Pathophysiology and classification of primary graft dysfunction after lung transplantation

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Abstract: The term primary graft dysfunction (PGD) incorporates a continuum of disease severity from moderate to severe acute lung injury (ALI) within 72 h of lung transplantation. It represents the most significant obstacle to achieving good early post-transplant outcomes, but is also associated with increased incidence of bronchiolitis obliterans syndrome (BOS) subsequently. PGD is characterised histologically by diffuse alveolar damage, but is graded on clinical grounds with a combination of PaO_2/FiO_2 (P/F) and the presence of radiographic infiltrates, with 0 being absence of disease and 3 being severe PGD. The aetiology is multifactorial but commonly results from severe ischaemia-reperfusion injury (IRI), with tissue-resident macrophages largely responsible for stimulating a secondary 'wave' of neutrophils and lymphocytes that produce severe and widespread tissue damage. Donor history, recipient health and operative factors may all potentially contribute to the likelihood of PGD development. Work that aims to minimise the incidence of PGD in ongoing, with techniques such as *ex vivo* perfusion of donor lungs showing promise both in research and in clinical studies. This review will summarise the current clinical status of PGD before going on to discuss its pathophysiology, current therapies available and future directions for clinical management of PGD.

Keywords: Primary graft dysfunction (PGD); oedema; ischaemia-reperfusion injury (IRI); inflammation; ex vivo lung perfusion (EVLP)

Definition of primary graft dysfunction (PGD)

PGD is a syndrome that encompasses a spectrum of mild to severe lung injury that occurs within the first 72 h after lung transplantation. PGD is a major cause of early morbidity and mortality in lung transplantation and is characterised by progressive hypoxaemia and alveolar infiltrates on a chest radiograph. Following ischaemia in the donor organ and reperfusion in the recipient, inflammatory and immunological injury-repair responses seem to be the key pathological mechanisms. PGD has significant impact on the short and longer-term outcomes for lung transplant patients, however strategies aimed at identifying and reducing PGD risk are being developed. Ex vivo lung perfusion (EVLP) is one strategy to improve assessment of the donor organ and has the potential to act as a platform for implementing interventions to reduce the risk of or prevent PGD. This review will provide an overview of the pathophysiology of PGD and its implications in the clinical setting.

In 2005, the International Society for Heart and Lung Transplantation (ISHLT) published their standardised definition for PGD (updated in 2016) (1). In this definition, the PaO_2/FiO_2 (P/F) ratio and the presence of bilateral

Table 1 ISHLT PGD definition and severity grading 2005 and updates from 2016

PGD stage	P/F ratio (mmHg)	Chest radiography	Updates from 2016 Consensus Group
0	>300	Normal	Any P/F ratio
1	>300	Diffuse allograft infiltration/pulmonary oedema	No changes
2	200–300	Diffuse allograft infiltration/pulmonary oedema	No changes
3	<200	Diffuse allograft infiltration/pulmonary oedema	No changes

ISHLT, the International Society for Heart and Lung Transplantation; PGD, primary graft dysfunction; P/F, PaO_2/FiO_2.

infiltrates on a chest radiograph consistent with non-cardiogenic pulmonary oedema are assessed, see *Table 1*. Assessment is carried out at specific time points after reperfusion; within the first 6 h (T0), post 24 h (T24), 48 h (T48) and 72 h (T72). Ideally, the P/F ratio is measured on a FiO_2 of 1.0 and positive end expiratory pressure (PEEP) of 5 cm H20. The radiographic findings of PGD are non-specific and include peri-hilar ground glass opacities, peri-bronchial, perivascular thickening and reticular interstitial and airspace opacities located in a dependent fashion. The 2016 Consensus Group has clarified that the PGD timing starts at the point of reperfusion following release of the second lung recipient pulmonary arterial cross clamp. The Consensus Group proposes that no changes be made to the grading time points of T0, T24, T48, and T72 and reaffirms this approach and now clarifies that "any" P/F ratio is to be considered grade 0 in the absence of radiographic diffuse pulmonary oedema.

Other contributory factors that can mimic, modify and confound the definition and grading need to be excluded, including cardiogenic oedema, pneumonia, hyper-acute rejection and pulmonary venous anastomotic obstruction.

Epidemiology and clinical outcomes

Historically it was difficult to accurately assess the incidence of PGD due to varying definitions and even with the introduction of the ISHLT standardised definition in 2005; there is still a dependence on accuracy in the timing and severity grading of PGD. Since the implantation of the standardised ISHLT definition, the incidence of severe PGD grade 3 (PGD3) within 48–72 h postoperatively has been reported at approximately 10–20% and the incidence of PGD3 at any time point within the first 3 days, approximately 30% (2-4). Diamond *et al.* demonstrated in a large, multi-centre, prospective cohort study of 1,255 lung transplant recipients between 2002 and 2010, that there was

an overall incidence of PGD grade 3 of 30.8% at any time point during the first 72 h of lung transplantation, while grade 3 PGD present at 48 or 72 h after reperfusion had an incidence of 16.8% (5).

PGD is associated with significant early and late post-transplant morbidity: patients with PGD3 have longer hospital and intensive care length of stays, duration of mechanical ventilation, increased short and longer-term mortality than those with lower grades of PGD. Whitson *et al.* analysed 402 lung transplant recipients at the University of Minnesota from 1992 to 2004, reporting that that the 90-day mortality rate associated with the occurrence of PGD grade 3 at any point within the first 48 h after transplantation was 17% versus 9% in the group without grade 3 PGD (6). In a study of the United Network for Organ Sharing (UNOS)/ISHLT database between 1994 and 2000, all-cause mortality at 1-year was 64.9% in recipients with PGD 3 beyond 48 h versus 20.4% in the non-PGD group (7). Diamond *et al.* showed that PGD was significantly associated with 90-day and 1-year mortality with grade 3 PGD at 48 or 72 h after transplant being associated with an absolute risk increase for death within 90 days compared to those without grade 3 PGD of 18% and 23 % for death within 1 year (5). PGD has also been associated with an increased risk of developing bronchiolitis obliterans syndrome (BOS) which is the main limiting factor for long-term survival after lung transplantation (8).

Whitson *et al.* showed that patients with PGD3 had a significantly reduced longer term survival compared with those PGD1 and PGD2; median survival grade 3: 4.6 years; grade 2: 6.6 years; grade 1: 7.5 years (6). Kreisel *et al.* reported significant association between PGD with decreased long-term survival in their single centre cohort study of 1,000 recipients. They showed 1-, 5- and 10-year survival to be 72.8%, 43.9 % and 18.7% in the PGD group compared with 87.1%, 59.8% and 35.7% in the non-PGD group respectively (9). PGD has also been shown to

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significantly impact on recipient functional status which ultimately will impact on quality of life. For example, survivors of PGD 3 at 12 months were seen to have a much shorter 6 min walk distance than those without (10).

Risk factors: pre-transplantation

With regard to the overall lung transplant procedure there are several places that can act as potential risk factors for subsequent PGD. Brain death leads to haemodynamic compromise, hormonal derangements, hypothermia and release of inflammatory cytokines (11,12). Warm ischaemia occurring after loss of circulation during organ retrieval and then cold ischaemia to preserve the organs causes a deterioration in tissue oxygenation. A release of inflammatory cytokines, namely IL-8, IL-12, IL-18, TNF-alpha and IFN-gamma triggers a cascade of tissue apoptosis, necrosis and results in organ dysfunction (11). The pathophysiology will be discussed in depth later in this review.

Hypoxia during ischaemic periods promotes coagulation through activation of endothelial cells, leading to a pro-coagulable environment. In an analysis of the UNOS database, an association with PGD was found with donor traumatic brain injury (TBI) as a cause of death (13). Diamond *et al.*, however reported in a large multicentre cohort study that cause of donor death was not a risk factor for PGD (5).

There are increasing numbers of lung transplantations following donation after circulatory confirmation of death (DCD), with differing opinions on the effect of type of donation, DCD versus to donation after brain death (DBD) on PGD risk. A meta-analysis of five studies found no difference in PGD incidence between DCD and DBD donors (14). A study published the same year, however conversely reported that there was a higher incidence of PGD and a trend towards greater need for extracorporeal life support (ECMO) in a series of 60 DCD transplant recipients compared to their score matched DBD recipients (15).

Risk factors: post-transplantation

Donor-related factors

Donor-related factors can be categorised as hereditary and acquired. PGD risk has been reported to increase in donors older than 32–45 years (16). Whitson *et al.* demonstrated that the relationship between the development of PGD3

and donor age in their series was linear from the age 35 years upwards and a 3% increased risk of PGD per one-year increased in donor age (6). More recent data from Baldwin *et al.* suggests that the age-related risk of PGD is restricted to the extremes of age and that donor age 55 to 64 years was not associated with a significantly increased risk of severe PGD after controlling for recipient, surgical and other donor factors (17).

Other donor risk factors reported include age, African American race, female gender, and history of tobacco exposure (6,12,13,16,18). Bonser *et al.* reviewed the effect of donor smoking after lung transplantation using a cohort study of a prospective registry and found that donor smoke exposure was associated with worse recipient outcomes (19). The survival probability, however, still exceeded that of remaining on the waiting list. Alcohol use also appears to increase PGD risk, with donors categorised as "heavy drinkers" displaying a 9-fold higher risk of severe PGD compared to those with no alcohol intake (20).

Potential donor risk-factors that are acquired include prolonged periods of mechanical ventilation, aspiration, pneumonia, excessive blood transfusion and trauma, however further definitive studies are needed to demonstrate their associations with PGD. The presence of fat embolism from the donor is said to increases the risk of PGD development by 25-fold (21).

Recipient-related factors

The primary diagnosis leading to the need for lung transplantation is an important modifier of the risk of developing PGD. The most significant recipient risk factors are obesity (22,23), pulmonary hypertension and diagnoses of pulmonary fibrosis (24) and/or sarcoidosis (6,25). In a systematic review of 10 studies, the incidence of PGD was 11.8% in patients with chronic obstructive pulmonary disease (COPD), 12.4% in cystic fibrosis, 18.0% in patients with idiopathic pulmonary fibrosis (IPF), 50% in sarcoidosis and 30.3% in patients with idiopathic pulmonary arterial hypertension (IPAH) (18). Several studies including a meta-analysis, large multicentre cohort study and a single centre study have shown IPAH was strongly associated with risk for PGD. This remains the case even after adjustment for recipient pulmonary artery systolic pressure, with reports highlighting that higher pulmonary arterial pressure has been shown to be strongly correlated with the development of PGD, with a 30% increased risk of PGD for every 10 mmHg increase in mean PAP (5,16,18). After adjustment

for multiple risk factors, obese and overweight recipient body mass index (BMI) were independent predictors of PGD. Lederer *et al.* demonstrated a link between plasma levels of leptin which is associated with adiposity and risk of PGD with obesity being associated with greater than a 2-fold increased risk of grade 3 PGD within 72 h postoperatively in adult lung transplant recipients with COPD or ILD (22).

Pre-transplantation biological levels of certain markers have also been shown to have an association with a higher risk of PGD development. Cantu and colleagues found that donor polymorphisms in the oxidant stress gene *NOX3* were associated with increased risk of PGD (26). Machuca and colleagues found that lungs that subsequently developed PGD had higher levels of IL-8, macrophage colony stimulating factor and growth-related oncogene-α compared to lungs that did not develop PGD (27). More PGD-associated biomarkers will be discussed later on in the review.

Operative/surgical factors

The operative risk factors for PGD reported are single lung transplant procedure, prolonged ischaemia time, cardiopulmonary bypass (CPB) use, red blood cell transfusion greater than 1 litre and reperfusion FiO_2 greater than 0.4 (5,13,28-30). Weber *et al.* reported that patients who received an intraoperative blood transfusion of greater than 4 units were seen to have an increase in PGD, renal replacement therapy, a post-operative ECMO requirement and mortality (31). CPB has been associated with an increased risk of PGD, reported by Aeba *et al.* in a retrospective study over a 2-year period in 100 lung transplant recipients. They found that the cohort of patients whom had CPB had a more prolonged period of intubation and more severe pulmonary infiltrates on radiography (32). Diamond *et al.* demonstrated in their large, multi-centre cohort study and meta-analysis that CPB was an independent risk factor for the development of PGD (5) and also reported the association between PGD and single lung transplantation. In a meta-analysis, however this did not seem to be the case (18).

Reperfusion FiO_2 was also seen to be an independent risk factor for PGD in a large, 10-centre prospective cohort study with an increased risk of PGD at 48 or 72 h reported in patients where the reperfusion FiO_2 had been >0.4, with an absolute risk increase of 6% when compared to a reperfusion FiO_2 of <0.4 (5).

Pathophysiology of primary graft dysfunction

The underlying causes of PGD remain poorly defined. However, the emergence of data from large, multicentre-derived lung transplant recipient outcome studies and genetic data sets has allowed for the development in the understanding of the PGD mechanisms at a cellular and molecular level.

Neutrophil activation and interactions with the endothelium

The hallmark of PGD pathophysiology is the migration of polymorphonuclear neutrophils (PMNs) from the pulmonary circulation into interstitium and the airways. These PMNs are attracted out of the circulation by chemotactic mediators such as CXCL8 and damage-associated molecular patterns (DAMPs) which have been released from apoptotic and necrotic lung tissue following ischaemia-reperfusion injury (IRI). This leads to the release of DAMPs into the circulation, with HMGB1 and ATP being well-known examples. A hyperoxic mouse model of acute lung injury (ALI) utilised by Entezari *et al.* identified high levels of HMGB1 in the bronchoalveolar lavage fluid (BALF) of these mice (33). Intra-tracheal administration of additional HMGB1 within this model served to further exacerbate the symptoms of ALI in this model by mediating leukocyte infiltration into the airways. Shah *et al.* used another model of pulmonary inflammation to identify extracellular ATP as another potent mediator of neutrophil recruitment into the lungs during ALI (34). Both HMGB1 and ATP are well-renowned DAMPs, thus IRI-mediated damage of the pulmonary airways serves to release these into the circulation and facilitate attraction of neutrophils to the lungs in the case of acute pulmonary diseases such as PGD.

Alongside neutrophil activation and migration, activation of pulmonary endothelium is another very important part of the pathophysiology of PGD. This activation occurs through the release of pro-inflammatory cytokines TNFα and IL-1β which enables up-regulation of adhesion molecules on the surface of the endothelium (35,36). E-selectin on the endothelial surface interacts with P- and L-selectin found on neutrophils to facilitate weak binding, as the latter roll along the endothelial surface through a cytokine 'gradient' before being arrested via integrin binding. Mac-1 and $\alpha_4\beta_1$ have been shown to play essential roles in recruiting neutrophils to the lungs during *S. Pneumoniae* infection and thus likely enable this process

in the case of inflammation produced in PGD (37). Once localised to the lungs, transmigration of PMNs into the interstitial space and airways is facilitated by up-regulation of adhesion molecules, notably ICAM-1 and PECAM-1 (36). Support for the significance of the models is demonstrated by the study conducted by Simms *et al.*, who identified heightened CD11b/CD18 cell surface expression as being associated with individuals who displayed acute respiratory distress syndrome (ARDS) (38). Finally, a study that utilised atopic asthma patients highlighted the fact that a CXCR1/2 agonist was able to down-regulate the amount of CXCL8-mediated neutrophil infiltration of the airways, indicating that this is a key pathway by which neutrophil trafficking in the context of inflammatory lung disease is enabled (39). Thus, the combination of *in vivo* models of neutrophil trafficking along with epidemiological studies of associated molecular entities highlight the enormous significance of this process in enabling heightened pulmonary inflammation.

Endothelial cells up-regulate adhesion markers and secrete cytokines in response to IRI, resulting in leukocyte recruitment out of the circulation. The act of interrupting blood flow before restoration is considered the major event in IRI that disrupts the homeostasis of the endothelium. This is a well-documented mechanism in which the sudden re-establishment of blood flow within the organ causes dramatic cell depolarization. A 2011 study of 126 PGD patients between the year 2002 and 2007 was carried out by Fang *et al.* that investigated the effect of pulmonary arterial pressure on the likelihood of developing grade 3 PGD. The authors found a statistically significant correlation between the two, indicating the effect of significant reperfusion force on endothelial integrity (24). Schnickel *et al.* analyzed the effects of a modified reperfusion technique involving the insertion of a catheter into the main or individual pulmonary artery after implantation on the incidence of PGD (40). The recipient blood was depleted of leukocytes; supplemented with nitroglycerin; adjusted for pH and calcium level; enriched with aspartate, glutamate, and dextrose; then administered into the pulmonary arteries of the newly transplanted lung(s) for the first 10 min of reperfusion. Severe primary graft dysfunction was defined as a PaO_2/inspired oxygen fraction of less than 150 with diffuse infiltrate on the radiograph in absence of other causes. One hundred patients underwent lung transplantation with the modified reperfusion technique. Forty-two patients underwent single-lung transplantation, of which 5 patients required CPB for the procedure. Fifty-eight patients underwent double-lung transplantation; all double-lung transplantation procedures were performed with patients on CPB. There were no technical complications associated with the modified reperfusion, with the mean PaO_2/inspired oxygen fraction at 6 h in this cohort being 252 ± 123 mmHg. The median number of days on the ventilator was 2, with incidence of severe primary graft dysfunction in this cohort being 2.0%. The early survival (30-day or in-hospital mortality) of this group of patients was 97%. They concluded that the technique of modified reperfusion in human lung transplantation is associated with a low incidence of severe primary graft dysfunction and favorable short-term outcomes. Another study by Porteous *et al.* highlights that diastolic dysfunction also plays a role in increasing the risk of PGD development (41). These studies provide substantial evidence for the key role that flow along the endothelium has in instigating lung damage, as IRI is essential in enabling PGD to develop.

Initiation of IRI provokes endothelial cells to up-regulate various adhesion markers on their surface, produce cytokines and begin actively contributing to inflammation (42-44). ICAM-1 and PECAM-1 have both been implicated in ALI and inflammation in general, with these facilitating leukocyte transmigration from the circulation into the airways (35). ICAM-1 levels can be seen to relate to patient outcome in human studies (45). The integrins Mac-1 and $\alpha_4\beta_1$ were shown in a mouse model of lung infection by Kadioglu *et al.* to not only be essential in allowing neutrophil and T lymphocyte movement into the airways, but also facilitated the immunological response that was directed against the pathogen by these cells (37).

The other major effect that stimulation of the endothelium causes is production of cytokines by these cells. Endothelial cells have been shown to directly participate in amplifying the inflammatory response and can produce PGD-associated cytokines including PAI-1 and IL-17. These are detrimental to the function of the lung, with levels of both of these examples having been linked with worsening outcomes of PGD in patients (46-48). Activation of endothelial cells primes the environment for the secondary 'wave' of leukocyte influx into the pulmonary airways. It is this secondary phase that is ultimately so damaging.

Many key mediators released as a result of inflammation can also directly affect the structural integrity and viability of the endothelium. IRI initiates generation of caspase-8 and -9 that have been shown in a rat model to sustain mitochondrial injury and overall contribute in a

Table 2 Neutrophilic enzymes

Neutrophil secretion	Example(s)	Function(s)	Role in pathogenesis of PGD
Proteases	Neutrophil elastase, cathepsins, gelatinases, collagenases	Peptide cleavage, cytokine release	Destruction of airways and pulmonary barriers
Hydrolytic enzymes	Alkaline phosphatase, lysozymes	Digestion/breakdown of pathogenic components	Destruction of airways, reduction of oxygenation
Reactive oxygen species	Superoxide ion (O_2^-), peroxide (O_2^{2-}), tyrosyl free radical	Catalysis of pathogenic structural components	Leukocyte recruitment, tissue disruption
Pro-inflammatory cytokines	IFNγ, IL-1β, IL-6, IL-8, TNFα	Various pro-inflammatory mediators	Establishment of pathology, recruitment of immune cells, tissue disruption

detrimental manner to the consequences of reperfusion. In lung injury, apoptosis has generally been shown to play a key role in mediating significant tissue damage (49,50). Li *et al.* highlight how TNF-alpha and LPS-mediated damage *in vitro* triggers activation of MAPK pathways that culminates in substantially increased vascular endothelial permeability, translating to impaired barrier functions within an *in vivo* setting and disrupting gaseous exchange within the airways. CXCL8, which is released in large quantities by a variety of cells to attract leukocytes out of circulation has been shown *in vitro* to directly regulate cell permeability by causing down-regulation of tight junctions. This was seen to operate in a time-dependent manner, putting forward the likely scenario that accumulated CXCL8 gradually wears down the endothelial integrity over time (51). This is in turn supported by human studies which have shown that IL-8 levels in the lungs of DBD donors correlate in a concentration-dependent manner to PGD severity and mortality (52). Vascular permeability is therefore one of the major facets in lung insult and thus plays a large role in PGD pathogenesis.

Once localised to the airways, neutrophil activation contributes to the significant inflammation in this microenvironment. Multiple studies highlight how platelet-neutrophil interactions are key to enabling PMN activation in a number of autoimmune disease pathologies, including acute lung conditions (53-55). This subsequent activation is associated with production of numerous protease enzymes. Neutrophil elastase (NE) can utilise a wide range of extracellular matrix (ECM) proteins as substrates and thus disrupting airways integrity. Furthermore; release of cytokines such as IL-1β have also been attributed to the action of this enzyme. A study by Ishii *et al.* used a bilateral nephrectomy model to induce ALI and identified that application of an NE antagonist reduced levels of cytokines such as IL-6, as well as numerous other inflammatory processes associated with this model (56). Matrix metalloproteinases (MMPs) target a wide range of substrates, with neutrophils being known to secrete these when activated. MMP-8 (neutrophil collagenase) levels in BALF were correlated with severity of disease in bronchiectasis patients, whilst another study found that blood levels of MMP-9 could be used to help differentiate between PGD grades 2/3 and individuals with no PGD present (57). These studies strongly support neutrophil degranulation being a major contributory factor to PGD disease progression.

Neutrophils are also responsible for the secretion of neutrophil extracellular traps (NETs) (58). These are strands of chromatin with the ability to bind a range of intercellular secretions and granules and ultimately ensnare extracellular pathogens. Sayah *et al.* used two experimental murine models to confirm that the presence of platelet-mediated NET formation contributed to PGD pathology. Prevention of NET formation by direct disruption with DNase I was seen to reduce the amount of lung injury present. Epidemiological evidence for this pathogenic role is provided in the study, as human BALF samples were analysed for the presence of NE-DNA complexes. Generally, these were present to a much higher degree within individuals with moderate to severe grades of PGD (59). *Table 2* outlines the enzymes secreted by activated neutrophils and the role that plays in PGD pathophysiology.

Alveolar macrophages (AMs) activation and leukocyte recruitment

AMs are a niche population that are located within the alveolar space and airways and are continuously surveying the airways for pathogen-associated molecular patterns

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(PAMPs). The pro-inflammatory functions of AMs are normally tightly regulated by epithelial signals, including CD200 binding and secretions of the cytokines TGF-β and IL-10 (60). Damage to the airways caused as a result of IRI provokes an initial wave of cellular apoptosis, followed by a subsequent secondary necrosis. This releases an array of previously detailed DAMPs such as RAGE and HMGB1, with the latter in particular stimulating AMs to migrate to the site of inflammation (61). Damage to the epithelium also has the effect of decreasing the concentrations of anti-inflammatory mediators that are continuously secreted into the airways. Lack of effective cellular regulation produces populations of cells with an inflammatory profile, characterised in the case of kidney IRI by heightened cytokine production and increased oxidative burst that resembles an 'M1' polarized phenotype (62,63). Several studies have highlighted the importance of AMs in establishing IRI (64-66). A study carried out in rat lungs utilised liposomal clodronate to knockdown resident macrophage populations. This not only reduced the concentration of several known inflammatory cytokines, including; MIP-1, MIP-2α and TNFα, but also protected against much of the epithelial damage that is such a hallmark of IRI (67). Balamayooran et al. used an MCP-1$^{-/-}$ mouse strain to demonstrate that these mice had a significantly impaired clearance of bacteria when infected with *Escherichia coli*, caused by lower cytokine levels, less cellular adhesion and a reduced capacity of neutrophil influx. Shah et al. correlate this in patients, noting that levels of MCP-1 at 24 h can be used as a biomarker for PGD (45,68). Other models of lung diseases such as IPF also display a similar dependency on AMs to initiate inflammation (66). Many of these studies further demonstrate that depletion of this niche macrophage population can often alleviate much of the damage observed in these attempts to model ALI. Finally, the evidence gathered by these models is supported by *in vitro* data, as AMs have been shown to produce levels of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNFα when stimulated, demonstrating directly relatable proof for these mechanisms of action (69,70).

Disruption of the tissues - loss of epithelial integrity

Multiple studies identifying epithelial injury markers having a high association with more severe grades of PGD. Patel et al. note the detrimental effect that TNF-mediated cell death has on epithelial integrity in their 2013 study, with the presence of TNFα already being well established in the pathology of ALI in general with regard to leukocyte recruitment into the airways (71). RAGE is a major DAMP in PGD and levels have been correlated with higher grades of disease and linked to lengthened hospital stays by multiple patient studies, underlining how disruption of epithelial integrity appears to be a major factor in post-transplant failure (34,72). Expression levels were found to correlate with increased length of hospitalization by Pelaez et al. in a 2010 study of PGD patient BALF samples (73). RAGE is of course known to be a receptor for the aforementioned DAMP HMGB1, thus higher levels of RAGE on the surface of leukocytes enables higher levels of activation through this pathway and thus worsens the pathology of PGD. This link is confirmed in a model by Sharma et al. (2013) who used an *in vivo* hilar clamp model to induce IRI in C57BL/6 wild-type mice. Upon treatment with recombinant HMGB1, symptoms of IRI considerably worsened, but this was not the case when the same model was applied to RAGE$^{-/-}$ mice (74).

One notable contributory factor towards PGD is the presence of pre-transplant anti-col(V) antibodies. Iwata et al. identified in a 2008 study that epithelial tissue and not endothelial tissue showed expression of this, which explained why exclusively epithelial cells were targeted by an anti-col(V), CD4+ mediated cytotoxic response. Examination of patient samples identified that individuals with pre-formed antibodies had strong likeliness of developing PGD, confirming this link (75). This autoantibody response has been confirmed as playing a significant role in a murine model by Bharat et al. Here, the authors note that in a syngeneic transplantation model, lung-restricted antibodies directed against col(V) showed a dose-dependent response, characterised by poor oxygenation compared to the isotype controls performed (76). Pre-formed anti-col(V) antibodies in patients are therefore a significant factor in potentially damaging the epithelium in acute lung injuries.

Whilst epithelial damage is indeed an early hallmark of primary graft dysfunction evidence supports the contributory role that these cells in fact have towards worsening of pathology in many cases. One study highlighted that once produced; IL-17 and TNFα act in synergy with one another to promote the epithelial up-regulation of CXCL1, which mediates potent migration of neutrophils to the site of expression, as well as being known to modulate leukocyte functions in immunity (47,48,77). This observation was also correlated with significantly less pulmonary dysfunction when this NADPH-mediated pathway was blocked *in vivo* in a study by Sharma et al., who

noted down-regulation in expression of CXCL1, as well as CCL2, CCL5 and IL-6 (78). The epithelial layers therefore are not only significant targets in IRI, but also actively support the progression of this injury to development of clinically relevant PGD.

Recent studies have started to map out the signalling pathways that lead to IRI. One such pathway involves toll-like receptors (TLRs); an important class of pattern-recognition receptor that are located in abundance on the surface of AMs. Several studies have attempted to model ALI and highlighted the importance of TLR4 in establishing ALI pathology. A study in 2012 utilised pulmonary arterial occlusion to highlight that mice could generate inflammatory mediators when exposed to a model of IRI, with this capability being abrogated if TLR4$^{-/-}$ mice were used (79). One *in vivo* model finding that using TLR4$^{-/-}$ mice was sufficient to not only reduce the amount of pro-inflammatory markers produced in the wild-type, but also prevented efficient neutrophil recruitment into the airways (79). Ding *et al.* also managed to identify that activated TLR4 can bring about activation of TLR3 via an NF-κβ-mediated pathway and that this leads to an up-regulation of PMN migration in the context of ALI (80). Further evidence for the importance of the role of TLR4 is demonstrated by Xu *et al.* who note that levels of micro RNA-21 (miR-21) have been noted as being reduced in patients with higher grades of PGD. This microRNA acts to down-regulate TLR4 expression, as well as levels of other signalling molecules, highlighting a pathway by which individuals with lower levels may be more susceptible to excess inflammation post-transplantation (81). AM populations by Dhaliwal *et al.* were seen to reduce neutrophil influx into the airways (67,79). The authors note that once this damage has been avoided, replenishment of macrophage populations is not sufficient to initiate damage to the airways, but this can be done via later administration of PMNs, effectively signalling the 'guiding' role that AMs perform within PGD and other pulmonary abnormalities. This provides evidence for the importance of AMs in modulating PMN migration in the context of ALI and subsequent development into severe grades of PGD. Lastly, TLR4 has been further implicated in PGD progression by activating NETosis in neutrophils, which also correlates with PGD grade. Knockdown of macrophages also prevented IRI development in this model, with lack of neutrophil infiltration into the airways remaining absent even after 3 h had elapsed (53). NETosis will be discussed in more depth later.

The role of CD4+ T lymphocytes in facilitating leukocyte recruitment

Lymphocytes are typically more involved in the latter stages of PGD development. Once the 'resident' AMs have detected and responded to IRI within the lungs, they can then initiate intercellular communication with donor T lymphocytes. CD4+ cells mediate these effects in PGD, and to a much lesser extent CD8+ cells. In a murine model of IRI; depletion of T lymphocytes was seen to be sufficient to alleviate the worst of the pathologically associated symptoms (82). The authors also note that depletion of CD4+ T lymphocytes within this system was sufficient to reduce the amount of PMN infiltration into the tissues. Evidence therefore points towards them having a 'chaperone' role; in that they stimulate and direct neutrophils to infiltrate the lung, which will then cause the majority of the damage to the pulmonary environment. The mechanisms of infiltration into the airways appear to be homologous with those utilised by PMNs in the case of lung disease. A combination of Th2 and Th17 responses have been identified in PGD and are synonymous with severity of disease in both cases. Levels of the cytokines IL-23 and especially IL-17 have been heavily linked with development of PGD pathology (83).

As highlighted in the section on epithelial damage; pre-formed anti col(V) antibodies are a significant factor in the outcome of a lung transplant, with immunity mediated by CD4+ T cells being identified by many studies as a significant observation within PGD. Circulating antibodies bind to these regions on epithelial cells and the exposed Fc regions will then bind to receptors on the surface of professional phagocytes, namely the AMs present in the airways. Numerous studies have documented the presence of anti-col(V) immunity present within PGD patients (75,84). This is further corroborated by the links between antibody presence and transplant outcome which again point towards a higher incidence being of great significance in development of graft injury.

Mechanisms involved in PGD progression and disease-associated biomarkers

Much of the work to identify biomarkers of PGD has come from the 'Lung Transplant Outcomes Group'. This was sponsored by the United States National Institutes of Health and collected data on lung transplant patients at 10 transplant U.S. centres between 2002 and 2010. Links have

Table 3 Biomarkers associated with primary graft dysfunction

Biomarker/molecule	Function(s)
RAGE/sRAGE	Intracellular DNA-binding protein, pattern-recognition receptor (PRR), up-regulation of inflammatory processes, auto-inflammatory disease
IL-8/CXCL8	Neutrophil/leukocyte chemoattractant, neutrophil activation, phagocytosis
IL-17	Pro-inflammatory cytokine, monocyte/neutrophil chemoattractant, autoimmune disease, allergic reactions
Ang2	Promotes apoptosis, devascularisation, autoimmune disease
MCP-1	Monocyte/basophil chemoattractant, degranulation, tumour formation
P-selectin	Endothelial/thrombocyte adhesion marker, leukocyte recruitment, thrombocyte aggregation, tumour metastasis
ICAM-1	Endothelial/leukocyte adhesion marker, leukocyte transmigration, pro-inflammatory signalling, rhinovirus entry site
PAI-1	Inhibition of thrombolysis, promotes progression to fibrosis, inhibits action of MMPs
Col(V)	Major structural protein component of connective tissues
SP-D	Chemoattractant for alveolar macrophages and neutrophils
Protein C	Major component of anticoagulation pathway, anti-inflammatory effects, anti-apoptotic factor, promotes endothelial barrier function
Pentraxin 3	Acute phase protein, assists clearance of apoptotic cells

MMP, matrix metalloproteinase.

been made between relative levels of the proteins listed in *Table 3* and risk of PGD development. Whilst much of this information on molecular associations with outcomes in PGD remains epidemiological, recent studies have begun to attempt to map out many of the signalling pathways that lead to exacerbation of IRI. Implicated pathways within the pathogenesis involve receptors such as the toll- and NOD-like receptors (TLRs and NLRs) and up-regulation of cytokines such as IL-1β. Continued research into these pathways remains ongoing in the hope of identifying novel targets for therapeutic intervention.

Shah *et al.* measured levels of a panel of biomarkers in the plasma of a cohort of 315 PGD patients. This contained molecules involved in a range of processes such as lung epithelial damage (sRAGE), cellular adhesion (ICAM-1) and coagulation (PAI-1). All were successfully linked with PGD grade and when used in combination with one of the others and the authors conclude that addition of any one of these to the clinical PGD grading significantly improved 90-day mortality prediction (85). Another detailed analysis of patient BAL fluid samples by Cantu *et al.* in 2013 revealed a wide range of immune pathways that associated heavily with enhanced cytokine release in the case of PGD development. IL-1, IL-6 and CCL4 all showed enhanced expression levels, with signalling pathways associated with

immune receptors such as the TLRs were all expressed to higher degrees as well (26).

These aforementioned receptors become activated to release substantial amounts of pro-inflammatory mediators. Notable examples in the case of acute lung pathologies are CXCL8 and IL-17. With regard to the former, a 2001 study by Fisher *et al.* highlighted how levels of IL-8 within donor lungs could be linked to graft failure early on post-transplantation (86). As previously detailed, this cytokine plays a key role in recruiting PMNs and other leukocytes into the pulmonary airways during inflammation (87). The indispensable role of IL-17 within auto-inflammatory diseases is well-renowned and has been closely linked to PGD progression. IL-17 receptor (IL-17R) polymorphism has also been shown to display a predisposition to PGD development post-lung transplantation, suggesting a role for assisting neutrophil recruitment to the lungs (47).

Prevention and treatment of PGD

Due to a lack of appropriately powered clinical studies, there is no clear, overall consensus on the treatment of PGD after lung transplantation. Given the similar clinical features and radiographic findings seen in both PGD and ARDS, many of the potential treatment options have been extrapolated

Table 4 Selected potential treatment strategies

Mechanism of action	Examples investigated
Replacing endogenous cytoprotective substances	Prostaglandins
	Nitric oxide
	Surfactant
	Adenosine
	Endothelium-derived relaxing factor
Inhibiting pro-inflammatory mediators	Platelet activating factor 1 inhibitor
	Inhibitor of ROS
Inhibiting neutrophil and neutrophil derived mediators	Cytokines (TNF-alpha, IL-1beta)
	Proteases
	Complement cascade

ROS, reactive oxygen species.

from the management of ARDS. Strategies used to prevent and minimise the development and severity of PGD include optimising donor selection, matching and management pre-operatively; improving lung preservation and storage techniques and improving reperfusion techniques. Several therapeutic agents have been studied in an effort to reduce the incidence of PGD, with a selection of these being depicted in *Table 4*. Soluble complement receptor 1 inhibitor, plasminogen activating factor antagonist and exogenous surfactant demonstrated beneficial effects on surrogates of PGD including the A-a gradient.

Surfactant is synthesised by type 2 pneumocytes and secreted into the alveolar space where it forms a stable monolayer, resulting in reduced surface tension in the alveoli, preventing atelectasis and alveolar oedema. Experimental studies have demonstrated the link between ischaemia, cold storage and reperfusion with alterations in surfactant composition and function leading to reduction in pulmonary compliance, atelectasis, pulmonary oedema and oxygenation (88). Amital *et al.* in a prospective randomised study demonstrated that delivery of surfactant through a bronchoscope after bronchial anastomosis is established could improve oxygenation and lead to reduced PGD grade, severe PGD rates, earlier extubation and shorter ICU length of stay (89).

Recommendations have emerged for lung protective ventilation (LPV) for PGD patients based on the pre-existing evidence for this strategy in ARDS.

Veno-arterial ECMO has been used for salvage of refractory hypoxaemia due to severe PGD following lung transplantation. VA-ECMO intraoperatively instead of CPB

has been associated with shorter duration of mechanical ventilation and ICU/hospital length of stay, and lower transfusion requirements, but no statistically significant difference in 90-day mortality (90).

Normothermic EVLP is an emerging evaluation technique for high risk donor organs and allows for a period of normothermic assessment and reconditioning (47,91-96). Steen *et al.* in Lund, Sweden were the first to successful carry out EVLP in 2001 using DCD donor lungs. Cypel and colleagues then published results of their landmark study in 2011 where they reported excellent clinical outcomes using donors considered unsuitable for transplantation (97). EVLP also offers a platform through which to deliver targeted therapeutic agents to enhance the quality of the donor lungs. The results of a randomised control clinical trial using a portable extracorporeal perfusion system with the aim to reduce ischaemic time are awaited (98,99).

Conclusions

PGD has significant impact on the short and longer-term outcomes for lung transplant patients, and understanding its underlying pathophysiology is crucial to developing novel strategies aimed at identifying and reducing PGD risk. The immunological and inflammatory cellular and molecular mechanisms involved in the pathogenesis of PGD are highly complex and future experimental models are needed to investigate these further. Findings from PGD patient multicentre outcome and genetic studies are now being analysed in experimental lung transplant models with the aim to develop new therapeutic approaches.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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