); mouse anti-Muc5AC, 1:300 (clone 45M1; NeoMarkers, Fremont, CA); rabbit anti-human Bcl-2, 1:300; and rat anti-mouse Mac3, 1:250 (clone M3/84) (both antibodies from BD Biosciences, San Jose, CA). For immunofluorescence, secondary antibodies used were conjugated with indocarbocyanine (CY3) (Jackson ImmunoResearch Laboratories, West Grove, PA) Alexa Fluor 488 (Molecular Probes, Carlsbad, CA) and mounted with media containing 4',6'-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). For peroxidase-based detection, sections were incubated with biotinylated secondary antibody (ABC Elite; Vector Laboratories), visualized with 3,3'-diaminobenzidine (DAB) substrate solution and counterstained with hematoxylin. No detectable staining was observed for isotype-matched or species-specific control antibodies. A Leica DM5000 microscope (Wetzlar, Germany) was used for image capture with a Retiga 200R charge-coupled device camera (Q Imaging, Surrey, BC, Canada) interfaced with QCapture Pro software (Q Imaging). Images were composed using Photoshop and Illustrator software (Adobe Systems, San Jose, CA).

Total RNA Isolation and RT-PCR Amplification

Total RNA was isolated from frozen lung and trachea samples using a guanidium thiocyanate-phenol-chloroform extraction. Levels of target mRNA were assayed by semiquantitative RT-PCR and compared with glyceraldehyde phosphate dehydrogenase (GAPDH) expression. The following primer pairs were used to amplify GAPDH and each of four genes of interest: GAPDH (452 bp) 5' ACC ACA GTC CAT GCC ATC AC 3' and 5' TCC ACC ACC CTG TTG CTG TA 3'; VEGF (826 bp) 5' GGA CCC TGG CTT TAC TGC 3' and 5' CGG GCT TGG CGA TTT AG 3'; VEGFR-1 (499 bp) 5' GAG AGC ATC TAT AAG GCA GCG GAT T 3'; and 5' CAC GTT TAC AAT GAG AGT GGC AGT G 3'; VEGFR-2 (456 bp) 5' TAC ACA ATT CAG AGC GAT GTG TGG T 3' and 5' CTG GTT CCT CCA ATG GGA TAT CTT C 3'; and PDGF (225 bp) 5' TGT GCC CAT TCG CAG GAA GAG 3' and 5' TTG GCC ACC TTG ACA CTG CG 3'. All PCR reactions began with a 4-minute incubation at 94°C and were optimized for individual primer sets as follows: for GAPDH, 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 45 seconds (28 cycles); for VEGFR-1 and VEGFR-2, 94°C for 45 seconds, 58°C for 30 seconds, 72°C for 1 minute (36 cycles); for VEGF, 94°C for 45 seconds, 65°C for 30 seconds, 72°C for 1.5 minutes (35 cycles); for PDGF-A, 94°C for 1 minute, 63°C for 1.5 minutes, 72°C for 2.5 minutes (34 cycles). All reactions concluded with a 10-minute extension at 72°C. PCR products were resolved by electrophoresis using an ethidium bromide-stained 2% agarose gel. The 826-bp VEGF product was resolved on a 1.5% agarose gel.

RESULTS

Histopathologic Changes after Lung Transplantation

Syngeneic lung grafts (B6 → B6) appeared normal 28 days after transplantation (Figure 1A). MR imaging demonstrated that the transplanted lung was well-aerated and similar to the native right lung at 28 days after transplantation (Figure 1B). At this time, on histologic examination syngeneic grafts had no evidence of inflammatory changes (Figure 1C). We have previously shown that normal oxygenation is maintained after the native lung has been clamped in syngeneic lung transplant recipients, indicating functional vascular and airway anastomoses of the graft (15). We have further extended these observations and have shown in preliminary experiments using a microsphere-based assay that flows to transplanted syngeneic and naïve left lungs are similar (data not shown).

In contrast to syngeneic transplants, allogeneic lung grafts (Balb → B6) could not be ventilated and appeared necrotic at

Figure 1. Changes in gross appearance and histopathology of allogeneic (Balb → B6) and syngeneic (B6 → B6) vascularized lung grafts. (A) Gross appearance of left syngeneic lung grafts 28 days after transplantation. Depicted here is posterior view of excised lung block. (Tx = transplanted lung). (B) Magnetic resonance image of mouse 28 days after syngeneic transplantation. (C) Histology of syngeneic lung grafts 28 days after transplantation. (D) Gross appearance of left allogeneic lung graft at 28 days after transplantation. (E) Representative histologic changes observed in allogeneic lung grafts 7, 14, 21, and 28 days after transplantation. (F) High-power view of histology of small airways at indicated days after transplantation. (G) Histologic changes in allogeneic lung graft at 106 days after transplantation treated with MR1 and CTLA4 Ig at Days 0 and 2, respectively. (L, left; Tx, transplanted lung; aw, airway; v, vessel; pl, pleura). Bars = 100 μ m.

28 days (Figure 1D). Lung allografts had evidence of progressively worsening perivascular and interstitial infiltrates from 7 to 14 days after transplantation (Figure 1E). These findings on Days 7 and 14 were analogous to A2 and A3 acute rejection as defined for human lung transplants, respectively. Subepithelial mononuclear infiltrates in the small airways were minimal at 7 and 14 days. By 21 days after transplantation the vascular rejection had led to marked necrosis of the allograft. By 28 days, these necrotic changes became progressively more extensive and were accompanied by pleural thickening by 28 days after transplantation. While immunosuppression resulted in prolonged allograft survival and prevention of necrosis (Figure 1G), most small vessels were completely occluded in nonimmunosuppressed recipients at 28 days. Remarkably, however, the airway inflammation remained minimal to mild, with sparse mononuclear infiltrates into the airway epithelium of both small and large airways (Figures 1E and 1F). Acellular proteinaceous material was observed within many airways.

Growth Factor Up-Regulation after Lung and Tracheal Transplantation

Vascular endothelial growth factor-A (VEGF) is an important mediator of angiogenesis and vasculogenesis, and its expression is up-regulated under hypoxic conditions (18). The two main receptors through which VEGF mediates its effect on target cells are VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1). In addition, it has also been shown to act as a proinflammatory cytokine. To this end, several investigations have focused on the role of VEGF in alloimmune responses. Its expression was found to be associated with both acute and chronic allograft rejection (19, 20). In acute rejection, VEGF plays an important role in mediating leukocyte trafficking, in part through regulating the expression of endothelial adhesion molecules and chemokines (21). VEGF has also been found to be a growth factor for epithelial cells and has been therefore linked to the development of fibrotic lesions that characterize chronic allograft rejection in various organs (22, 23). In addition, platelet-derived growth factor-A (PDGF) is believed to be one of the most potent chemotactic and proliferative factors for cells of mesenchymal origin and has been implicated in the pathogenesis of

several fibroproliferative diseases (24). Furthermore, in experimental studies of lung transplantation using heterotopically transplanted tracheal allografts, VEGF and PDGF have been suggested to be critical for the development of obliterative airway disease. At 10 days after transplantation tracheal allografts were found to express high levels of VEGF in inflammatory cells of the airway wall, and VEGFR-1 and VEGFR-2 expression was observed in smooth muscle cells and endothelial cells of small blood vessels, respectively (25). Similarly, PDGF was found to be expressed in allogeneic murine tracheal grafts (26). Overexpression of VEGF after adenoviral transfer of a VEGF gene has been reported to increase the degree of airway occlusion (27). Interestingly, simultaneous blockade of VEGF and PDGF receptors by administration of protein tyrosine kinase inhibitors at the time of transplantation prevented luminal occlusion of tracheal allografts (25).

In the present study, we compared the expression levels of VEGF, VEGFR-1, VEGFR-2, and PDGF in syngeneic and allogeneic lung grafts to those in syngeneic and allogeneic tracheal grafts (Figure 2A). We have extended the findings of previous investigations and demonstrated progressive up-regulation of VEGF, VEGFR-1, VEGFR-2, and PDGF in tracheal allografts, while we could not detect the expression of these markers in syngeneic grafts. At the same time points, we observed similar patterns of upregulation of VEGF, VEGFR-1, VEGFR-2, and PDGF in rejected lung allografts.

Macrophages have the capacity to produce and secrete a variety of growth factors and, to this end, have been shown to be one of the main cellular sources of PDGF in lung transplant patients suffering from bronchiolitis obliterans (28). Administration of gadolinium chloride, a macrophage-depleting agent, prevents the development of obliterative airway disease in heterotopically transplanted tracheas, demonstrating the importance of this cell population in the pathogenesis of this process (29). To characterize the infiltration of macrophages into tracheal and lung allografts, we performed immunohistochemical analyses of tracheal and lung allografts harvested at 7 and 14 days after transplantation (Figure 2B). Macrophages were present within vessels of allogeneic lung grafts. Remarkably, however, while tracheal allografts were significantly infiltrated by macrophages,

Figure 2. Growth factor up-regulation, macrophage infiltration, and collagen deposition in heterotopic tracheal and vascularized lung grafts. (A) Representative RT-PCR analysis of vascular endothelial growth factor (VEGF), VEGFR-1, VEGFR-2, platelet-derived growth factor, and glyceraldehyde phosphate dehydrogenase in syngeneic (B6 → B6) and allogeneic (Balb → B6) heterotopic tracheal grafts and orthotopic lung grafts. Syngeneic tracheal and lung grafts were analyzed 28 days after transplantation; allogeneic tracheal and lung grafts were analyzed 14 and 28 days after transplantation. (B) Representative immunostaining for Mac3 in allogeneic tracheal and lung grafts at 7 and 14 days after transplantation (aw, airway; v, vessel). Bars = 10 μ m. (C) Representative trichrome staining of syngeneic and allogeneic lung grafts at 28 days after transplantation (aw, airway; v, vessel). Bars = 100 μ m.

the lumina of airways in the lung allografts were relatively free of macrophage infiltration.

Both syngeneic tracheal and lung grafts did not demonstrate significant fibrosis at 28 days after transplantation (Figure 2C). As previously described, the lumen of tracheal allografts was occluded at 28 days after transplantation with evidence of collagenous tissue both subepithelially and intraluminally (12) (data not shown). In contrast, we observed a severe interstitial fibrotic pattern in lung allografts at 28 days after transplantation with deposition of collagen surrounding vessels and airways. The pattern of collagen deposition in the lung allografts is nonspecific, but, strikingly, there was no evidence of collagenous tissue within the lumina of small or large airways. Thus, despite marked up-regulation of growth factors and growth factor receptors that have been suggested to be critical for the pathogenesis of obliterative airway disease in heterotopic tracheal allografts, there is no evidence of fibrotic remodeling resulting in occlusion of the airways in lung allografts.

Airway Epithelial Cells Remain Intact in Rejected Lung Allografts

Damage of airway epithelial cells has been shown to be a critical event for the development of obliterative airway disease (30). In fact, enzymatic denudation of epithelial cells can lead to fibrotic obliteration of syngeneic tracheas (31, 32). Luminal obliteration can be prevented by seeding epithelial cells in denuded syngeneic tracheal grafts. In syngeneic tracheal grafts, the airway epithelium displayed a normal architecture and there was no evidence of luminal narrowing at 28 days after transplantation (Figure 3A). In tracheal allografts, the airway epithelial cells underwent apoptosis with progressive flattening, a feature well characterized by others (12). At 28 days after transplantation, no airway epithelium could be detected and the airway lumen was completely obliterated. These changes were in contrast to findings in the mouse orthotopic lung transplant model.

In syngeneic lung grafts the airway epithelium of both small and large airways appeared intact (Figure 3B). Similarly, in lung allografts—despite severe vascular rejection with extensive necrosis—the airway epithelial cells were preserved. To further assess morphologic changes, we evaluated the expression of molecular markers of airway epithelial differentiation. For this purpose, syngeneic and allogeneic lung grafts that were harvested 28 days after transplantation were stained for the cilia-specific protein β -tubulin-IV, the nonciliated cell protein Clara cell secretory protein (CCSP) and the mucous cell protein Muc5AC, which has been suggested to be expressed under inflammatory conditions (33) (Figure 3C). β -tubulin-IV and CCSP were expressed in the airway epithelial cells of both syngeneic and allogeneic lung grafts at comparable levels. Of note, we did not detect expression of Muc5ac in either syngeneic or allogeneic lung grafts or the presence of mucous cells by Alcian blue stain.

Bcl-2 is an antiapoptotic protein that resides in the outer mitochondrial membrane. It has been shown to inhibit the release of cytochrome c from the mitochondria into the cytosol, thereby preventing the activation of the caspase cascade and apoptotic cell death. Previous animal studies have shown that inflammatory stimuli that are associated with the development of mucous cell hyperplasia lead to increased expression of Bcl-2 in metaplastic mucous cells (34, 35). To determine the expression of Bcl-2 in airway epithelial cells after lung transplantation we immunostained syngeneic and allogeneic lung grafts 28 days after transplantation (Figure 3D). While only few airway epithelial cells expressed Bcl-2 in syngeneic grafts, Bcl-2 was expressed in the majority of both ciliated and nonciliated airway epithelial cells in lung allografts. These results suggest that

Figure 3. Airway epithelial cell differentiation in heterotopic tracheal and vascularized orthotopic lung grafts. (A) Representative photomicrographs demonstrating preservation of airway epithelial cells in syngeneic (B6 → B6) heterotopic tracheal grafts at 28 days after transplantation. Flattening and destruction of airway epithelial cells in allogeneic (Balb → B6) heterotopic tracheal grafts shown at 21 and 28 days after transplantation, respectively. (B) Representative photomicrographs demonstrating preservation of airway epithelial cells in small airways of syngeneic and allogeneic lung transplants 28 days after transplantation. (C) Representative photomicrographs of airway sections obtained from syngeneic and allogeneic lung transplants 28 days after transplantation subjected to immunofluorescent staining for β -tubulin (β -tub, green) and CCSP (red). (D) Representative photomicrographs of airway sections obtained from syngeneic and allogeneic lung transplants 28 days after transplantation subjected to immunostaining for Bcl-2. Bars = 10 μ m.

up-regulation of cell survival genes may promote the survival of airway epithelial cells in lung allografts that are undergoing acute vascular rejection.

In conclusion, we demonstrate that in a novel model of orthotopic vascularized lung transplantation airway epithelial cells remain intact despite severe acute vascular rejection that leads to severe necrosis and interstitial fibrosis of the allograft. We did not observe significant changes in airway epithelial cell differentiation in these acutely rejected allografts. This is in marked contrast to the progressive loss of airway epithelial cell differentiation, apoptosis, and airway remodeling with fibrotic occlusion of the lumen observed in heterotopic tracheal allografts. As the heterotopic tracheal graft is not vascularized, ischemia is likely to play an important role in the development of these changes. Thus, the orthotopic lung transplant model may be a more physiologic tool to study mechanisms of lung allograft injury, since it mimics human lung transplantation with reestablishment of pulmonary arterial blood flow and aeration through anastomosis of the mainstem bronchi. Furthermore, various organs are known to differ with regard to the number of graft-resident donor-derived antigen-presenting cells. These cells migrate to the recipient's secondary lymphoid organs after transplantation, where they interact with recipient T cells, a process referred to as direct allorecognition (36, 37). While such donor-derived antigen-presenting cells are abundant in lungs, they are relatively sparse in tracheal grafts. These differences in graft-resident donor-derived antigen-presenting cells may contribute to variations between immunologic responses to lung and tracheal allografts.

Future studies will be required to define mechanisms that contribute to the damage of airway epithelium in vascularized lung transplants. It is widely believed that airway epithelial injury is important for the development of bronchiolitis obliterans (38). Therefore, our findings that airway epithelial cells up-regulate antiapoptotic genes in lung allografts that undergo severe acute rejection provide support to the notion that

additional noxious stimuli such as infectious agents or bile acids are necessary to initiate the airway epithelial damage that ultimately leads to fibrous remodeling (7, 8). The ability to successfully immunosuppress these animals provides an opportunity to study the effects of epithelial cell-specific injury at later time points. The vascularized mouse lung transplantation model thus provides a new experimental tool to study the interplay between alloimmune mechanisms and nonimmune factors in the pathogenesis of airway rejection. Compared with existing large animal models of lung transplantation, the mouse model offers the advantage of abundant knockout and transgenic technology that will allow for a better understanding of processes that contribute to lung allograft injury. As obliterative bronchiolitis has been observed in swine lung allografts, future studies will have to determine whether obliterative airway disease can be observed in immunosuppressed mouse lung transplant recipients (39). Ultimately, findings in this mouse model will have to be validated in preclinical large animal models of lung transplantation (40).

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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