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Novel approaches for long-term lung transplant survival

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Allograft failure remains a major barrier in the field of lung transplantation and results primarily from acute and chronic rejection. To date, standard-of-care immunosuppressive regimens have proven unsuccessful in achieving acceptable long-term graft and patient survival. Recent insights into the unique immunologic properties of lung allografts provide an opportunity to develop more effective immunosuppressive strategies. Here we describe advances in our understanding of the mechanisms driving lung allograft rejection and highlight recent progress in the development of novel, lung-specific strategies aimed at promoting long-term allograft survival, including tolerance.

KEYWORDS

lung transplantation, acute cellular rejection (ACR), chronic lung allograft dysfunction (CLAD), immunosuppression, *ex vivo* lung perfusion (EVLP), tissue-resident memory T-cells, mesenchymal stromal cells (MSCs), antibody-mediated rejection (AMR)

Introduction

Lung transplantation has evolved significantly since its introduction in 1963 and is now commonly performed for a variety of end-stage lung diseases. Despite this, survival after lung transplant remains poor and has not significantly improved over the past several decades. Standard-of-care immunosuppressive regimens utilized in lung transplantation have failed to achieve acceptable long-term graft and patient survival. The median 6.7-year post-transplant survival represents one of the lowest among solid organs and is limited primarily by allograft failure due to acute and chronic rejection (1, 2). Recent insights into the unique immunologic properties of lung allografts have provided a framework to better understand the limitations of conventional immunosuppression in lung transplantation and provide an opportunity to develop novel strategies that take advantage of these properties (3).

In this review, we describe recent advances in our understanding of the mechanisms driving lung allograft rejection, particularly in the context of limitations related to

conventional immunosuppression. We then highlight existing and emerging lung-specific strategies aimed at promoting long-term allograft survival. Specifically, we describe novel preservation methods, cellular therapies, anti-inflammatory agents, strategies targeting memory T-cells, and tolerance induction (Figure 1).

Mechanisms of lung allograft failure

Acute inflammation

Beginning immediately after transplantation, lung allografts are at risk of primary graft dysfunction (PGD), a form of acute lung injury that can result in severe intra-graft inflammation. It is widely accepted that PGD, which occurs in up to 25% of lung transplants (4), is mediated by ischemia reperfusion injury (IRI) and represents an independent risk factor for the subsequent development of chronic allograft lung dysfunction (CLAD) (5–7).

Adaptive immunity

Adaptive immune activation resulting in acute cellular and antibody-mediated rejection contribute significantly to early graft failure, with acute cellular rejection (ACR) occurring in 30-50% of recipients in the first year after transplantation (8, 9). Numerous studies have supported a role for ACR in subsequent development of CLAD, with increased risk associated with greater frequency and histologic severity of ACR (10–15). Antibody-mediated rejection (AMR) has emerged as one of the most vexing challenges in lung transplantation and is characterized by allograft dysfunction, the presence of circulating donor-specific antibodies (DSA), capillary endothelial C4d deposition and pathological findings characteristic of acute lung injury (16). This condition often leads to acute graft failure and can also predispose to the development of CLAD (17).

Chronic lung allograft dysfunction

CLAD occurs in up to half of recipients within five years of transplantation and represents the principal life-limiting factor for lung transplant patients (18). The development of CLAD is likely multifactorial and related to the complex interaction of immune and non-immune factors, including but not limited to acute rejection, pre-transplant allosensitization, bacterial infection and colonization, acute viral infection, and gastroesophageal reflux disease; in a subset of patients, no clear risk factors for CLAD are identified and it is presumed to result from chronic rejection (19–22). CLAD is marked by fibrotic remodeling within the pulmonary allograft, with described phenotypes include bronchiolitis obliterans syndrome (BOS), restrictive allograft syndrome (RAS), and a

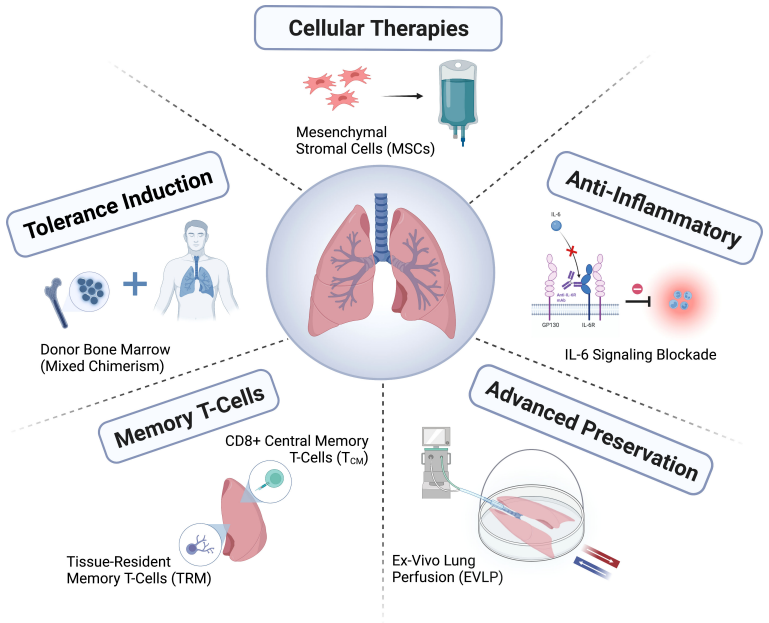

FIGURE 1
Novel approaches to achieve long-term lung transplant survival.

mixed BOS-RAS phenotype (23). Histologic changes of BOS include dense submucosal fibrosis in membranous and respiratory bronchioles resulting in luminal occlusion and vasculopathy, whereas RAS is characterized by fibrosis of the alveolar interstitium, visceral pleura, and interlobular septa (14, 24).

Unique immunobiology of lung allografts

Lung allografts possess a number of distinctive features that contribute to poor outcomes including 1) a large total surface area of vascular endothelium, 2) constant exposure to environmental antigens, and 3) an abundance of lymphoid tissue patrolled by a robust innate immune system. The expansive vascular endothelium of lung allografts results in increased susceptibility to ischemia reperfusion and complement-mediated injury, leading to an influx of neutrophils/macrophages into the graft with early T-cell activation and consequent rejection (25, 26). Due its role as a barrier organ, the lung is continually exposed to environmental antigens resulting in stimulation of toll-like receptors (TLRs) which activate proinflammatory innate and adaptive immune responses, thereby promoting rejection (27). Moreover, there exists abundant intragraft lymphoid tissue containing resident monocytes that can interact with and prime T-cells within the lung itself (28). Other important properties of lung allografts include the existence of immunomodulatory tissue-resident memory T-cells (TRMs) and CD8+ memory T-cells shown to promote allograft tolerance (29–33).

Similar to lung allografts, the skin harbors tissue-resident immune cells (including T-effector memory cells) and is continually exposed to the environment. As with the lung, the presence of local T-effector memory cells enables a more potent response to alloantigen than would occur following activation of naïve T cells. When compared to solid organ transplantation, recipients of vascularized composite allografts experience a much higher incidence of acute rejection (85% during the first year after transplantation) (34).

It is increasingly recognized that immune pathways driving lung allograft rejection and tolerance differ from those of other solid organs, likely due to the unique immunobiology of lung allografts (3, 28–31). One primary difference is regulation of alloimmune responses at the level of the lung allograft, which is in contrast with other transplanted organs that depend on cell trafficking to secondary lymphoid organs for activation of allorecognition pathways (28–30, 35–37). Early graft injury resulting from IRI and infection has been shown to activate innate immune pathways within the lung allograft, which in turn trigger alloantigen-specific T-cell expansion (19, 25, 38). During IRI, resident donor monocytes in the lung elaborate chemotactic and proinflammatory cytokines that facilitate neutrophil entry into lungs grafts, enhancing $CD4^+$ T-cell responses to donor antigens and resulting in PGD (39–41). Additionally, lung monocytes can interact with Th17 cells and contribute to the development of CLAD (42).

The design of conventional immunosuppression has focused on controlling T-cell mediated responses. However, it is increasingly clear that inflammation, along with humoral and innate immunity, also play critical roles in lung allograft survival. As such, it is not surprising that current immunosuppressive regimens are unsuccessful in achieving acceptable long-term graft and patient survival. Moreover, conventional immunosuppression fails to account for the unique properties of lung allografts and as a result may negatively impact tolerogenic cell populations while allowing for the propagation of immune pathways leading to rejection. Taken together, these findings highlight the need for novel, lung-specific therapies that promote long-term allograft and patient survival.

Novel preservation methods

Ischemia reperfusion injury is a critical mediator of PGD, which affects up to 25% of lung transplant recipients and is associated with the development of CLAD and late mortality (4, 43–45). Novel perseveration strategies aimed at reducing the incidence of IRI and subsequent PGD are critical to promoting long-term graft and patient survival. Moreover, the shortage of acceptable lung allografts calls for innovative methods that enable expansion of the donor organ pool.

Ex-vivo lung perfusion

Conventional cold static preservation is performed by flushing donor lungs with a specialized preservation solution followed by hypothermic storage on ice (~4°C). The goal of hypothermic storage is to sustain cellular viability by reducing cellular metabolism, with maximum accepted preservation times limited to <8 hours with cold static preservation (46). During this process, a lack of arterial blood supply results in anaerobic metabolism, failure of ion-exchange channels, cell swelling, and impaired enzymatic activity (47). Upon donor lung reperfusion, oxidative stress from mitochondrial damage and electrolyte imbalance promotes local inflammation and results in release of reactive oxygen species, pro-inflammatory cytokines, proteases, and expression of damage-associated molecular patterns (DAMPs) (48–52). Consequent activation of the innate immune system further contributes to the inflammatory cascade and promotes harmful adaptive immune responses driven by alloreactive T-cells (53, 54). The culmination of these events results in the tissue damage that characterizes IRI.

EVLP involves machine preservation of donor lungs in a perfused, ventilated, normothermic condition, thereby reducing tissue injury resulting from anaerobic metabolism and

hypothermia (55). EVLP represents a novel method for the assessment and treatment of donor lungs, with the ultimate goal of expanding the donor organ pool and improving long-term outcomes. Since first used clinically in 1991 (56), EVLP has undergone system modifications primarily relating to technical parameters of the platform (static *vs*. portable) and composition of the perfusate (cellular *vs*. acellular). Other proposed modifications involve donor lung positioning (supine *vs*. prone) (57), mode of ventilation (positive *vs*. negative-pressure) (58), and perfusate temperature (normothermic *vs*. subnormothermic) (59, 60). Currently, three EVLP platforms are approved for clinical use and include the Organ Care System Lung (OCS, Transmedics, Andover, MA), Vivoline LS1 (Vivoline Medical, Lund, Sweden), and the XPS XVIVO Perfusion AB system (XVIVO Perfusion, Gothenburg, Sweden). The major difference between these platforms is that they are either mobile, enabling donor lungs to be placed on EVLP immediately after procurement, or fixed, requiring transport of donor lungs to a specialized perfusion center.

EVLP offers several advantages over cold static preservation, including 1) the opportunity for evaluation of marginal donor lungs prior to transplantation, 2) safe extension of preservation time, and 3) the potential to improve both marginal and standard grafts through targeted administration of therapeutics (61). EVLP provides insight into the function of a marginal donor organ by enabling assessment of physiological parameters including pulmonary vascular pressures, perfusate oxygen content (PaO2), lung edema, and PaO2:FiO2 ratios, as well as novel parameters such as interstitial fluid metabolite composition (62). Extended duration of donor lung preservation through use of EVLP offers the advantage of greater flexibility in timing of graft implantation, reduction in the physical limitations related to organ allocation, and enables performance of advanced diagnostics and therapeutics that require longer periods of perfusion (63). EVLP using the Toronto Protocol has been shown to safely maintain lungs for >12 hours (46), with extended preservation to 24 hours using perfusate modifications that reduce metabolite accumulation and electrolyte imbalances known to occur during prolonged EVLP (64).

Perhaps the most significant advantage of EVLP relates to its use as a therapeutic platform for administration of localized, targeted lung-specific therapies (61, 65, 66). Even without modifications, EVLP is associated with alterations in the donor lung environment from diminished release of inflammatory mediators and augmentation of anti-inflammatory signaling pathways (67–70). EVLP has been shown to alter the inflammatory signaling profile of the donor lung, with a global profile of cellular survival and anti-apoptotic signature (67). Experimental studies have investigated administration of agents aimed at augmenting the anti-inflammatory properties of EVLP, many of which have shown promise in preventing inflammatory cytokine release and reactive oxygen species (ROS) generation during IRI. Such agents include alpha1-anti-trypsin (71), adenosine A1A-receptor agonists (72, 73) and A2B-receptor antagonists (74), K (ATP) channel modulators (75), ROS scavengers and PARP inhibitors (76–79). By removing donor leukocytes prior to transplantation, EVLP alters immunogenicity of the graft resulting in reduced allorecognition, T-cell priming, and T-cell infiltration in the recipient (68). EVLP has been used for targeted delivery of immunosuppressive agents including perfusate-based methylprednisolone (80), intrabronchial adenoviral human IL-10 (81), and drugs targeting leukocyte activation and function (82, 83). Further applications of EVLP involve modification of lung properties (84), anti-microbial treatment (85), and administration of cellular-based therapies (86–91).

Clinical trials have evaluated the impact of EVLP on outcomes in standard and extended criteria donors, as well as conversion rates for grafts initially deemed unsuitable for transplant. To date, EVLP has failed to demonstrate superior survival in clinical studies of standard criteria donor lungs (92, 93). The randomized INSPIRE trial evaluated EVLP outcomes with the OCS platform compared to traditional cold storage in standard criteria lungs, demonstrating a 50% reduction in rate of grade 3 PGD (PGD3) with EVLP but no statistically significant difference in short- and long-term survival (92). Similarly, a randomized trial using the XVIVO platform for standard criteria donors demonstrated no significant difference in 30-day survival compared to standard donor lung preservation, and similar short-term clinical outcomes between groups (duration of intubation, length of intensive care unit (ICU) and hospital stay) (93).

Remarkably, trials using EVLP for extended criteria donors have shown equivalent survival compared to standard criteria donors without EVLP (94, 95). The EXPAND trial applied the OCS system in extended criteria donors (donation after circulatory death (DCD), >age 55, PaO2:FiO2 ≤300, expected ischemic time >6 hours), with a 99% 30-day survival but a 44% rate of PGD3 at 72 hours (94). Additional studies of extended criteria donors using EVLP have shown similar clinical outcomes compared to standard criteria donors regarding rates of PGD3, duration of ICU and hospital stay, and 30-day or 1-year survival (95). Other prospective multicenter studies using the Vivoline perfusion system for extended criteria lungs initially declined for transplantation showed similar 1-year survival despite inferior short-term parameters (higher rate of extracorporeal membrane oxygenation (ECMO) support, duration of ICU stay/time to extubation) when compared to conventional donor lungs (96, 97). Importantly, in the prospective, multi-center NOVEL trial, lungs initially deemed unacceptable for transplant were screened using EVLP, resulting in a 50.9% conversion rate and equivalent short- and long-term outcomes compared to standard criteria lungs (98).

In sum, EVLP has been shown to reduce tissue inflammation and downregulate harmful immune responses, thereby

improving the function of donor lungs. Current evidence from clinical trials supports the use of EVLP in extended criteria donors and as a mechanism to screen for viable grafts among the unused donor pool, enabling equivalent short- and long-term outcomes compared to standard criteria donor lungs. To date, there is a lack of demonstrated superiority using EVLP in clinical trials of standard criteria lungs. However, there exists great potential for further modifications to EVLP to enhance outcomes of both standard and marginal/extended criteria lungs with the overall effect of improved graft and patient survival.

Xenogenic cross-circulation

Cross-circulation represents an advanced method of *ex vivo* lung preservation which, unlike machine perfusion, provides full physiologic support to donor lungs. In a porcine lung model, allogeneic cross-circulation with a host swine has been shown to successfully regenerate severely injured lungs and support lungs *ex vivo* for up to four days (99–101). Based on these results, the concept of xenogeneic cross-circulation of injured human lungs with living swine has been proposed with successful functional and histologic recovery of severely injured human lungs after 24 hours of xenogeneic cross-circulation (102). Cross-circulation with xenogeneic support may result in superior outcomes compared to isolated machine perfusion by providing systemic physiologic regulation in the *ex vivo* setting (103). However, the clinical applicability of xenogeneic cross-circulation as an approach for lung preservation and rehabilitation is limited by immunologic and ethical barriers as well as feasibility.

Modifications to cold static preservation

Despite the advantages of *ex vivo* perfusion, considerable limitations exist including complexity and cost; as such, cold static storage remains the clinical standard of donor lung preservation. There have been few major changes in the technique of cold static preservation in the past decades, but several modifications have been proposed. One such modification involves use of a temperature-controlled preservation device, the Paragonix LUNGguard™ Donor Lung Preservation System (Paragonix, Braintree, Mass). This device enables continual monitoring of storage temperature and maintenance in the range of 4-8°C for prevention of freeze injury or inadvertent warming during donor lung transport and storage. Use of increased storage temperature of 10°C rather than 4°C during static preservation has also been proposed, with a recent study demonstrating improved mitochondrial health after prolonged preservation at 10°C compared to the standard 4°C (104). While the optimal lung preservation solution has yet to be established, Perfadex (XVIVO Perfusion AB, Gothenburg, Sweden) is a commonly used, low-potassium dextran solution. Recently, the use of a hydrogen-rich preservation solution comprised of dissolved hydrogen in Perfadex has been proposed as a mechanism to mitigate lung IRI by reducing levels of inflammatory cytokines, oxidative stress markers, and vascular endothelial dysfunction (105, 106).

Cellular therapies

The use of cellular-based therapies in solid organ transplantation continues to evolve, with emerging roles for regulatory T-cells, B-cells, macrophages, dendritic cells, and genetically-modified CAR T-cells in prevention/treatment of IRI and rejection. The majority of such therapies have been explored in kidney and liver transplantation, however, mesenchymal stromal cells (MSCs), as well as epithelial progenitor cells (EPCs) and regulatory T-cells (Tregs) have shown promise in lung transplantation.

Mesenchymal stromal cells

Mesenchymal stromal cells (MSCs, also seen as mesenchymal stem cells) are multipotent cells with a fibroblast-like morphology that were first isolated from bone marrow and spleen in 1970 (107). MSCs are a heterogenous population, but according to the International Society for Cellular Therapy Standards they must: 1) adhere to plastic, 2) be CD105+CD90+CD73+ and CD45-CD34-CD14-CD11b-CD79a-CD19-HLA-DR-, and 3) have the capacity to differentiate into the mesenchymal lineages (either bone, cartilage, or fat) (108). MSCs have been predominantly isolated from bone marrow, but umbilical cord MSCs and adipose-derived MSCs are also widely used.

Since their discovery, MSCs have also been found as resident cells in many organs throughout the body, including the lungs (109). In bone marrow, MSCs produce a host of trophic and regulatory factors to create a niche to support stem cell hematopoiesis (110), and it is thought that lung resident MSCs serve to similarly support bronchoalveolar stem cell populations and help direct the maintenance and repair of lung tissues (111).

MSCs also have several unique features that make them particularly attractive candidates for cell therapy in lung transplantation. First, they are either very long-lived or capable of self-renewal; in gender-mismatched lung transplantation, MSCs have been shown to retain a donor's gender even years post-transplant (112). Second, they express little to no co-stimulation or MHC markers and are poorly immunogenic, to the extent they have been successfully transplanted across not only HLA barriers but also across

species (113). This has led many groups to transition away from bespoke cultured MSCs and toward more shelf-stable allogeneic cell lines, often derived from neonatal stem cells, that do not require *de novo* MSC isolation and GMP culture. Third, MSCs have been found to be possess a potent immunomodulatory capacity, which may differ based on their source (114). *In vitro* studies show that they inhibit effector T-cell activation (115) and may promote regulatory T-cells over pro-inflammatory Th17 cells (114, 116). In the presence of inflammation as occurs in the setting of transplantation, MSCs may be primed to possess an even greater capacity for immunosuppression *via* TNF-TNFR2 dependent signaling (114, 117). MSCs secrete a variety of cytokines, chemokines, and inflammatory factors that regulate the immune system and have anti-oxidative and anti-apoptotic functions (118–122) (reviewed in (123, 124)). Fourth, because of their large size after *in vitro* culture, MSCs may become trapped in the lungs following intravenous infusion (125). The effect of MSCs can be seen throughout the body due to paracrine and secreted factors, but MSCs can also migrate to sites of tissue injury, and it is possible that lungs derive additional benefit from the presence of MSCs locally in the graft. Finally, MSCs have already been tested in human studies for a wide variety of indications including acute respiratory distress syndrome (ARDS), with doses ranging up to 100 million cells/kg without evidence of significant adverse effects (126–129).

MSC-derived extracellular vesicles

Of note, some of the effects of MSCs do not require cell-cell contact *in vitro*, and MSCs have been found to exert effects on distant organs *in vivo*. It is thought that MSCs release not only paracrine signaling molecules, but also extracellular vesicles (EVs). EVs are small, membrane-bound, non-nucleated particles actively assembled and released by cells including MSCs. EVs can contain proteins, mRNA, and miRNA which can be transmitted to another cell. As the field evolves, guidelines have been proposed by the International Society for Extracellular Vesicles to more formally categorize these EVs by origin (whether plasma membrane or endosome), size, density, etc. (130). EVs are also being investigated as a means of inducing the beneficial effects of MSCs without the risk of alloreactivity to cells or the entrapment of cells in the lungs.

Preclinical studies of MSCs in lung IRI

MSCs, MSC-derived EVs, and MSC-derived conditioned media (which contain EVs) have been evaluated in dozens of studies of lung IRI. Most studies used rodent models with IRI induced by hyperoxia, ventilator damage, E. coli bacteria/LPS, or chemicals such as bleomycin [reviewed in (123)]. Consistently, treatment with MSCs has been found to result in increased survival and decreased lung injury and edema.

Nearly all studies found that MSC-treated subjects had more favorable, anti-inflammatory cytokine and growth factor profiles. Neutrophil infiltration was decreased with MSC treatment (87, 131–135) while there was an increase in M2-like macrophages and regulatory T-cells (121, 136). The p38 MAPK is inhibited and Bcl-2 is translocated to the nucleus, preventing apoptosis (137). In other organ models, MSCs have been found to transfer mitochondria and glycolytic enzymes, ameliorating mitochondrial dysfunction that can lead to excessive ROS generation and metabolic dysfunction (138). After treatment with MSCs, autophagy was markedly decreased (139). In rodents with elastase-induced emphysema and bleomycin-induced fibrosis, both emphysema and fibrosis were decreased as well (136, 140).

MSC delivery using EVLP

Most preclinical studies of MSC treatment for lung IRI use intravenous or intraperitoneal infusion to deliver the cells, however, studies in lung transplantation have the unique opportunity to use EVLP for cell delivery. Combining MSC treatment with EVLP may enable the targeting of IRI before it occurs and reconditioning of lungs without the theoretical risks of microvascular embolism from high dose intravenous infusion of MSCs [reviewed in (141) and (142)].

In one large animal transplant study, swine lungs were perfused with human umbilical cord MSCs after 24 hours of static cold storage and an additional 12 hours of EVLP. Once transplanted and reperfused, lungs treated with MSCs had significantly reduced acute lung injury scores, improved wet-to-dry ratios, lower levels of inflammatory cytokines, and higher levels of growth factors than the control group, suggesting an amelioration of IRI associated with the transplantation process (86). Previous studies from the same group confirmed that intravascular perfusion led to better retention of cells in the parenchyma than intrabronchial delivery of MSCs, and that larger numbers of cells perfused offered little benefit over the optimum dose of 5 million cells/kg (143, 144).

MSCs may also reduce the risk of pulmonary edema and PGD by increasing alveolar fluid clearance (AFC) rates, which can be negatively impacted by IRI-associated damage to the alveolar epithelium. A handful of studies have been published evaluating human lungs treated with MSCs or multipotent adult progenitor cells (MAPCs) *ex vivo*. After 4-12 hours of EVLP, IL-8, IL-10, TNFa, pulmonary vascular resistance (PVR), and oxygenation were not demonstrably changed in most studies. However, one study found that histologic lung injury scores were decreased, and studies that evaluated AFC consistently found that AFC was improved or restored to normal levels. Two additional studies have also evaluated microvesicles derived

from bone marrow MSCs and similarly found that AFC rates were improved (Table 1).

MSC-based cellular therapy in human lung transplantation

Based on the results from early studies demonstrating the safety and feasibility of MSC administration in human lung transplant recipients (128, 129), there are currently three clinical trials of allogeneic MSC infusion for lung transplantation on clinicaltrials.gov (Table 2). Two of the trials aim to evaluate the effect of MSCs on lung transplant recipients with BOS or CLAD, while one aims to evaluate the effects of MSCs on PGD in all lung transplant recipients.

Unresolved questions remain about the subtle differences between MSCs derived from different sources (i.e., bone marrow *vs*. umbilical cord blood), their performance and viability suspended in acellular perfusate compared to blood-based perfusate (as is used in normothermic machine perfusion) or culture media, and the possible toxic effects of dimethyl sulfoxide used as a cryoprotectant for frozen MSCs. Additionally, MSCs are a heterogeneous population and autologous cells can vary significantly between patients. The development of standardized allogeneic cell preparations can alleviate some of these concerns, as can the use of EVs instead of cells.

In sum, there exists mounting evidence demonstrating the beneficial effects of MSCs in treating lung injury in animal models and on *ex vivo* perfused human lungs. As a result, there exists great potential for use of MSC-based therapies to help expand the donor pool by reconditioning marginal lungs and by limiting or treating lung injury after transplantation.

Endothelial progenitor cells

Endothelial injury represents a critical component in the pathogenesis of lung IRI, wherein release of proinflammatory cytokines from hypoxemia and reoxygenation results in compromised endothelial integrity and increased alveoli-capillary permeability. During reperfusion, overproduction of ROS and activation of cell adhesion molecules causes endothelial swelling and detachment from the basement membrane, resulting in increased vascular permeability that enables leukocyte entry into the tissues (151–153). Release of proinflammatory mediators by activated macrophages causes lung injury characterized by damage to pulmonary and alveolar endothelial cells (154–156). Strategies aimed at protecting the lung endothelium include inhibition of ROS, targeting of endothelial adhesion molecules, and endothelial glycolax stabilization (153).

More recently, the use of endothelial progenitor cells (EPCs) has been explored as a mechanism by which to attenuate lung injury and promote vascular regeneration. EPCs possess anti-inflammatory properties and a capacity for re-endothelialization; in models of acute lung injury, EPCs have been shown to preserve

TABLE 1 Studies of mesenchymal stromal cells (MSCs), multipotent adult progenitor cell (MAPCs), and MSC-derived extracellular vesicles (EVs) in human lung *ex vivo* lung perfusion (EVLP).

Lead Author	Year	Cells	Dose	Lung injury model	EVLP time	Outcome
Lee (143)	2009	BM MSC from NIH repository, Tulane Center for Gene therapy	5 million	<30h ischemic time plus E. coli bacteria/endotoxin	4h	AFC restored, IL-8, IL-10, TNFa unchanged
Lee (145)	2013	GMP BM MSC from University of Minnesota	5 million	<48h ischemic time plus endotoxin or E. coli bacteria	6-10h	AFC restored, IL-8 and TNFa decreased, IL-10 increased *in vitro*
McAuley (146)	2014	GMP BM MSC from University of Minnesota	5 million	>30h cold ischemia	4h	No change
La Francesca (147)	2014	MAPC	10 million	8h cold ischemia	4h	Reduced lung injury score, reduced neutrophils and eosinophils
Gennai (148)	2015	BM MSC-derived EV	MV from 10-20 million MSCs	Lungs rejected for transplant (<48h cold ischemia, no parenchymal lesions, and AFC >0% but <10%/h)	8h	Improved AFC, restored tracheal pressure, increased compliance relative to baseline, reduced PAP or PVR. No significant difference in oxygenation
Park (149)	2019	BM MSC-derived MV	MV from 20-40 million MSCs	E. coli bacteria	6h	Improved AFC, no change in PAP, PVR, compliance, or oxygenation
Nykanen (150)	2021	UC MSC modified to produce IL-10	40 million	<10 hours cold ischemia	12h	No difference in PVR, oxygenation, compliance, airway pressure

BM, Bone marrow; MSC, mesenchymal stromal cell; GMP, Good manufacturing process; AFC, Alveolar fluid clearance; MAPC, Multipotent adult progenitor cell; EV, Extracellular vesicle; MV, Microvesicle; PAP, Pulmonary artery pressure; PVR, Pulmonary vascular resistance; UC, Umbilical cord.

TABLE 2 Currently registered clinical trials of mesenchymal stromal cells (MSCs) in lung transplant recipients.

Lead institution	Type	Phase	Patients	MSC source	Intervention	Primary outcome	NCT	Status
Rigshospitalet, Denmark	Double blind	1/2	All lung transplant	Allogeneic adipose	100 million vs 200 million vs placebo	PGD	NCT04714801	Recruiting
Mayo clinic, MN	Non-randomized	1	BOS+	Allogeneic bone marrow	0.5 million vs 1 million +/- 1 million booster	Safety, changes in PFTs	NCT02181712	Completed 2021
University of Queensland, Australia	Randomized	2	CLAD+	Allogeneic bone marrow	8 million/kg vs placebo	Progression-free survival from CLAD	NCT02709343	Recruiting

PGD, Primary graft dysfunction; BOS, Bronchiolitis obliterans syndrome; PFT, Pulmonary function test; CLAD, Chronic lung allograft dysfunction.

pulmonary endothelial function and prevent increased permeability of the pulmonary alveolar-capillary barrier (157, 158). The potential for EPCs in attenuating lung IRI has been demonstrated in animal models of lung transplantation, in which administration of autologous EPCs improved lung allograft survival and function in the setting of prolonged ischemia (159). Additional findings support the ability for EPCs to ameliorate lung IRI through downregulation of inflammatory and endothelial adhesion molecule expression *via* the endothelial NOS (eNOS) pathway (160). Further research will help to elucidate the role for administration of EPCs as a therapeutic strategy to mitigate lung IRI.

Regulatory T-cells

Tregs are known to mediate alloimmunity are known to mediate alloimmunity in solid organ transplantation and play a distinct role in lung transplantation due to local immunoregulation within the allograft. The formation of bronchus-associated lymphoid tissue (BALT), a tertiary lymphoid organ enriched in T_{regs}, is associated with the development of lung transplant tolerance (30). T_{regs} not only participate in local mechanisms of tolerance induction within the lung allograft, but can also regulate peripheral immune responses *via* lymphatic egress (161). Further support for the tolerogenic role of graft resident T_{regs} is evidenced by development of AMR after selective depletion of intragraft T_{regs} from tolerant lung allografts (162). In human lung transplant recipients, reduced percentage of T_{regs} in BAL fluid correlates with rejection (163), and increased levels of circulating T_{regs} are associated with improved graft survival (164, 165).

Only recently have T_{regs} been investigated as a cellular therapy in lung transplantation. Pre-transplant administration of *in-vitro* expanded recipient T_{regs} using EVLP was performed in a porcine model of lung transplantation and discarded human lungs (166); T_{regs} were shown to enter the lung parenchyma in both models and retain suppressive function, and thereby represent a promising strategy for local immune regulation prior to transplantation. However, the administered T_{regs} only remained in the graft for 3 days after transplantation with no significant difference in acute rejection between the control and T_{regs} -treated groups by day 7, suggesting that lung allograft residency may be required for T_{regs} to exert their tolerogenic effects.

Anti-inflammatory strategies

IL-6 signaling blockade

IL-6 is a pleiotropic, pro-inflammatory cytokine that has been linked to lung allograft inflammation and immune -mediated graft injury, with elevated IL-6 levels shown to be predictive of PGD and 30-day mortality (167–169). Evidence from multiple model systems mechanistically link IL-6 to the inflammatory cascades inherent to IRI and downstream dendritic cell and T-cell activation/infiltration that result in graft rejection (28, 54, 170, 171). Moreover, studies of PGD in lung transplantation support a role for IRI-induced proinflammatory gene expression including IL-6 (167, 169). Monocytes/macrophages including alveolar macrophages express the IL-6 receptor (IL-6R) and generate pro-inflammatory responses comprised of inflammasome activation, production of toxic reactive oxygen and nitrogen species, and release of cytokines including IL-6, culminating in acute and chronic allograft immunopathology (172, 173). Thus, initiation of agents aimed at IL-6 signaling inhibition prior to reperfusion in lung transplantation offers the potential to limit innate inflammation downstream of IRI and thereby reduce the development of PGD.

The potential benefits of IL-6 signaling inhibitors in lung transplantation surpass their anti-inflammatory effects, given the role of IL-6 in the adaptive immune responses responsible for development of acute and chronic lung transplant rejection (174–178). Agents currently approved for clinical use or in development include primarily those that target IL-6 (clazakizumab, siltuximab) or the IL-6 receptor (tocilizumab, sarilumab) (179, 180). Our group previously achieved long-term lung allograft survival in non-human primates (NHPs) by supplementing conventional triple drug immunosuppression with anti-thymocyte globulin induction therapy and a short post-operative course of anti-IL6R therapy with tocilizumab (181). Recent clinical trials in kidney transplantation have focused on the

use of IL-6 signaling blockade for desensitization and for the treatment of acute and chronic AMR, with promising initial results (182–187). In addition, a multicenter phase II clinical trial investigating the efficacy of tocilizumab in cardiac transplantation has been initiated (NCT03644667), with clinical trials in lung transplantation forthcoming.

JAK inhibitors

The JAK/STAT pathway is essential for cytokine signaling involved in upregulation of acute inflammatory pathways, including those associated with lung allograft rejection. JAK-dependent cytokines including IFN gamma and IL-5 have been shown to be upregulated in CLAD (188), and elevated levels of CXCR3 chemokines downstream from JAK-dependent IFN gamma signaling have been associated with worse outcomes in lung transplant recipients (189). Use of systemic JAK inhibitors for prevention of allograft rejection has shown efficacy in kidney transplantation but was associated with increased rates of infection and posttransplant lymphoproliferative disease (190).

Recently, lung-specific, inhaled (non-systemic) JAK inhibitors have been developed and offer potential for use in the prevention of acute and chronic lung allograft rejection. The use of inhaled, lung-specific JAK inhibitors provides local drug delivery with minimal systemic effects and has shown efficacy in corticosteroid-resistant pulmonary inflammation (191). The inhaled pan-JAK inhibitor nezulcitinib (TD-0903, Theravance Biopharma) was used for treatment of COVID-19 associated lung injury in a Phase II clinical trial (NCT04402866) but failed to meet its primary endpoint; however, the drug was well-tolerated and showed a trend towards decreased mortality and duration of hospitalization (192).

Azithromycin

Azithromycin is a macrolide antibiotic with diverse antibacterial, antiviral, and anti-inflammatory effects, and has been shown to attenuate airway and systemic inflammation after lung transplantation (193–195). A randomized-controlled trial demonstrated that use of prophylactic azithromycin combined with conventional immunosuppression improved post-transplant outcomes including lung allograft function and risk of CLAD; its efficacy was attributed to reduced airway and systemic inflammation based on lower C-reactive protein levels in patients receiving azithromycin (194). Although it has been shown to decrease the rate of CLAD and improve long-term survival after lung transplantation (196–198), post-transplant azithromycin did not result in improved early allograft function in a randomized-controlled trial (199). However, this study confirmed the known anti-inflammatory properties of azithromycin with lower bronchoalveolar lavage (BAL) neutrophilia and IL-8 levels at 30 and 90 days post-transplant (199).

Perfusion-based strategies

The process of EVLP using normothermic machine perfusion with an acellular perfusate has been shown in experimental studies to mitigate lung allograft inflammation by downregulating the release of pro-inflammatory mediators and promoting anti-inflammatory pathways. Specifically, use of EVLP is associated with diminished release of DAMPs including mitochondrial DNA (mt-DNA) (67, 200) and decreased levels of pro-inflammatory cytokines such as IL-6, IL-1 beta, IL-18, and TNF-alpha (69, 70, 201). Correspondingly, EVLP induces the expression of genes encoding for anti-inflammatory pathways, including feedback inhibitors of TLRs and regulatory cytokines (i.e., IL-10) (69, 200).

Beyond its intrinsic anti-inflammatory effects, EVLP enables targeted delivery of anti-inflammatory agents as additives to the perfusate. In an experimental model, reconditioning of lung allografts with Cyclosporine A during EVLP attenuated proinflammatory changes and mitigated mitochondrial dysfunction, resulting in improved early graft function after transplantation (202). Administration of anti-inflammatory adenosine A2A-receptor agonists and pro-inflammatory A2B-receptor antagonists was also shown to improve lung quality and was associated with diminished expression of pro-inflammatory cytokines including CXCL1, CCL2, TNF-alpha, IFN gamma, and IL-12 (72–74, 203). Other perfusion-based therapeutics aimed at diminishing cell death and inflammation include ROS scavengers/PARP inhibitors and alpha1-anti-trypsin (71, 76, 79). Finally, implementation of a cytokine adsorber to the EVLP system for cytokine removal has shown promise in limiting the inflammatory response in experimental models (204).

Tissue-resident and central memory T-cells

Tissue-resident memory T-cells

Tissue-resident memory T-cells (TRMs) represent a unique population of non-circulating T-cells with memory features that serve a critical role in the defense against infectious pathogens at barrier surfaces (205, 206). TRMs are defined by their commitment to non-lymphoid peripheral tissues and lack of recirculation and are distinct from circulating central memory and effector memory T-cells (207–210). As a key immunological barrier organ, the lung is highly enriched for TRMs that have the

potential to mediate rapid, *in-situ* immune responses (211, 212). TRMs therefore serve an important role in localized immunity with significant implications regarding allogeneic responses within lung allografts.

Donor-derived T-cells have been identified in lung allografts as TRMs for >1 year after transplantation, and their long-term persistence is associated with reduced incidence of PGD and ACR (32). In contrast, lung TRMs derived from infiltrating recipient T-cells gradually acquire TRM markers in the months after transplant and may mediate allograft rejection (32, 213, 214). Longitudinal analysis of lung transplant recipient BAL has demonstrated lower levels of donor CD4+ and CD8+ T-cell chimerism in recipients who developed PGD compared to those who did not (32). Similarly, BAL samples from recipients with ACR demonstrated lower levels of donor T-cell chimerism, indicating that infiltrating recipient T-cells may mediate development of ACR. Taken together, these findings suggest that future therapies aimed at maintaining donor-derived TRMs and preventing their replacement by recipient TRMs may improve post-transplant outcomes.

More recently, the existence of recirculating TRMs has been identified, implying that TRMs may be capable of participating in systemic recall responses (215–217). Furthermore, the observation that graft-infiltrating recipient T-cells gradually acquire TRM markers suggests that these cells may actually represent repopulation of donor lung tissue by circulating effector memory T-cells that then acquire TRM phenotypes or by recirculating ex-TRMs (218). The ability of TRM cells to influence the immune environment within lung allografts through rapid *in-situ* and potentially also systemic immune responses represents yet another avenue for targeted interventions to prevent allograft injury and rejection.

CD8+ central memory T-cells

Memory T-cells are generally viewed as pathogenic in the context of solid organ transplantation, with early infiltration of CD8+ memory T-cells into allografts shown to result in accelerated rejection (219–223). CD8+ alloreactive memory T-cells generated through heterologous immunity are considered a barrier to long-term graft survival due to a relative resistance to traditional immunosuppression. As a result, lymphoablative strategies to globally deplete T lymphocytes or specifically eliminate memory T-cells have been developed.

However, there exists emerging evidence that under certain circumstances memory T-cells maintain a regulatory capacity and suppress deleterious pro-inflammatory immune responses (224). Recent findings in lung transplantation support a critical role of $CD8^+$ central memory T-cells (T_{CM}) in the induction of transplant tolerance (29, 31). In a murine model of lung transplantation, it was shown that CCR7-expressing CD8+ T_{CM} cells promote costimulatory blockade-mediated lung allograft acceptance *via* IFN gamma-dependent nitric oxide production. The induction of tolerance by CD8+ T_{CM} cells was dependent on CCR7 expression, which enabled prolonged and stable interaction with intragraft antigen-presenting cells resulting in IFN gamma production, induction of nitric oxide, and downregulation of immune responses (29). The tolerogenic effect of CD8+ T_{CM} cells was shown to be dependent on expression of programmed cell death 1 (PD-1) by the CD8+ T_{CM} cells; in the absence of PD-1, these cells instead differentiated into an effector memory phenotype with subsequent allograft rejection (31). These findings suggest that indiscriminate T-cell depletion or targeting of pathways critical to the function of tolerogenic CD8+ T_{CM} cells may interfere with lung allograft acceptance and promote rejection. As such, a critical reevaluation of current immunosuppressive strategies is warranted to ensure optimization of protective cell populations in lung transplantation.

Tolerance induction

Induction of immune tolerance remains the ultimate goal in the field of transplantation, as it would enable indefinite graft survival in the absence of ongoing immunosuppression. In contrast to kidney and liver allografts, lung allografts are considered along with other "tolerance-resistant" organs due to their unique immune characteristics (225). While tolerance to kidney allografts has been achieved in human patients through mixed chimerism with donor bone marrow transplantation (226), such an approach has yet to reach the clinical realm for lung transplant recipients. Our group was the first to demonstrate the successful induction of lung allograft tolerance in a NHP model using a mixed chimerism strategy with anti-IL6R therapy (181). Four cynomolgus NHPs underwent MHC-mismatched lung transplantation followed by 4-month delayed donor bone marrow transplantation using a non-myeloablative mixed chimerism conditioning regimen (including anti-IL6R therapy). Three of the four NHP recipients achieved tolerance with long-term lung allograft survival off immunosuppression (Table 3); the tolerant NHPs had no evidence of acute or chronic allograft rejection, exhibited donor T-cell unresponsiveness, and did not develop donor-specific alloantibody (Figure 2). These findings represent significant progress towards clinical lung transplant tolerance, with tolerance induction representing yet another promising approach for improved outcomes following lung transplantation.

Moreover, long-term graft survival without the need for immunosuppression in the setting of tolerance induction would obviate the need for patient adherence to immunosuppressive regimens. Patient adherence remains a major barrier in achieving long-term survival following solid

TABLE 3 Outcomes of four NHP lung transplant recipients that underwent delayed donor bone marrow transplantation for induction of lung allograft tolerance. *Adapted from* (181).

NHP Recipient	MHC Mismatch		Mixed Chimerism	ACR at last biopsy	Chronic rejection	Allo-antibody	Graft survival
	MHC I	MHC II					
M4012	2/4	2/4	Permanent	ACR 0 Day 610 Post-LTx	None	None	299 days post-BMT (euthanized with no signs of rejection)
	Haploidentical						
M2411	2/4	2/4	Permanent	ACR 0 Day 939 Post-LTx	None	None	813 days post-BMT (euthanized with no signs of rejection)
	Haploidentical						
M912	2/4	2/4	Transient until Day 75 post-BMT	ACR 0 Post-mortem	None	None	464 days post-BMT (euthanized with no signs of rejection)
M4711	4/4	4/4	None	ACR 3 Post-mortem	Severe OB	Developed post-lung tx	176 days post-BMT

ACR, Acute cellular rejection; BMT, Bone marrow transplantation; LTx, lung transplant; OB, Obliterative bronchiolitis.

organ transplantation, including the lung (227–229). A recent meta-analysis evaluating the efficacy of interventions aimed at improving adherence identified the importance of a multidisciplinary team, a comprehensive intervention approach, and mobile health monitoring (230). Indeed, optimization of strategies that increase patient adherence represents an important aspect for improved outcomes following lung transplantation.

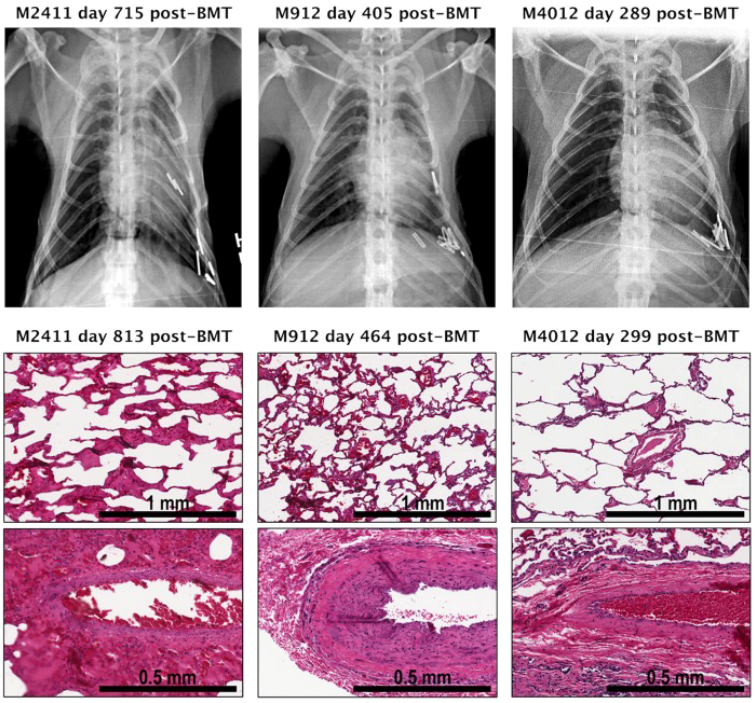

FIGURE 2
Pathology and chest radiographs from three tolerant NHP lung transplant recipients that underwent delayed donor bone marrow transplantation. Shown are photomicrographs (hematoxylin and eosin (H&E) staining) of lung biopsies performed at the time of euthanasia and chest radiographs obtained at indicated time points from each recipient. The chest radiographs of NHP recipients M2411, M912, M4012 displayed well-aerated lung allografts in the left thoracic space. No signs of rejection were seen in the lung graft biopsy of M2411, M912, and M4012. *Adapted from* (181). BMT, Bone marrow transplantation.

Summary and conclusion

Although the field of lung transplantation has evolved significantly in the past several decades, there exists an urgent need for the development of more effective strategies to improve graft and patient outcomes. An improved understanding of the unique immunologic properties of the lung has helped to elucidate the mechanisms by which conventional immunosuppression fails to achieve long-term lung transplant survival. Promising future strategies include targeted delivery of lung-specific therapeutics using *ex vivo* lung perfusion, novel anti-inflammatory approaches (i.e., IL-6 signaling blockade), application of cell-based therapies (i.e., MSCs), harnessing of the regulatory potential of lung-specific memory T-cell populations, and tolerance induction. The ongoing development of advanced therapies in lung transplantation provides hope for a better future for lung transplant recipients.

Author contributions

CM and JO performed the literature search and wrote the manuscript. JM and JA supervised, revised, and provided critical review of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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