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Review **Immunology** **Transplantation**

Lung transplantation, a cure for a number of end-stage lung diseases, continues to have the worst long-term outcomes when compared with other solid organ transplants. Preclinical modeling of the most common and serious lung transplantation complications are essential to better understand and mitigate the pathophysiological processes that lead to these complications. Various animal and in vitro models of lung transplant complications now exist and each of these models has unique strengths. However, significant issues, such as the required technical expertise as well as the robustness and clinical usefulness of these models, remain to be overcome or clarified. The National Heart, Lung, and Blood Institute (NHLBI) convened a workshop in March 2016 to review the state of preclinical science addressing the three most important complications of lung transplantation: primary graft dysfunction (PGD), acute rejection (AR), and chronic lung allograft dysfunction (CLAD). In addition, the participants of the workshop were tasked to make consensus recommendations on the best use of these complimentary models to close our knowledge gaps in PGD, AR, and CLAD. Their reviews and recommendations are summarized in this report. Furthermore, the participants outlined opportunities to collaborate and directions to accelerate research using these preclinical models.

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Lung transplantation, a cure for a number of end-stage lung diseases, continues to have the worst long-term outcomes when compared with other solid organ transplants. Preclinical modeling of the most common and serious lung transplantation complications are essential to better understand and mitigate the pathophysiological processes that lead to these complications. Various animal and in vitro models of lung transplant complications now exist and each of these models has unique strengths. However, significant issues, such as the required technical expertise as well as the robustness and clinical usefulness of these models, remain to be overcome or clarified. The National Heart, Lung, and Blood Institute (NHLBI) convened a workshop in March 2016 to review the state of preclinical science addressing the three most important complications of lung transplantation: primary graft dysfunction (PGD), acute rejection (AR), and chronic lung allograft dysfunction (CLAD). In addition, the participants of the workshop were tasked to make consensus recommendations on the best use of these complimentary models to close our knowledge gaps in PGD, AR, and CLAD. Their reviews and recommendations are summarized in this report. Furthermore, the participants outlined opportunities to collaborate and directions to accelerate research using these preclinical models.

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Introduction

Preclinical models are essential to address knowledge gaps in three of the most vexing and serious lung transplantation complications: primary graft dysfunction (PGD), acute rejection (AR), and chronic lung allograft dysfunction (CLAD). Each one of these complications significantly curtails lung allograft and patient survival, and cannot be addressed by patient-based research alone. While preclinical models for these lung complications have been developed, the usefulness and robustness of these models needs to be better clarified. The National Heart, Lung, and Blood Institute (NHLBI) of the NIH convened a workshop to review the current state of preclinical models of PGD, AR, and CLAD and to provide consensus

recommendations to the broader research community on how to use and improve these models to facilitate research efforts in lung transplant research and to improve patients' outcomes. A summary of the models discussed in this workshop report is represented in Figure 1.

PGD

PGD is a spectrum of acute lung allograft injuries that range from a mild capillary leak in alveoli to severe diffuse alveolar damage occurring within the first 72 hours after lung transplantation (1). PGD is characterized by radiographic evidence of pulmonary edema with or without infiltrates and consolidation with progressive hypoxemia without other identifiable causes. PGD is a form of the acute respiratory distress syndrome that has previously been described by several terms including ischemia-reperfusion injury (IRI), reimplantation response, reperfusion edema, noncardiogenic pulmonary edema, early graft dysfunction, primary graft failure, and posttransplant acute respiratory distress syndrome (1). Numerous PGD models can be used to explore different aspects of the lung transplant's unique pathobiology. The advantages and disadvantages of these models are described below and summarized in Table 1.

In vitro modeling of PGD. In vitro modeling of IRI involves exposure of relevant pulmonary cells in cultures (or cocultures) to acute hypoxia followed by reoxygenation (2) or using stop-flow methods to test effects of endothelial shear stress during IRI (3). Further modifications of the hypoxia/reoxygenation model may include the use of 4°C preservation solution (e.g., Perfadex) before allowing the cells to warm to room temperature followed by reoxygenation in 37°C culture media (4, 5). In vitro models have demonstrated that longer cold aerobic times enhance apoptosis, cytoskeletal remodeling, permeability, as well as upregulation of innate and adaptive immune pathways (2, 6–12). In vitro systems are an economical way to decipher cellular responses to certain conditions that follow lung transplant (13–15); however, these models must be complemented by in vivo models of IRI.

In vivo modeling of PGD. The IRI component of PGD can be modeled in vivo using multiple well-accepted approaches in animals. The easiest approach involves unilateral hilar occlusion followed by reperfusion, and the more technically challenging models involve orthotopic lung transplantation. The well-characterized hilar clamp rodent model (16, 17) involves mechanical ventilation, a thoracotomy to expose the left lung hilum, and occlusion of all hilar structures with a microvascular clamp or lasso versus selective occlusion of the pulmonary artery for a variable period of warm ischemia (18, 19), followed by unclamping allowing for reperfusion (20, 21). Sham-operated controls go through a similar procedure to expose the hilum without occlusion. This model requires attention to the mechanical ventilation aspect, as this may lead to ventilation-induced lung injury that can add to the IRI (22, 23). However, using the combination of ventilation-induced injury and IRI may parallel what can occur during human lung transplantation (21, 24, 25).

Another variation of the hilar clamp model involves isolating and clamping the pulmonary artery alone to preserve gas exchange, and is referred to as the nonhypoxic lung ischemia model (19). Additionally, IRI has been studied using isolated, perfused rodent lung models in which the lungs are manipulated either ex vivo or in situ, and continuously perfused with synthetic media and ventilated in a temperature-controlled chamber (19, 26). The orthotopic, single-lung transplant model has also been used to model PGD. In this approach, the cold ischemic time of the donor lung is intentionally prolonged (up to 18 hours) to produce lung injury upon reperfusion (27, 28). It can also be performed as an allograft, allowing a unique ability to assess the effects of varying periods of cold ischemia on subsequent development of PGD, as well as the contributions of early antigen-independent events to the subsequent development of AR and potentially later chronic injury.

Key endpoints used in these models include lung edema and permeability using the gravimetric (wet/dry) ratio (20, 27, 29), the translocation of exogenously administered Evan's blue dye or radiolabeled or fluorescently labeled proteins, or by measuring the accumulation of endogenous proteins (total protein, albumin, IgM) in the bronchoalveolar lavage fluid (20, 21, 27). Testing for cardiopulmonary hemodynamics, lung function, and oxygenation using indwelling devices, surface probes, and arterial blood gases can also be performed (20, 21, 27). These IRI models involve lung oxidative stress with lipid peroxidation (30) and have been associated with reduced arterial oxygenation (6), reduced compliance, increased airway resistance, and high pulmonary artery pressures (31, 32). Lungs also develop increased pulmonary vascular fibrin deposition and elevated expression of plasminogen activator inhibitor-1 (PAI-1) (6). Furthermore, there is a robust extravasation and recruitment of innate and adaptive immune cells into the lung (20), increased levels of high mobility group box 1 protein (HMGB1) (7), myeloperoxidase (a marker of

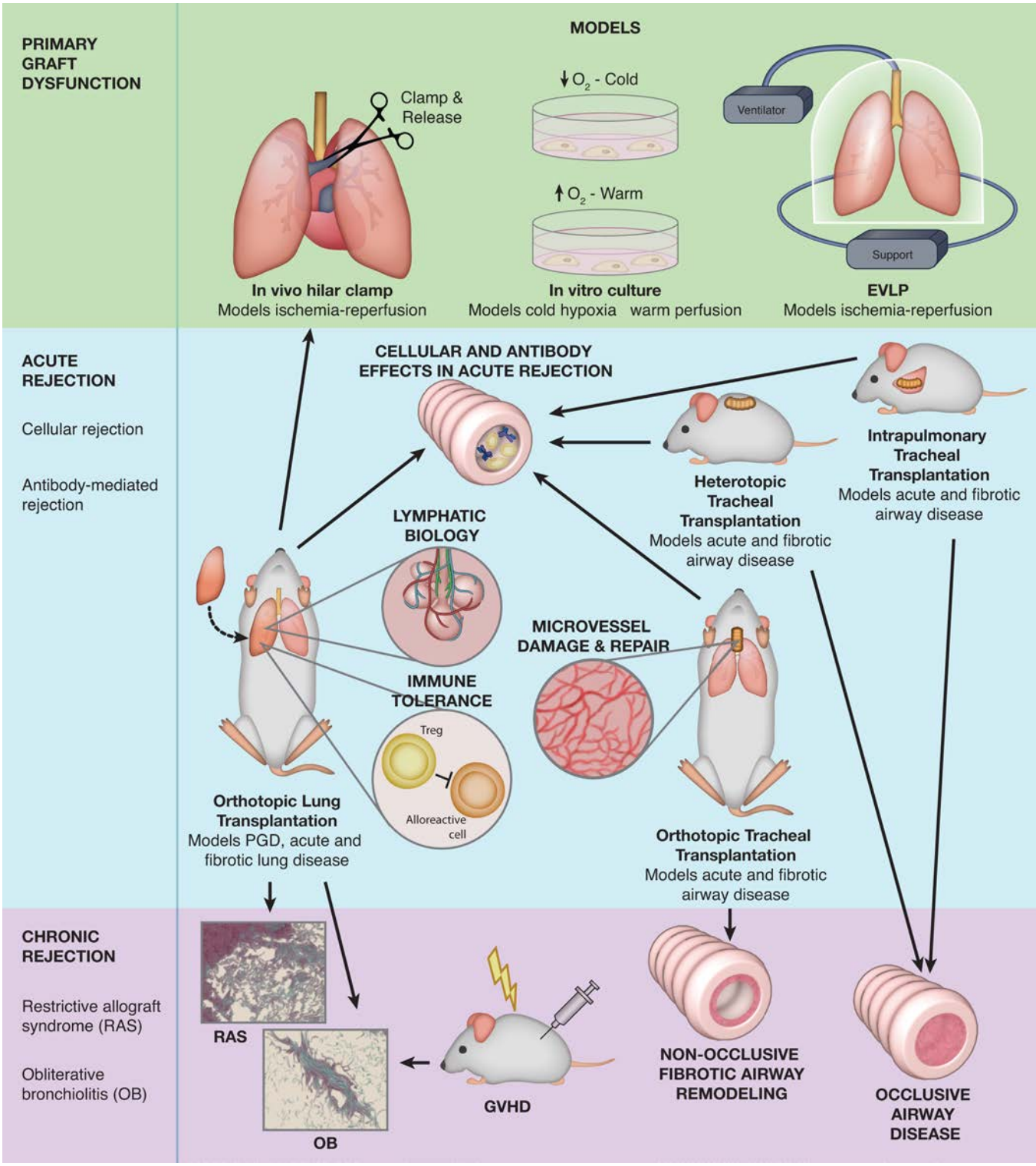

Figure 1. A summary of preclinical models used for lung transplant research. There are a number of preclinical models (right) to study the three major complications of lung transplantation (left): (i) Primary graft dysfunction (PGD), which occurs as a consequence of organ procurement, cold storage and implantation; (ii) acute rejection (AR), which is mediated through cell- and antibody-mediated immune responses; and (iii) chronic rejection or CLAD, which pathologically manifests as RAS and OB. PGD can be modeled through in vitro culture systems to assess cellular responses to cold hypoxia and warm reperfusion. Ischemia-reperfusion injury associated with PGD can be modeled by the hilar clamp model in vivo. Limiting PGD and prolonging transplant survival can be studied through the use of ex vivo lung perfusion (EVLP) in animal models. Orthotopic lung transplantation in animal models is a highly rigorous approach that can be used to study PGD as well as AR, immune tolerance, lymphatic biology, obliterative bronchiolitis (OB), and restrictive allograft syndrome (RAS). Heterotopic and intrapulmonary tracheal transplant models are high-throughput procedures useful for modeling AR processes. Orthotopic tracheal transplantation models large airway changes in acute and chronic rejection and is especially useful for studying microvascular changes. The graft-versus-host disease (GVHD) model, which relies on bone marrow transplantation of MHC-mismatched cells, produces OB-like lesions. Illustrated by Rachel Davidowitz.

Table 1. Models for investigating primary graft dysfunction (PGD)

Model	Advantages	Disadvantages
In vitro cell culture	Inexpensive, short duration, multiple cell types can be evaluated individually or in coculture conditions.	Lacks many conditions found during lung transplant ischemia-reperfusion. Does not account for all cell types involved in ischemia-reperfusion.
Hilar occlusion	Less technically challenging than the orthotopic lung transplant model and is a fast, high-throughput in vivo model.	Not an orthotopic model, requires expensive equipment such as a ventilator and dissecting microscope.
Orthotopic lung transplant model	Simulates most aspects of lung transplantation.	Expensive and time consuming. Very few individuals can master the surgery in a timely fashion.

neutrophil/mononuclear phagocyte activation and infiltration) (30), and proinflammatory cytokines and chemokines (7, 8, 15, 20, 24), all exemplifying multiple complex inflammatory pathways involved in PGD.

An advantage of the hilar clamp model for studying the IRI component of PGD is that it is less technically demanding than the orthotopic single-lung transplant models (33) and, as such, is a suitable platform for testing therapeutic delivery, rehabilitative potential, and biomarkers predictive of PGD. Orthotopic lung transplantation is a more technically demanding model that may be more informative for issues relevant to surgical implantation and innate immunity. Significant interest has been generated in allograft optimization through ex vivo lung perfusion (EVLP). Most studies involving EVLP have utilized human lungs (34, 35) or large animal (mainly porcine) models (36–39) with or without transplantation. Nonetheless, rodent studies using EVLP and lung transplantation have been performed (40–42). A recent study introduced a murine model of EVLP and demonstrated that Steen solution improved lung function as compared with cold static–preserved lungs (43). The addition of adenosine receptor agonist/antagonist in the Steen solution augmented lung function while reducing proinflammatory cytokines, deleterious innate immunity pathways, and neutrophil vascular margination (43, 44). Similar results have been shown in larger animals and human lungs using similar systems (45). Moreover, the delivery of IL-10 by EVLP adenoviral gene therapy to injured human donor lungs (46) resulted in improved lung function in pigs receiving therapy (47). These studies demonstrate that animal models provide a reproducible and effective means for experimental and mechanistic studies of EVLP. Combined with transplantation, these models could rapidly advance the potential of EVLP in rehabilitating marginal donor lungs for successful transplantation.

Imaging in animal models of PGD. Transplantation results in a highly predictable period of immune activation associated with the implantation of a solid organ expressing foreign antigens. Methods to image mobilization and activation of the immune system during this period are currently limited, but emerging technologies may provide new opportunities to study this period. With the emergence of molecular imaging, new sensitive and selective imaging probes have been developed that can be translated from animal models to patients. Single photon emission computed tomography (SPECT) and positron emission tomography (PET) imaging can detect and monitor a variety of pathophysiological processes such as T cell activity by glucose uptake and neutrophil activation by binding of the peptide cinnamoyl-F-(D)L-F-(D)L-F (cFLFLF) to the formyl peptide receptor 1 (FPR1) (48–50). Multiphoton intravital microscopy can also be used to assess specific cell populations and functions during murine IRI (51–53). These studies provide encouraging results to elucidate underlying pathways or monitor for PGD.

AR

The two major forms of AR are acute cellular rejection (ACR) and antibody-mediated rejection (AMR). ACR is well characterized and is classified into two subtypes: type A is a lymphocytic inflammatory cellular infiltrate that ranges from a mild perivascular infiltrate with no obvious tissue injury, to infiltrates that also involve the interstitium and air-spaces with prominent acute lung injury with vasculitis; type B airway inflammation, namely lymphocytic bronchitis, is currently classified as either low-grade, with no tissue injury, or high-grade, in which there is a more extensive infiltrate associated with injury to the airway (54). Both A and B types of ACR are thought to increase the risk of development of bronchiolitis obliterans syndrome (BOS). The other form of AR, AMR, is not as well characterized but has been described as including C4d deposition in capillaries, neutrophilic capillaritis, intravascular macrophages, and acute lung injury (55, 56). Preclinical models have been useful for exploring mechanisms of both ACR and AMR

Table 2. Animal models for investigating acute lung transplant rejection

Model	Advantages	Disadvantages
Heterotopic tracheal transplantation (HTT)	High throughput. Progresses from AR[A] to an occlusive airway disease that models OB. Lymphocytic bronchitis studies.	Non-native positioning under skin or in omentum, without luminal air flow. Changes in large airway of unclear significance for distal airways. No information provided about parenchymal lung injury during AR.
Orthotopic tracheal transplantation (OTT)	High throughput. Native position. Planar anatomy conducive to study of vascular systems and tracking cell populations. Good for tissue engineering, lymphocytic bronchitis, and anastomosis studies.	Changes in large airway of unclear significance for distal airways. No information provided about parenchymal lung injury during AR.
Intrapulmonary tracheal transplantation	High throughput. Pulmonary positioning. Useful for evaluating de novo lymphoid tissue.	Although intrapulmonary, trachea is ectopically placed.
Orthotopic lung transplant model	Most completely simulates the human AR response.	Low throughput. Technically challenging.

[A]AR, acute rejection; OB, obliterative bronchiolitis.

(summarized in Table 2). Understanding the pathophysiology and limiting the extent of injury of AR is an important need in the lung transplant field.

Modeling lung transplantation AR in mice and rats. Since the 1960s, experiments using canine and rat orthotopic lung transplant models have examined various aspects of AR. For example, in the rat model, the onset of AR was more rapid in lungs when compared with heart grafts (57). The most commonly used mouse model to study lung rejection in the 1990s and early 2000s was heterotopic tracheal transplantation (HTT) (58). In this model, rejecting allografts demonstrate early inflammation, epithelial necrosis, and fibroproliferation in the airway lumen, which were not observed in the isografts. While the histological changes make this model especially valuable for studying chronic rejection observed in the airways of human lungs (as described below), it has also been useful as a high-throughput model for studying the cellular requirements for rejection (59). Later, the orthotopic tracheal transplantation (OTT) model was developed that conferred the added advantage of being a functional transplant in its native position. This model has provided useful insights about early events in AR (e.g., see ref. 60) and has been developed as an especially useful model for studying microvascular changes that occur in AR, as described in detail below. The intrapulmonary tracheal transplant model as a model of AR has been useful to show the importance of intrapulmonary de novo lymphoid tissue (61). In 2007, the mouse orthotopic lung transplant model was introduced (33), and while highly technically challenging, the histological changes observed as early as three days after transplantation in major histocompatibility complex–mismatched (MHC-mismatched) strain combinations closely resembled perivascular and peribronchial mononuclear infiltrates seen on transbronchial biopsies of lung transplant patients suffering from AR.

With the aid of genetic tools available in mice and novel imaging approaches, the mouse orthotopic lung transplant model has yielded some fresh insights into mechanisms of acute lung allograft rejection. For example, recipient T cells rapidly infiltrate lung allografts where they interact with donor dendritic cells (62). In fact, the lung graft provides a suitable environment for the priming of alloreactive T cells, and lungs can be rejected in the absence of secondary lymphoid organs (62), in contrast to other solid-organ grafts such as hearts. Unlike the case for hearts, AR of lungs is not dependent on $CD4^+$ T cells (63). Furthermore, interaction between innate and adaptive immune cells within pulmonary grafts can trigger AR. To this end, neutrophilic graft infiltration, a hallmark of PGD, can trigger IL-12 production by dendritic cells that skews the T cell response towards Th1 (53). Orthotopically transplanted mouse lungs are exposed to the environment, allowing for the design of experiments to evaluate the role of respiratory pathogens on alloimmunity and AR responses. *Pseudomonas aeruginosa* respiratory infections can break immunosuppression-mediated lung allograft tolerance (64). This model is a suitable platform to evaluate new diagnostic modalities for AR. For example, fluorodeoxyglucose PET (FDG-PET) can be used to monitor acute lung allograft rejection owing to a high rate of metabolism of graft-infiltrating T cells (65). Thus, the orthotopic mouse lung transplant model is an effective experimental platform to study mechanisms that contribute to AR, test noninvasive diagnostic modalities as well as to study immune tolerance (66), and evaluate strategies to prevent or treat this complication.

Table 3. Animal models for investigating chronic rejection

Model	Advantages	Disadvantages
Heterotopic tracheal transplantation (HTT)	High throughput. Develops occlusive airway disease with possible (though uncertain) relevance to OB[A]	Ectopic position without normal air/environment interface which is likely relevant to development of OB.
Intrapulmonary tracheal transplantation	High throughput. Pulmonary positioning. Occlusive and fibrotic luminal pathology.	Although intrapulmonary, trachea is ectopically placed and large airways do not develop obstruction in native position.
Orthotopic tracheal transplantation (OTT)	High throughput in native position with normal environmental interface. Subepithelial fibrosis of large airways.	Does not model terminal airway obliteration characteristic of OB.
Bone marrow transplantation	Models OB observed in graft-versus-host disease with possible relevance to lung transplantation. High throughput.	Bronchial restriction is reproducible but OB has variable penetrance.
Orthotopic lung transplantation	Small airway obliteration reminiscent of OB in mouse model.	OB lesion has variable penetrance depending on strain combination and laboratory.

[A]OB, obliterative bronchiolitis.

AMR. Elicitation of immune responses to the mismatched donor human leukocyte antigen (HLA) and breakdown of tolerance to tissue-restricted self-antigens pose a significant challenge to acceptance and continued graft function following organ transplantation (67, 68). While the mechanisms of AMR are not firmly established, de novo donor-specific antibodies against HLA have been shown to predispose to the development of immune responses to lung self-antigens and BOS (69–71). To define mechanisms leading to anti-MHC–mediated development of rejection, a preclinical murine model was developed in which exogenous anti-MHC was administered into the native lungs and elicited production of antibodies and T cell responses specific for lung-associated self-antigens, type V collagen [col(V)], and K-α 1 tubulin, culminating in fibrotic pathology (72, 73). Human lung transplant recipients develop antibodies against col(V), a protein mainly located in the lung interstitium and not ordinarily exposed to the immune system. In the rat lung transplant model, allografts in minor histocompatibility complex–mismatched recipients induce col(V)-specific T and B immunity after transplantation and appear to be an important source of autoantigen that autoantibodies ligate (74). Better understanding of the exact roles of allo- and autoantibodies in AR, like chronic rejection, will clearly benefit from improved modeling and biomarkers that delineate disease phenotypes and also incorporate orthotopic lung transplant mouse models.

Microvascular injury and large airway disease. The Papworth Autopsy Study evaluated patients who died with BOS and correlated the development of chronic rejection with a dropout of airway microvessels (75); such a relationship between microvascular destruction during AR and subsequent chronic rejection has been suggested with all solid-organ transplants. The OTT model is useful for the study of airway microvessels because the tracheal vasculature can be easily visualized in one tissue plane (76). The model is performed through an interposition transplant in which the recipient trachea is transected and a donor trachea is sewn into the space created by the naturally retracted airway; alternatively, the donor trachea can be sewn in parallel to the native airway (60). During AR, this model has revealed that the airways undergo a transient loss of a functional microcirculation accompanied by localized tissue hypoxia and ischemia (77); although a functional microcirculation returns, these grafts cannot be rescued with immunosuppression once the vascular bed is transiently lost, suggesting an anatomic basis for the development of chronic rejection. The OTT model can incorporate fiberoptic bioprobes that detect tissue oxygenation and perfusion over time (76). Another facet of a compromised circulation occurs at the time of transplantation in the airway anastomosis that does not include a restored bronchial circulation and is susceptible to dehiscence and infection (78). The relative ischemia of the airway anastomosis is associated with a proclivity to infection, especially with *Aspergillus* and *Pseudomonas*. The OTT model appears to be a useful model for this process (79, 80).

Tracheal allografts are proving an exciting, but currently controversial (81), platform to test tissue bioengineering concepts in the clinic, as these transplants can be decellularized and repopulated with recipient-derived cells prior to surgical implantation, an approach that should prevent AR and limit the need for chronic immunosuppression (82). A more limited repopulation of donor-derived cells can be observed in

vivo using the mouse OTT model. This line of study may have value in determining how both destructive and reparative processes occur through the migration of cell populations from the recipient to the donor (77, 80, 83). OTT also facilitates lineage fate mapping studies to track the movement and transformation of various cell types in the allograft recipient (83–85). The mouse orthotopic lung transplant model also holds promise as an effective platform for evaluating the relative contribution of recipient cells in both the disease and repair of small airways and pulmonary parenchyma.

Lymphatic contribution to AR. In the normal lung, there is a highly complex network of lymphatics consisting of subpleural lymphatics largely over the lower lobes, and a deeper lymphatic network running along the major airways and the blood vasculature in the interstitial spaces. The visceral pleura and the neighboring lung tissue are drained through the superficial network into the hilar area of the lung where they connect with the deeper plexus of lymphatics (86). At the time of transplant, the bronchus, bronchial artery, pulmonary artery, and vein as well as the lymphatics are severed at the level of the hilum. However, only the bronchus, pulmonary artery, and vein are reanastomosed. A recent clinical study has revealed that, unlike chronic organ failure in kidney transplantation, lymphangiogenesis is not altered in CLAD patients (87), which raises questions about the role of altered lymphatics in lung transplantation. Using lymphoscintigraphy, the fate of the lymphatic vasculature after transplantation has been investigated in a canine lung transplant model. These studies have shown that functional lymphatic drainage is restored at seven days after transplantation in isografts. In allografts treated with immunosuppression, a functional lymphatic bed is observed between two and four weeks after transplantation (88). Lymphatic biology can also be effectively studied in the mouse orthotopic lung transplantation model, which revealed a marked decline in the density of lymphatic vessels, accompanied by accumulation of low-MW hyaluronan in mouse orthotopic allografts undergoing AR (89). Work in this model has suggested a protective role for the promotion of lymphangiogenesis in the posttransplant period. The role of the lymphatic vessels and their contribution to acute and chronic rejection in lung transplants are poorly understood and deserves greater study.

CLAD

A high rate of chronic graft failure continues to be the most significant hurdle in improving long-term survival after lung transplant. In the 1980s, obliterative bronchiolitis (OB) was identified as a common pathology in chronically failing lung transplants; OB was subsequently discovered to also be a complication of bone marrow transplant recipients (90). Histologic features of lung transplant–associated OB include anatomic restriction to membranous and respiratory bronchioles, presence of both constrictive and proliferative subtypes, and potential association with mononuclear cell infiltration (91). Graft vasculopathy with progressive myointimal thickening of the pulmonary arteries and veins is also described in association with OB. Clinically, OB presents as a progressive obstructive decline in lung function termed BOS. BOS has a very high prevalence in the lung transplant population with approximately 50% of patients demonstrating this syndrome by five years after transplant. Clinical studies demonstrate a strong link between AR, specifically airway involvement with lymphocytic bronchitis, and BOS. Other complications in the posttransplant period such as PGD and infections have also been linked to BOS.

While BOS remains the predominant cause of CLAD, more recently a restrictive allograft syndrome (RAS) has been described. Patients presenting with RAS have been demonstrated to exhibit distinct histologic phenotypes such as pleural and subpleural fibrotic changes, intra-alveolar fibrinous exudate, and acute fibrinous (and organizing) pneumonia (92). Most animal models to date have modeled OB and were the primary focus of the Workshop and this consensus statement. However, a recent model has been developed utilizing fully MHC-mismatched orthotopic lung transplants treated with chronic immunosuppression and evaluated ten weeks after transplant; these mice develop some features of RAS and offer promise as a new model of chronic rejection (93). It may be that a closer evaluation of the orthotopic lung transplant models will reveal features of RAS such as subpleural and interstitial fibrosis not previously appreciated (e.g., see ref. 94). We summarize the advantages and disadvantages of various preclinical models of chronic rejection below and in Table 3.

Modeling OB. While in vitro models of OB have utility in showing how individual populations, such as bronchial epithelial cells, mesenchymal stromal cells, and airway smooth muscle cells, may contribute to disease (95–98), animal models can provide a window into the events from transplant to the final fibrotic obliteration of small airways and is key to investigating the pathogenesis of OB and conducting preclinical studies of potential therapeutic interventions. Utilized models of post-lung-transplant OB are based on allogeneic lung tissue transplantation with a goal of reproducing fibrotic airway remodeling.

Technically, these models are similar to those utilized for studying AR and include HTT and OTT as well as orthotopic single-lung transplantation.

Tracheal transplant models of chronic rejection. Initial discoveries in posttransplantation OB have been fueled primarily by the HTT model in which a harvested donor trachea is transplanted into a subcutaneous pouch in the dorsal surface of the neck or omentum. Following the IRI and AR phases of injury, these transplants undergo a fibroproliferative phase with partial denuding of the epithelium at day 14 and fibroobliteration of the allograft trachea at day 21 (24, 99, 100). Conversely, the isografts have a healing airway graft at day seven with remnants of epithelial cell hyperplasia; however, this is followed by essentially normal isografts by days 14 and 21 (24, 99, 100). This model has been utilized in both rats and mice, although more consistent fibrosis is noted in rat versus mouse tracheas.

HTT offers a reproducible model to study alloantigen-associated fibrosis in an airway. Airway luminal obliteration can be quantified morphometrically and at various time points. Fibrosis can be evaluated by staining with trichrome or by smooth muscle actin staining of myofibroblasts. Multiple tracheas can be combined to measure soluble collagen quantitatively by hydroxyproline assay. The small tracheal segments make evaluation of cellular components and protein isolation challenging. However, careful single-cell digestion allows for evaluation of cellular composition by flow cytometry, and investigators usually combine two or more tracheal grafts for protein isolation. The ability to dissect a trachea in its entirety allows for evaluation of microvasculature and lymphatics, providing an avenue to study their role in OB development.

The major criticism of the tracheal transplantation model is that fibrotic obliteration is being modeled in a large cartilaginous airway that is histologically distinct from the small airways that are the site of human OB (101). Its relevance is also somewhat limited by the absence of a normal air interface and native mediastinal lymphatic drainage. Most importantly, human OB develops in a complex in vivo environment with distinct cellular niches that cannot be reproduced in a tracheal transplant placed in an extrapulmonary environment. Thus, the HTT model is useful as a high-throughput screen for alloimmunity-induced fibrosis, but findings obtained with this procedure must be considered with the appropriate caveat that its non-native positioning may affect results.

In the OTT model, epithelial regeneration from migration of recipient-derived epithelial cells limits the development of fibrotic occlusion or OB (102). Although obliterative lesions are not observed, OTTs develop lymphocytic bronchitis (a large airway precursor of BOS) as well as subepithelial fibrosis (103). Findings from the OTT studies have been cautiously extrapolated to explain how occlusive fibrosis may evolve following rejection in higher generation bronchioles (77, 80, 83, 104, 105).

Attempts to simulate airway fibrosis in the in vivo lung environment have also been made by placing the tracheal graft in the lung tissue through a transthoracic approach termed the intrapulmonary tracheal transplant model (61, 106, 107). Another potentially powerful model takes advantage of humanized mice by transplanting human airways and peripheral blood mononuclear cells into an immunodeficient mouse (108, 109). These models are promising for studying peripherally derived human leukocytes but have challenges due to the limited supply of human bronchi available.

Orthotopic lung transplant models of chronic rejection. As noted above, a significant step forward in animal modeling to understand posttransplant complications has come with establishment of surgical methods for single-lung transplant surgery in small animals. However, while rat lung transplantation has been successfully utilized extensively to investigate early complication such as AR, use of rat lung transplant to study chronic graft failure has been limited by a failure to establish a robust, reproducible model of OB. Transplantation across minor histocompatibility complex–mismatched combination (Fischer 344→Lewis [F344→LEW]) and across MHC mismatch (Brown Norway [BN]→LEW) in combination with pharmacologic immunosuppression has been shown to recapitulate some aspects of OB pathology in allografts (110). There is some disagreement in the transplant community about how closely OB-like lesions generated in orthotopic lung transplants recapitulate the human lesion, and there is agreement that appropriate caveats should be made when presenting data from these models. Other donor-recipient combinations, such as F344→Wistar Kyoto (WKY), have also been demonstrated to develop OB-like lesions at late time points of two to three months after transplantation (111). However, there appears to be a difference in animals obtained from different vendors as well as concerns of reproducibility across centers (77). Thus, at present, a consensus on a definitive rat lung transplant model to study OB pathogenesis has not emerged. An intriguing model of lung transplantation in ferrets also develops OB-like lesions (112). Finally, the role of innate immune activation in augmenting OB has also been evaluated using the rat lung transplant model with intratracheal gastric fluid challenge (113).

The advancement of surgical techniques to accomplish single-lung transplantation in mice has permitted access to resources such as transgenic mice that can facilitate cellular tracking and pathway targeting (33, 114, 115). However, while the AR model was again easily established by using MHC-mismatched mice, developing a robust model of OB has been more challenging. Strains that are disparate at MHC antigen loci (H-2^{d}→H-2^{b}; BALB/c→C57BL/6) develop severe AR by seven days after transplantation, and this nearly complete destruction of the lung prevents longer time-point evaluation for development of OB (116). This issue was circumvented by Fan et al. (117, 118), and a transplant involving minor histocompatibility complex mismatch (C57BL/10→C57BL/6) developed only mild rejection within one week. Grafts could be followed for longer time points, and peribronchial and intraluminal fibrotic lesions were described at days 21 and 28 (117). However, these airway fibrotic lesions were noted in only 50% of the transplanted mice and were limited to a small number of airways in the allografts. Use of immunosuppression (cyclosporine + steroids) to prolong graft life in MHC mismatch (BALB/c→C57BL/6) has also allowed for investigation of the development of OB pathology (119). This model only generates OB-like lesions in 25%–50% of the mice, with many mice demonstrating no evidence of OB or regaining normal histology after 12 weeks. The difference was related to variability in immunosuppression achieved with lower cyclosporine levels noted in mice that developed OB. Taken together, these studies suggest that a moderate mismatch with continued but contained allo-insult is crucial to development of chronic fibrotic airway lesions.

More recently, investigators at the University of Michigan examined transplant of an F1 to a parent mouse to obtain moderate MHC mismatching that was less antigenically disparate (120). A similar strategy has been previously employed in the field of bone marrow transplantation to study chronic graft-versus-host disease (GVHD) with injection of parental cells into F1 recipients or complete MHC mismatch (121–126). These studies demonstrate how the interplay between local innate immunity and alloreactive T cell subsets and alloantibody deposition contribute to chronic pulmonary GVHD with fibrotic (including OB-like) pathology. The specific combination of transplantation of B6D2F1/J (cross between C57BL/6J and H-2-$^{b/d}$) lungs into DBA/2J (H-2-^{d}) recipients results in consistent OB-like pathology with peribronchial and perivascular fibrosis by day 28 (120). These grafts, which are discordant at the class I allele (H-2D^{b}) with the recipient, demonstrate an evolution from immune cell infiltration to epithelial cell injury and development of fibrosis over time that is reminiscent of human disease. The B6D2F1/J→DBA/2J transplant model also had very high penetrance, with 100% of allografts exhibiting fibrotic remodeling in the majority of airways, allowing it to be utilized for investigation of therapeutic targets (127). However, using this combination lends itself to the disadvantage that transgenic mice are not readily available on B6D2F1/J and DBA/2J genetic backgrounds.

Fibrotic remodeling of the allograft is the predominant cause of CLAD; hence, a relevant animal model for investigating CLAD must recapitulate allograft fibrogenesis and allow for meaningful targeting of specific pathways. As a primary effector cell of fibrogenesis, mesenchymal cells play a key role in CLAD arising from OB or parenchymal fibrosis. While mesenchymal cells orchestrating fibrosis can potentially be derived from various sources, the mesenchymal population in the transplanted lung retains its donor origin and these graft-derived cells appear to be the predominant contributors to OB lesions (98, 128–131). Investigation of fibrotic lesions in the HTT model have shown them to be derived from the recipients (132). Utilizing the expression of H-2D^{b} to differentiate donor versus recipient origin of the mesenchymal cell population in lung allografts, approximately 90% of collagen I–expressing cells in the B6D2F1/J→DBA/2J orthotopic model were of donor origin (120). These investigations suggest that the whole-lung transplant model holds an advantage over tracheal transplantation in studies of mesenchymal cell recruitment and activation, as it is more reflective of mesenchymal populations involved in pathogenesis of OB in human lungs.

Conclusion and recommendations

This NHLBI Workshop report on models of lung transplantation provides an overview of the preclinical strategies for researching the major complications involved in this procedure including PGD, AR, and CLAD. There is significant opportunity for advancing the field through effective collaboration between investigators utilizing different experimental approaches. Cancer is a major complication of lung transplantation that is not adequately addressed by current animal models and deserves greater consideration. The NHLBI Workshop consensus recommendations, which were organized around three major complications (PGD, AR, and CLAD), are intended only to provide general guidance in the choice of animal models and collaborative possibilities and are as follows.

PGD research. In vitro approaches are useful in discerning basic, cell-specific mechanistic information that will gain impact through in vivo models, beginning with the hilar clamp technique in nontransplanted animals and advancing into orthotopic lung transplant models, which may incorporate EVLP. Opportunities for collaboration exist between basic science labs that focus on in vitro models and bring mechanistic questions into laboratories with skilled microsurgeons and incorporating novel imaging procedures.

AR research. ACR research continues to employ the HTT model, which offers the benefit of technical feasibility. OTT, while more technically challenging than HTT, is favored for being a functional transplant and is also useful for studying microvascular changes during rejection. Orthotopic lung transplantation requires the greatest microsurgical expertise but offers the most relevant platform for evaluating changes of small airways and lung parenchyma. Immune tolerance, a process that directly counters AR, is also effectively studied in the orthotopic lung transplant model. AMR, self-antigens that trigger autoimmunity, and disrupted lymphatic biology are important areas in development that will benefit through greater collaboration by investigators using different surgical models.

CLAD research. OB research has employed the HTT model, which provides a facsimile of terminal airway fibrosis and is useful because of high throughput and the ease of pathological readout. While OTT is useful for examining large airway changes of chronic rejection, occlusive airway disease does not develop. The more demanding modeling of OB is in the orthotopic lung transplant model in mice; however, this technique has been limited by variably penetrant pathology and remains technically challenging. Because of the low-throughput nature of this surgery in mice and the relatively limited number of laboratories that successfully achieve consistent results, a significant opportunity for collaboration exists between groups to test their most promising mechanistic concepts in laboratories that regularly employ this technique. The contribution of the microbiome to host immunity is only beginning to be understood, and modulating the microbiome to be protective against CLAD is a promising area of research that will be facilitated by studies using the whole lung (133, 134). Finally, there is currently an unmet need for new models of RAS, an increasingly recognized manifestation of CLAD. Further work needs to be done in modeling other histologic manifestations of CLAD such as pleural and parenchymal upper lobe–dominant fibrosis that has been demonstrated in patients presenting with restrictive CLAD and the recently described acute fibrinous pneumonitis (135).

Author contributions

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