Primary Graft Dysfunction after Lung Transplantation

An Experimental Study

Niels van der Kaaij

Primary graft dysfunction after lung transplantation. An experimental study
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Primary Graft Dysfunction after Lung Transplantation
An Experimental Study

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Contents

1

General Introduction

1 | The lung

1.1 Macroscopic anatomy of the lung

The human body requires oxygen to perform aerobic processes [1-3]. The lungs are the respiratory organs that not only supply oxygen to the blood, but also remove carbon dioxide from the body. The right lung is most commonly made up of three lobes (superior, middle and inferior) relatively separated by fissures, while the left lung consists of two lobes (superior and inferior). Every lobe is again divided in several bronchopulmonary segments, which are the largest subdivisions of a lobe and named according to the segmental bronchi supplying them. The bronchial tree consists of a conducting zone (bronchi, bronchioles and terminal bronchioles) and a respiratory zone (respiratory bronchioles and the alveolar duct). The alveolar ducts terminate in the alveolar sac which gives rise to several pulmonary alveoli. The conducting zone not only transports gases, it also plays a role in the host defense system, and it warms and humidifies inspired air. It has its own circulation, known as the bronchial circulation, which comes from the descending aorta and drains into the pulmonary veins. Gas exchange occurs in the respiratory zone. A pair of normal lungs contains between 400 and 700 million alveoli. If stretched, this contains an area of 70 m^2. The pulmonary circulation has its origin in the pulmonary trunk, that carries deoxygenated but carbon dioxide rich blood to the lungs. The pulmonary trunk divides in a right and left pulmonary artery that enter the pulmonary cavities at the pulmonary hilum. After branching several times, the pulmonary arteries terminate in capillaries that surround the alveolus. After gas exchange by diffusion, oxygenated blood enters the pulmonary veins that terminate in the left atrium, flows into the left ventricle, where it is pumped into the systemic circulation.

1.2 Microscopic anatomy of the alveolus

The alveolus is mainly made up of flat type I alveolar epithelial cells (80-90%) and 10-20% cuboidal type II cells [1-3]. Type I cells are easily injured, while type II cells are more resistant to injury. Type II cells are important in surfactant secretion and recycling, ion transport, and may differentiate into type I cells [4]. Surfactant lines the alveolar epithelium and is essential in normal breathing [4]. Capillaries surround the alveolus, so that air and blood are in close contact, but separated by the very thin (<0.5 μm) alveolar-capillary membrane.

1.3 Pulmonary physiology

Several pressures play an important role in normal breathing and thus movement of air: Alveolar pressure (pressure inside the alveolus (P_{ALV})), pleural pressure (pressure in the pleural cavity between lung and chest wall (P_{PL})), airway pressure (P_{AW}), atmospheric pressure (P_{ATM}) and the transmural pressure (pressure difference across an airway or pulmonary wall) [1-3]. The pleural pressure is negative or subatmospheric due to elastic recoil of the lung inward and recoil of the thoracic cage outward resulting in opposing forces. In normal breathing, two transmural pressures are important: 1) The transpulmonary pressure (P_{TP}), which is the pressure difference across the lung wall ($P_{TP}=P_{ALV}-P_{PL}$); and 2) The transairway pressure (P_{TA}), which is the pressure difference across the airways ($P_{TA}=P_{AW}-P_{PL}$). In order to ensure airflow into the lungs during inspiration, a pressure gradient must be developed. Alveolar pressure is equal to the atmospheric pressure in the absence of airflow. When the inspiratory muscles contract, the thoracic cage is enlarged and the thoracic volume is increased resulting in a decrease in pleural pressure. As a consequence the transpulmonary pressure increases, the lung inflates, the alveolar pressure becomes lower than the atmospheric pressure resulting in airflow into the lung until the alveolar pressure equals the atmospheric pressure. At expiration, the respiratory muscles relax, so that the thoracic cage decreases in size, the pleural pressure becomes less negative, the transpulmonary pressure decreases, the alveoli deflate, resulting in a supra atmospheric pressure in the alveolus, so that air is pushed out of the lungs until the alveolar pressure equals the atmospheric pressure. The transairway pressure is essential in keeping the airways open during forced expiration.

1.3.1 Pulmonary compliance

Pulmonary compliance can be divided in dynamic or static lung compliance [3]. Static lung compliance is the change in lung volume per unit change in the transpulmonary pressure, which corresponds with the slope of the pressure-volume curve at a particular point. Dynamic lung compliance is the compliance of the lung at any given time point during actual movement of air. Dynamic compliance is determined by dividing the tidal volume by the difference between the airway pressure at full inspiration and full expiration. Static compliance monitors elastic resistance of the lungs only, while dynamic compliance is an indication of the lung and airway resistance. Pulmonary compliance is affected by lung volume, lung size, elastic recoil and by pulmonary disease. For example, in pulmonary fibrosis, compliance is reduced, while in emphysema patients static compliance is increased.

1.4 Surfactant

Surfactant lines the alveolar epithelium and is essential in normal breathing [4]. Surfactant is responsible for decreasing the surface tension at the air-liquid interface at the alveolar-capillary membrane [4,5]. It hereby prevents the alveoli from collapsing at the end of expiration, resulting in inflation of the lung with minimal effort and balanced fluid homeostasis in the lung. Surfactant also suppresses inflammation by dampening the production of reactive oxygen species (ROS), macrophage migration, lymphocyte proliferation and cytokine production [6]. Finally, surfactant protects the lung against micro-organisms and serves as a functional barrier in the alveolus, so that the transfer of molecules across the alveolo-capillary membrane is limited [6]. Surfactant is composed of lipids (90%) (mainly dipalmitoyl-phosphatidylcholine) and surfactant associated proteins (10%). Surfactant associated proteins play an important role in maintaining the quality of surfactant and have immunoregulatory functions. In this regard, SPA and SPD are able to regulate toll-like receptors, resulting in inhibition of the production of inflammatory mediators [7]. Furthermore, surfactant preparations containing surfactant B and C reduce proinflammatory cytokine production by stimulated human alveolar macrophages and peripheral blood monocytes in vitro [7]. The surface lowering capacity of surfactant is predominantly due to dipalmitoyl-phosphatidylcholine and SPB.

2 | End stage lung disease

Despite improving medical resources, progressive pulmonary diseases, like chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), (idiopathic) pulmonary fibrosis, and pulmonary hypertension, may end up in irreversible pulmonary failure [8]. These patients with end-stage lung disease require a lung transplantation to survive, although lung transplantation should be regarded as a palliative rather than a curative treatment option.

3 | Lung transplantation

3.1 Indications and contra-indications for lung transplantation

The International Society for Heart and Lung Transplantation has developed guidelines for (contra) indications for lung transplantation [9, 10]. To be eligible for pulmonary transplantation, the patient must have deteriorating lung function with a limited prognosis despite optimal therapy for his disease, if available. Absolute contra-indications for transplantation include malignancies in the last 2 years with some supporting a 5-year cancer free interval, active chronic hepatitis B/C or human immunodeficiency virus, untreatable advanced dysfunction of another significant organ system, important deformities of the spine or thorax, substance abuse, proven non-adherence of medical therapy, untreatable psychiatric or psychological condition resulting in uncooperative behaviour or an unstable or absent support system. Relative contra-indications involve an age beyond 65 years, a body mass index over 30 kg/m^2, critical clinical state at the time of transplantation, severely reduced functional status, severe osteoporosis, colonization with resistant or very virulent bacteria or fungi, or mechanical ventilation at the time of transplantation.

3.2 Lung allocation

Historically, if patients were accepted for transplantation, they were placed on a waiting list for transplantation. If a lung became available for transplantation, recipients were matched to donors, based on time on the waiting list, blood type, size (total lung capacity), recipient urgency status and the number of lungs the recipient needed. Due to a shortage of organs and a relatively high waiting list mortality, the lung allocation score (LAS) was introduced [11]. The objectives were to reduce the waiting list mortality, to increase transplant benefit for recipients and to ensure efficient and equitable allocation of lungs to transplant candidates. The LAS is a weighted calculation based on the predicted risk to die on the waiting list during the following year and the predicted chance to survive after lung transplantation. The score ranges from 0 to 100 such that the sickest patient has the highest LAS.

3.3 Donor acceptance

A donor may be accepted for lung donation if the age is below 75 years, there is no underlying lung disease, malignancy, septic shock, an active viral infection (rabies, herpes zoster, rubella, or HIV), active tuberculosis, anencefalie, or an episode of aspiration just before transplantation [9, 10]. Organ assessment at the time of procurement includes review of chest X-rays, flexible bronchoscopy, PaO_2/FiO_2 ratio (10 minute ventilation with 100% oxygen with a positive end expiratory pressure of 5 cm H_2O) and inspection and palpation of the lungs. Donor lungs are considered as marginal if patients are older than 55 years, if they have a significant history of smoking, if there is a suggestion of infection (infiltrates on chest X-ray, presence of purulent secretions on bronchoscopy, and/or positive sputum culture), if there are signs of aspiration or pulmonary contusion, or when the PaO_2/FiO_2 ratio is lower than 300-350 mm Hg [12].

3.4 Number of lung transplantations

The number of lung transplantations performed worldwide still increases [13]. From the year 2000 to 2011 the total number of transplantations performed almost doubled from 2,000 to 4,000 per year with the number of single lung transplantations more or less stable around 1000 transplantations. In the last decade increasingly more recipients beyond 60 and even 65 years old were transplanted with a reduction in the number of transplantations performed in the 30-49 year old group. Nevertheless, more than 30% of the recipients are still between 50 and 59 years old, while 30% of the donors are between 18 and 29 years old. The indications for lung transplantation performed between 1995 and 2012 were COPD/emphysema (34%), idiopathic pulmonary fibrosis (24%), cystic fibrosis (17%), alpha-1 deficiency (5.8%), idiopathic pulmonary arterial hypertension (3.1%), bronchiectasis (2.7%) sarcoidosis (2.5%) and other (12%). Over the years, the percentage of single lung transplantations is decreasing due to a shift to bilateral lung transplantations.

3.5 Outcome of lung transplantation

The outcome of lung transplantation remains limited with a 5-year and 10-year survival of respectively 53% and 31% [13]. Development of primary graft dysfunction (PGD) is the main cause for early morbidity and mortality after lung transplantation, resulting in a 1-year survival of approximately 80% [13-15]. The major impediment to long-term survival is

development of chronic transplant dysfunction, also known as the bronchiolitis obliterans syndrome (BOS) [13,16].

4 | Primary graft dysfunction

PGD is a form of acute lung injury that occurs within the first days after allograft reperfusion [14, 15]. PGD is defined by diffuse radiological infiltrates of the lung allograft and a decreased PaO_2/FiO_2 ratio within the first 72 hours after transplantation after excluding other causes of pulmonary failure, like hyperacute rejection, pneumonia, venous anastomotic obstruction, and cardiogenic edema. PGD is divided into three grades with increasing severity (Table 1). PGD grade III occurs in 10-30% of lung transplant recipients and is the main cause for early morbidity and mortality after lung transplantation. Symptoms clinically resemble the symptoms of the acute respiratory distress syndrome (ARDS) and consist of hypoxemia, which cannot be corrected by supplemental oxygen, non-cardiogenic pulmonary edema, increased pulmonary artery pressure, and decreased pulmonary compliance. Histologically, PGD is characterized by diffuse alveolar damage. The development of primary graft dysfunction after lung transplantation is the end result of multiple lung injuries that the lung may sustain during the lung transplantation procedure. Lung ischemia-reperfusion injury (LIRI) has been suggested to be a major risk factor for PGD, although other factors like donor brain death, mechanical ventilation, pneumonia, hypotension, aspiration, donor thoracic trauma and allo-immunity have been found to interplay with LIRI in PGD development.

Table 1. Classification of PGD.

Grade	**PaO_2/FiO_2 ratio** (mm Hg)	Chest X-ray
0	>300	Normal
1	>300	Pulmonary edema
2	200-300	Pulmonary edema
3	<200	Pulmonary edema

The presence and severity of PGD is assessed on several time points after reperfusion. T0 corresponds with P/F ratio and chest X-ray assessment within 6 hours of lung reperfusion while the patient is still being ventilated (FiO_2 of 1.0 and PEEP set at 5 cm H_2O). The P/F ratio and chest X-ray assessment are repeated 24 hours, 48 hours and 72 hours after reperfusion, recognizing that after 72 hours other factors may confound the definition. There are several limitations to the grading scheme. Although this scheme can be used for all transplants, the type of transplant may influence the P/F ratio. Absence of infiltrates on chest radiograph is sufficient for PGD grade 0, even if the P/F ratio is less than 300 mm Hg. If the subject is on nasal canula for oxygen or if the FiO_2 is lower than 0.3, the patient is graded as having PGD grade 0 or 1, based on chest radiograph. Any patient on extracorporeal life support is automatically regarded as having PGD grade 3 and any patient mechanically ventilated with a FiO_2 greater than 0.5 and with nitric oxide beyond 48 after reperfusion should be considered as having PGD grade 3. If multiple blood gas values are available, the worst P/F ratio should be used.

4.1 Lung ischemia-reperfusion injury

Ischemia is defined by an oxygen deficiency in the lung caused by an obstruction of the blood flow to the lung [17, 18]. Reperfusion occurs when the blood flow to the organ is restored. During ischemia, adenosine triphosphate (ATP) is decreased causing inactivation of ATP-dependent membrane pumps, accumulation of intracellular calcium, formation of eicosanoids and reactive oxygen species (ROS), inflammation and potential cell death. A central feature of LIRI is the inflammatory cascade. LIRI causes a direct release of proinflammatory cytokines by macrophages. Consequently, neutrophils and lymphocytes are recruited into the lung. Because of expression of adhesion molecules on endothelium and leukocytes, leukocytes roll, adhere, and extravasate into the lung tissue. Macrophages and neutrophils contribute to cellular damage by the production of ROS and several other mediators, such as proteolytic enzymes, lysozyme, and lactoferrin. The superoxide anion, hydrogen peroxide and hydroxyl radical, which all are part of the ROS family, are very unstable and damage cellular membranes by lipid peroxidation.

4.2 Pathophysiology of primary graft dysfunction

Experimental and clinical studies have identified considerable overlap in the pathophysiology of ARDS and PGD [14, 15, 19]. A key feature of both syndromes is disruption of the alveolar-capillary barrier. ROS, phospholipases and proteolytic enzymes directly injure epithelial and endothelial cells, but also their junctions leading to increased capillary permeability and thus flooding of the alveolus with protein-rich fluid and leakage of surfactant components into the bloodstream. Direct injury to epithelial type II cells results in impaired fluid removal from the alveolar space and decreased recycling of surfactant. To repair the damage, protein-rich hyaline membranes are formed on the basement membrane. Other important features of PGD include abnormalities in fibrinolysis and coagulation, altered angiogenesis and dysregulation of the innate immune pathways.

4.2.1 Surfactant and primary graft dysfunction

Experimental data generated in lung transplantation models have provided evidence for surfactant abnormalities and depletion after LIRI and PGD [20-22]. The combination of surfactant inactivation and increased capillary permeability results in protein rich alveolar edema. These proteins further dose-dependently inhibit surfactant leading to a self-triggering mechanism of surfactant inactivation. This is partly visible by conversion of the surface active large aggregate subform of surfactant into the non-surface active subform of surfactant. In 1998, Hohlfeld and colleagues demonstrated surfactant alterations in human lung transplant recipients, more than 1 year after transplantation [23].

5 | Bronchiolitis obliterans syndrome

Long-term survival after lung transplantation is impaired by development of the bronchiolitis obliterans syndrome (BOS) [13, 16, 24]. PGD is suggested to be one of the factors contributing to BOS, which affects 50% of patients surviving beyond 3 months after transplantation. The exact etiology of BOS is not fully understood, but its pathogenesis appears to involve a “response to injury” type of pattern. BOS may develop as a result of multiple injuries the

allograft sustains, like brain death, PGD, rejection and infection [24, 25].

6| Room for improvement

Although important improvements have been made in the last decades in pre- peri- and postoperative care for lung transplantation, several issues that could have significant impact on the outcome can be improved. Some of these issues form the basis of this thesis and are mentioned below.

6.1 Lung ischemia-reperfusion injury and primary graft dysfunction

Even though a positive correlation between cold ischemia time and PGD development has been suggested [26-29], other studies found that the length of cold ischemia did not predict outcome after lung transplantation and suggested that other factors interplay with LIRI in PGD development [30-35]. The question whether LIRI is an independent risk factor for the development of PGD is difficult to answer. In clinical studies, often multiple interfering factors are examined simultaneously and a satisfactory experimental model is missing.

6.2 Experimental model

The best experimental model to study LIRI and PGD is a lung transplantation model in which both cold and warm ischemia can be investigated. However, such a model is expensive, time-consuming and technically difficult, resulting in an unacceptable high mortality, especially in small animals. Therefore, the majority of experimental studies use ex vivo models, like the Langendorff system, which is a non-physiological model. Also it is impossible to investigate reperfusion times beyond the first hours. A reproducible, technically less demanding, relatively cheap experimental model, in which symptoms comparable to PGD symptoms can be studied up to months after reperfusion, is missing.

6.3 Detailed characterization of primary graft dysfunction parameters

Since most studies have only investigated the early hours of reperfusion, the effect of severe LIRI up to months after reperfusion is unknown [22, 36-48]. Furthermore a detailed description and the time course of several parameters, like pulmonary function, capillary permeability, inflammation, surfactant composition and histological changes, up to months after LIRI is lacking.

6.4 Effect of lung ischemia-reperfusion injury on the non-ischemic lung

The changes in the native lung after contralateral lung transplantation are not well established, especially on the long term.

6.5 Surfactant treatment for primary graft dysfunction

To control the disturbed intra-alveolar fluid homeostasis after LIRI, surfactant replacement therapy has been investigated [21, 22, 43, 47, 49, 50]. The administration of exogenous surfactant just before or after reperfusion resulted in improved lung function within hours after reperfusion in experimental models. Most of these studies have a short follow-up time of maximal 120

minutes after reperfusion. Studies regarding the effect of surfactant treatment with longer follow-up are scarce. A study by Erasmus and colleagues demonstrated that surfactant treatment at reperfusion enhanced recovery from LIRI at one week postoperatively [49, 51]. However, treatment with surfactant before the onset of ischemia may be more beneficial [50]. The effect of surfactant treatment before ischemia up to months after reperfusion is unknown. Secondly, surfactant replacement therapy for patients with PGD grade 3 could be considered up to days after reperfusion. It is, however, questionable whether surfactant treatment still makes sense at this time. Since the cost of surfactant is the most important limitation for clinical use, it is also important to determine the lowest surfactant dose that reduces LIRI or prevents PGD. Finally, surfactant also has shown strong immunomodulatory properties in vitro [7, 52-54]. Despite these clear effects of surfactant in vitro, data on the immunomodulatory effect of surfactant in vivo are scarce and contradictory. For instance, Vreugdenhil and colleagues showed exogenous surfactant to restore lung function, but with no effect on proinflammatory cytokine expression in the lung [55]. In line with these data, exogenous surfactant therapy had no effect on proinflammatory mediators in several experimental models [56-58]. However, Stamme and coworkers reported increased tumor necrosis factor alpha and interleukin-6 production after surfactant therapy for ventilation-induced lung injury, while Rasaiah and colleagues reported decreased tumor necrosis factor alpha and Interleukin-6 production after surfactant therapy for sepsis-induced lung injury [59, 60].

6.6 Alveolar preservation strategies to minimize primary graft dysfunction

Although organ preservation in transplantation has been studied extensively with the majority of studies focussing on the composition of the preservation solution, the optimal inflation pressure and oxygen concentration are relatively underexposed and the best alveolar inflation protocol to preserve pulmonary grafts during the ischemic period is unclear. Some studies have demonstrated adverse effects of inflation pressures between 11 and 27 cm H_2O [61-63], while others have provided evidence for a protective effect of static inflation with pressures up to 30 cm H_2O [64- 68]. Furthermore, inflation with 100% oxygen may protect the lung against LIRI [69], but it may also aggravate LIRI [61, 70] or has no effect [66, 68, 71]. Disadvantages of these studies are that they make use of ex vivo models or use short reperfusion times up to 360 minutes while lungs are still being ventilated, which by itself interferes with the outcome of LIRI [72]. Studying the effect of alveolar inflation on PGD parameters measured later in the reperfusion period without these interfering effects, is more valuable.

6.7 Ventilation strategies to reduce primary graft dysfunction

Patients who develop PGD after lung transplantation are at increased risk of prolonged mechanical ventilation, putting them at risk for ventilator-induced-lung injury on top of PGD [72]. Studies on mechanical ventilation in ARDS patients and models have demonstrated that a reduction in tidal volume from 12 to 6-8 ml/kg decreases mortality and improves pulmonary function [73, 74]. Since then, the ARDS network proclaims to minimize lung strain while maintaining minimal acceptable gas exchange in these patients [74]. Ventilator settings are adjusted to a tidal volume as low as 4-6 ml/kg and a combination of positive-end-expiratory-pressure (PEEP) and FiO_2 so that the inspiratory pressure is below 30 cm H_2O and

oxygen saturation between 88 and 95%. This approach is non-individual, non-physiological and is associated with permissive atelectasis and consequently shear stress. An alternative approach is open lung ventilation (OLV), which is a more individual approach based on physiologic principles [75-80]. The aim of OLV is to minimize cyclic alveolar collapse and reopening. An open lung is characterized by minimal atelectasis, resulting in a low rate of pulmonary shunting and thus optimal gas exchange, corresponding with a PaO_2/FiO_2 ratio above 450 mm Hg [75]. Therefore, the first step is to recruit the closed alveoli. Once the lung is open, the next step is to prevent closing by applying the minimal PEEP level needed to keep the alveolus open. Finally, the risk of alveolar overdistension is reduced by decreasing the tidal volume to an as low as possible level while the required gas exchange is secured. The clinical setting of mechanical ventilation in lung transplantation patients is special, since the start of a potential PGD can exactly be predicted. Therefore an optimal ventilator approach can be started early in the process so that ventilator-induced-lung injury can be kept to a minimum. In addition, the use of early onset OLV may prevent PGD grade 3.

7 | References

1. Shields TW, Locicero III J, Reed CE, Feins RH. General Thoracic Surgery 2009.
2. Moore KL, Dalley AF. Clinically Oriented Anatomy 2006.
3. Rhoades RA, Tanner GA. Medical Physiology 1995.
4. Griese M. Pulmonary surfactant in health and human lung diseases: State of the art. Eur Respir J 1999;13:1455-1476.
5. Haitsma JJ, Papadakos PJ, Lachmann B. Surfactant therapy for acute lung injury/acute respiratory distress syndrome. Curr Opin Crit Care 2004;10:18-22.
6. Reid KB, Clark H, Palaniyar N. Surfactant and lung inflammation. Thorax 2005;60:620-622.
7. Wright JR. Immunoregulatory functions of surfactant proteins. Nat Rev Immunol 2005;5:58-68.
8. Arcasoy SM, Kotloff RM. Lung transplantation. N Engl J Med 1999;340:1081-1091.
9. Orens JB, Boehler A, de Perrot M, et al. A review of lung transplant donor acceptability criteria. J Heart Lung Transplant 2003;22:1183-1200.
10. Orens JB, Estenne M, Arcasoy S, et al. International guidelines for the selection of lung transplant candidates. 2006 J Heart Lung Transplant 2006;25:745-755.
11. McShane PJ, Garrity Jr ER. Impact of the lung allocation score. Semin Respir Crit Care Med 2013;34:275-280.
12. Schiavon M, Falcoz PE, Santelmo N, Massard G. Does the use of extended criteria donors influence early and long-term results of lung transplantation? Interact Cardiovasc Thorac Surg 2012;14:183-187.
13. Yusen RD, Christie JD, Edwards LB, et al. The Registry of the International Society for Heart and Lung Transplantation: Thirtieth Adult Lung and Heart-Lung Transplant Report. J Heart Lung Transplant 2013;32:965-978.
14. Lee JC, Christie JD. Primary graft dysfunction. Proc Am Thorac Soc 2009;6:39-46.
15. Suzuki Y, Cantu E, Christie JD. Primary graft dysfunction. Semin Respir Crit Care Med 2013;34:305-319.
16. Todd JL, Palmer SM. Bronchiolitis obliterans syndrome: The final frontier for lung transplantation. Chest 2011;140:502-508.
17. de Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia-reperfusion-induced lung injury. Am J Respir Crit Care Med 2003;167:490-511.
18. Grace PA, Mathie RT. Ischemia-Reperfusion-Injury 1999.
19. Ware LB, Matthay MA. The acute respiratory distress syndrome. N Engl J Med 2000;342:1334-1349.
20. Veldhuizen RA, Lee J, Sandler D, et al. Alterations in pulmonary surfactant composition and activity after experimental lung transplantation. Am Rev Respir Dis 1993;148:208-215.
21. Erasmus ME, Petersen AH, Oetomo SB, Prop J. The function of surfactant is impaired during the reimplantation response in rat lung transplants. J Heart Lung Transplant 1994;13:791-802.
22. Novick RJ, Veldhuizen RA, Possmayer F, Lee J, Sandler D, Lewis JF. Exogenous surfactant therapy in thirty-eight hour lung graft preservation for transplantation. J Thorac Cardiovasc Surg 1994;108:259-268.
23. Hohlfeld JM, Tiryaki E, Hamm H, et al. Pulmonary surfactant activity is impaired in lung transplant recipients. Am J Respir Crit Care Med 1998;158:706-712.
24. Boehler A, Estenne M. Post-transplant bronchiolitis obliterans. Eur Respir J 2003;22:1007-1018.
25. Tullius SG, Tilney NL. Both alloantigen-dependent and -independent factors influence chronic allograft rejection. Transplantation 1995;59:313-318.
26. Thabut G, Mal H, Cerrina J, et al. Graft ischemic time and outcome of lung transplantation: A multicenter analysis. Am J Respir Crit Care Med 2005;171:786-791.
27. Meyer DM, Bennett LE, Novick RJ, Hosenpud JD. Effect of donor age and ischemic time on intermediate survival and morbidity after lung transplantation. Chest 2000;118:1255-1262.
28. Sleiman C, Mal H, Fournier M, et al. Pulmonary reimplantation response in single-lung transplantation. Eur Respir J 1995;8:5-9.
29. Snell GI, Rabinov M, Griffiths A, et al. Pulmonary allograft ischemic time: An important predictor of survival after lung transplantation. J Heart Lung Transplant 1996;15:160-168.
30. Novick RJ, Bennett LE, Meyer DM, Hosenpud JD. Influence of graft ischemic time and donor age on survival after lung transplantation. J Heart Lung Transplant 1999;18:425-431.
31. Sommers KE, Griffith BP, Hardesty RL, Keenan RJ. Early lung allograft function in twin recipients from the same donor: Risk factor analysis. Ann Thorac Surg 1996;62:784-790.
32. Kshettry VR, Kroshus TJ, Burdine J, Savik K, Bolmon RM. Does donor organ ischemia over four hours

affect long-term survival after lung transplantation? J Heart Lung Transplant 1996;15:169-174.
33. Glanville AR, Marshman D, Keogh A, et al. Outcome in paired recipients of single lung transplants from the same donor. J Heart Lung Transplant 1995;14:878-882.
34. Gammie JS, Stukus DR, Pham SM, et al. Effect of ischemic time on survival in clinical lung transplantation. Ann Thorac Surg 1999;68:2015-9; discussion 2019-2020.
35. Fiser SM, Kron IL, Long SM, et al. Influence of graft ischemic time on outcomes following lung transplantation. J Heart Lung Transplant 201;20:1291-1296.
36. Burne MJ, Daniels F, El Ghandour A, et al. Identification of the CD4(+) T cell as a major pathogenic factor in ischemic acute renal failure. J Clin Invest 2001;108:1283-1290.
37. de Perrot M, Young K, Imai Y, et al. Recipient T cells mediate reperfusion injury after lung transplantation in the rat. J Immunol 2003;171:4995-5002.
38. Deeb GM, Grum CM, Lynch MJ, et al. Neutrophils are not necessary for induction of ischemia-reperfusion lung injury. J Appl Physiol 1990;68:374-381.
39. Eppinger MJ, Deeb GM, Bolling SF, Ward PA. Mediators of ischemia-reperfusion injury of rat lung. Am J Pathol 1997;150:1773-1784.
40. Eppinger MJ, Jones ML, Deeb GM, Bolling SF, Ward PA. Pattern of injury and the role of neutrophils in reperfusion injury of rat lung. J Surg Res 1995;58:713-718.
41. Fiser SM, Tribble CG, Long SM, et al. Lung transplant reperfusion injury involves pulmonary macrophages and circulating leukocytes in a biphasic response. J Thorac Cardiovasc Surg 2001;121:1069-1075.
42. Fiser SM, Tribble CG, Long SM, Kaza AK, Kern JA, Kron IL. Pulmonary macrophages are involved in reperfusion injury after lung transplantation. Ann Thorac Surg 2001;71:1134-1138; discussion 1138-1139.
43. Friedrich I, Borgermann J, Splittgerber FH, et al. Bronchoscopic surfactant administration preserves gas exchange and pulmonary compliance after single lung transplantation in dogs. J Thorac Cardiovasc Surg 2004;127:335-343.
44. Gunther A, Balser M, Schmidt R, et al. Surfactant abnormalities after single lung transplantation in dogs: impact of bronchoscopic surfactant administration. J Thorac Cardiovasc Surg 2004;127:344-354.
45. Maitra G, Inchley K, Novick RJ, Veldhuizen RA, Lewis JF, Possmayer F. Acute lung injury and lung transplantation influence in vitro subtype conversion of pulmonary surfactant. Am J Physiol Lung Cell Mol Physiol 2002;282:L67-74.
46. Nakamura T, Abu-Dahab R, Menger MD, et al. Depletion of alveolar macrophages by clodronate-liposomes aggravates ischemia-reperfusion injury of the lung. J Heart Lung Transplant 2005;24: 38-45.
47. Novick RJ, Gilpin AA, Gehman KE, et al. Mitigation of injury in canine lung grafts by exogenous surfactant therapy. J Thorac Cardiovasc Surg 1997;113:342-353.
48. Steimle CN, Guynn TP, Morganroth ML, Bolling SF, Carr K, Deeb GM. Neutrophils are not necessary for ischemia-reperfusion lung injury. Ann Thorac Surg 1992;53:64-72; discussion 72-73.
49. Erasmus ME, Hofstede GJ, Petersen AH, Haagsman HP, Oetomo SB, Prop J. Effects of early surfactant treatment persisting for one week after lung transplantation in rats. Am J Respir Crit Care Med 1997;156:567-572.
50. Hausen B, Rohde R, Hewitt CW, et al. Exogenous surfactant treatment before and after sixteen hours of ischemia in experimental lung transplantation. J Thorac Cardiovasc Surg 1997;113:1050-1058.
51. Erasmus ME, Petersen AH, Hofstede G, Haagsman HP, Bambang Oetomo S, Prop J. Surfactant treatment before reperfusion improves the immediate function of lung transplants in rats. Am J Respir Crit Care Med 1996;153:665-670.
52. Antal JM, Divis LT, Erzurum SC, Wiedemann HP, Thomassen MJ. Surfactant suppresses NF-kappa B activation in human monocytic cells. Am J Respir Cell Mol Biol 1996;14:374-379.
53. Thomassen MJ, Meeker DP, Antal JM, Connors MJ, Wiedemann HP. Synthetic surfactant (Exosurf) inhibits endotoxin-stimulated cytokine secretion by human alveolar macrophages. Am J Respir Cell Mol Biol 1992;7:257-260.
54. Baur FM, Brenner B, Goetze-Speer B, Neu S, Speer CP. Natural porcine surfactant (Curosurf) down-regulates mRNA of tumor necrosis factor-alpha (TNF-alpha) and TNF-alpha type II receptor in lipopolysaccharide-stimulated monocytes. Pediatr Res 1998;44:32-36.
55. Vreugdenhil HA, Lachmann B, Haitsma JJ, et al. Exogenous surfactant restores lung function but not peripheral immunosuppression in ventilated surfactant-deficient rats. Exp Lung Res 2006;32:1-14.
56. Kinniry P, Pick J, Stephens S, et al. KL4-surfactant prevents hyperoxic and LPS-induced lung injury in mice. Pediatr Pulmonol 2006;41:916-928.

57. Krause MF, Wiemann T, Reisner A, Orlowska-Volk M, Köhler H, Ankermann T. Surfactant reduces extravascular lung water and invasion of polymorphonuclear leukocytes into the lung in a piglet model of airway lavage. Pulm Pharmacol Ther 2005;18:129-139.
58. van Kaam AH, Lutter R, Lachmann RA, et al. Effect of ventilation strategy and surfactant on inflammation in experimental pneumonia. Eur Respir J 2005;26:112-117.
59. Stamme C, Brasch F, von Bethmann A, Uhlig S. Effect of surfactant on ventilation-induced mediator release in isolated perfused mouse lungs. Pulm Pharmacol Ther 2002;15:455-461.
60. Rasaiah VPA, Malloy JL, Lewis JF, Veldhuizen RAW. Early surfactant administration protects against lung dysfunction in a mouse model of ARDS. Am J Physiol Lung Cell Mol Physiol 2003;284:L783-790.
61. Haniuda M, Hasegawa S, Shiraishi T, Dresler CM, Cooper JD, Patterson GA. Effects of inflation volume during lung preservation on pulmonary capillary permeability. J Thorac Cardiovasc Surg 1996;112:85-93.
62. Aoe M, Okabayashi K, Cooper JD, Patterson GA. Hyperinflation of canine lung allografts during storage increases reperfusion pulmonary edema. J Thorac Cardiovasc Surg 1996;112:94-102.
63. Patel MR, Laubach VE, Tribble CG, Kron IL. Hyperinflation during lung preservation and increased reperfusion injury. J Surg Res 2005;123:134-138.
64. Fukuse T, Hirata T, Nakamura T, Kawashima M, Hitomi S, Wada H. Influence of deflated and anaerobic conditions during cold storage on rat lungs. Am J Respir Crit Care Med 1999;160:621-627.
65. Hausen B, Ramsamooj R, Hewitt CW, et al. The importance of static lung inflation during organ storage: The impact of varying ischemic intervals in a double lung rat transplantation model. Transplantation 1996;62:1720-1725.
66. Sakuma T, Takahashi K, Ohya N, et al. Ischemia-reperfusion lung injury in rabbits: mechanisms of injury and protection. Am J Physiol 1999;276:L137-145.
67. Puskas JD, Hirai T, Christie N, Mayer E, Slutsky AS, Patterson GA. Reliable thirty-hour lung preservation by donor lung hyperinflation. J Thorac Cardiovasc Surg 1992;104:1075-1083.
68. Kao SJ, Wang D, Yeh DY, Hsu K, Hsu YH, Chen HI. Static inflation attenuates ischemia/reperfusion injury in an isolated rat lung in situ. Chest 2004;126:552-558.
69. Weder W, Harper B, Shimokawa S, et al. Influence of intraalveolar oxygen concentration on lung preservation in a rabbit model. J Thorac Cardiovasc Surg 1991;101:1037-1043.
70. Fukuse T, Hirata T, Ishikawa S, et al. Optimal alveolar oxygen concentration for cold storage of the lung. Transplantation 2001;72:300-304.
71. Watanabe A, Kawaharada N, Kusajima K, Komatsu S, Abe T, Takahashi H. Influence of oxygen in inflation gas during lung ischemia on ischemia-reperfusion injury. J Thorac Cardiovasc Surg 1997;114:332-338.
72. de Perrot M, Imai Y, Volgyesi GA, et al. Effect of ventilator-induced lung injury on the development of reperfusion injury in a rat lung transplant model. J Thorac Cardiovasc Surg 2002;124:1137-1144.
73. Halter JM, Steinberg JM, Gatto LA, et al. Effect of positive end-expiratory pressure and tidal volume on lung injury induced by alveolar instability. Crit Care 2007;11:R20.
74. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network. N Engl J Med 2000;342:1301-1308.
75. Lachmann B. Open up the lung and keep the lung open. Intensive Care Med 1992;18:319-321.
76. Spieth PM, Güldner A, Carvalho AR, et al. Open lung approach vs acute respiratory distress syndrome network ventilation in experimental acute lung injury. Br J Anaesth 2011;107:388-397.
77. Spieth PM, Carvalho AR, Pelosi P, et al. Variable tidal volumes improve lung protective ventilation strategies in experimental lung injury. Am J Respir Crit Care Med 2009;179:684-693.
78. van Kaam AH, Haitsma JJ, De Jaegere A, van Aalderen WM, Kok JH, Lachmann B. Open lung ventilation improves gas exchange and attenuates secondary lung injury in a piglet model of meconium aspiration. Crit Care Med 2004;32:443-449.
79. van Veenendaal MB, van Kaam AH, Haitsma JJ, Lutter R, Lachmann B. Open lung ventilation preserves the response to delayed surfactant treatment in surfactant-deficient newborn piglets. Crit Care Med 2006;34:2827-2834.
80. Reis Miranda D, Gommers D, Struijs A, et al. Ventilation according to the open lung concept attenuates pulmonary inflammatory response in cardiac surgery. Eur J Cardiothorac Surg 2005;28:889-895.

Outline of the thesis

The aim of this thesis is:

- To develop a preclinical in vivo model of primary graft dysfunction.
- To assess the relation between warm lung ischemia-reperfusion injury and primary graft dysfunction related symptoms.
- To characterize primary graft dysfunction days to months after reperfusion in terms of inflammation, pulmonary function, surfactant alterations and histology.
- To evaluate treatment strategies at the time of harvest that can prevent primary graft dysfunction.
- To evaluate treatment strategies after the development of primary graft dysfunction.

Literature review and development of a preclinical in vivo model

Chapter 2 reviews the pathophysiology of lung ischemia-reperfusion injury, its effect on the endogenous pulmonary surfactant system, and the considerations for surfactant treatment. Chapter 3 describes the development of a preclinical PGD model, determines the effect of warm LIRI on PGD development, characterizes PGD up to months after reperfusion and demonstrates the effect of LIRI on the contralateral lung. Chapter 4 is an invited commentary on the interaction between mediators produced by hypoxia exposed alveolar macrophages and the subsequent inflammatory response of type 2 pneumocytes to oxidative stress. Chapter 5 is an invited commentary on the relationship between viral infection and development of BOS.

Surfactant treatment options

This part of the thesis describes the effect of surfactant treatment on PGD. In Chapter 6 we determine whether surfactant treatment of the donor is effective in reducing symptoms seen with PGD up to 7 days after the operation. The experiment in Chapter 7 was conducted to test whether surfactant pretreatment was able to optimize pulmonary function up to months after reperfusion. Since the cost of surfactant limits its use in the clinical setting, the experiment in Chapter 8 was performed to test the lowest possible surfactant dose that reduces PGD and to investigate whether surfactant treatment after reperfusion still has a beneficial effect compared to pretreatment. In Chapter 9 we examined the effect of exogenous surfactant on additional inflammatory and apoptotic factors in the lung.

Mechanical ventilation strategies

The influence of alveolar preservation pressure and oxygen concentration on the development of PGD is determined in Chapter 10. In Chapter 11, we compared mechanical ventilation strategies started at the time of reperfusion to evaluate their effect on PGD development.

2

Surfactant alterations and treatment in lung transplant ischemia-reperfusion injury

Based on
NP van der Kaaij, RA Lachmann, AJJC Bogers, B Lachmann
Journal of Organ Dysfunction 2006;2:221-229

Summary

This review addresses surfactant alterations in and treatment options for lung transplant ischemia-reperfusion injury. Lung ischemia-reperfusion injury damages the endogenous surfactant system by the production of reactive oxygen species, proteolytic enzymes and (phospho)lipases. Surfactant is composed of phospholipids and proteins and its main function is to reduce the surface tension inside the alveolus. Impairment of surfactant will cause atelectasis, influx of serum proteins, pulmonary edema, decreased lung compliance, and impaired gas exchange. Surfactant therapy restores the quantity and composition of surfactant and reduces the inhibitory effect of serum proteins. Other effects are that it serves as an anti-oxidant and anti-inflammatory agent. Pretreatment may be more beneficial than treatment after the development of lung ischemia-reperfusion injury. However, the cost of surfactant must be weighed against clinical outcome.

1 | Lung transplantation

Lung transplantation is nowadays a well-accepted treatment option for patients with end-stage pulmonary diseases. Although improvements in lung preservation, peri– and post-operative care and surgical techniques have been made, the outcome of lung transplantation remains limited [1, 2]. Development of primary graft dysfunction (PGD) is the main cause for early morbidity and mortality after lung transplantation, resulting in a one-year survival of approximately 80% [1, 2]. Moreover, long-term prognosis is also limited (5-year survival <60%) due to the development of bronchiolitis obliterans syndrome (BOS) [2]. Lung ischemia-reperfusion injury (LIRI) is thought to significantly contribute to both PGD and BOS [2-4].

1.1 Primary graft dysfunction

PGD, which symptomatically resembles the acute respiratory distress syndrome (ARDS), develops within 72 hours after transplantation [5]. Symptoms of PGD consist of non-cardiogenic pulmonary edema, increased pulmonary artery pressure, decreased lung compliance and impaired gas exchange [5-9]. Histological analysis of PGD lungs shows diffuse alveolar damage with micro-atelectasis. Although approximately 97% of the recipients show some degree of reperfusion edema on chest X-ray, severe LIRI occurs in 15-30% of lung transplant recipients [6]. Experimental LIRI studies have shown that abnormalities and depletion of pulmonary surfactant (surface active agent) significantly contribute to the symptoms seen with PGD [7-10]. Furthermore, surfactant obtained through bronchoalveolar lavage (BAL) of human lung transplant recipients, demonstrated surfactant dysfunction up to seven years after transplantation, thereby contributing to lung malfunction [11]. Pulmonary surfactant is essential for normal breathing, since it diminishes the surface tension at the air-fluid interface inside the alveolus [12]. As a result, the alveolus is kept open at the end of expiration and fluid homeostasis is preserved [12].

1.2 Bronchiolitis obliterans syndrome

Although severe LIRI considerably contributes to the development of PGD, the impact of LIRI on long-term mortality is subject of debate. The major obstacle to long-term survival is the development of post lung transplant BOS [2, 13]. BOS affects about 50% of patients who survive beyond 3 months after transplantation [13]. The pathogenesis of BOS is not completely understood, but appears to involve a "response to injury" type of pattern, where multiple injuries may finally result in BOS (Figure 1). Both donor characteristics and transplant procedure complications may result in early lung injury. Reperfusion injury may aggravate early lung injury, whereafter rejection and allo-antigen independent factors (pneumonia, cytomegalovirus) can act as subsequent injuries and increase the risk of BOS [13, 14].

2 | Lung ischemia-reperfusion injury

LIRI predominantly occurs in lung transplantations. However, LIRI symptoms have also been described after cardio-pulmonary bypass, isolated lung perfusion, and pulmonary sleeve resection. Since severe LIRI plays a significant role in the development of PGD after lung transplantation and since LIRI is an early actor in the multiple hit theory of BOS,

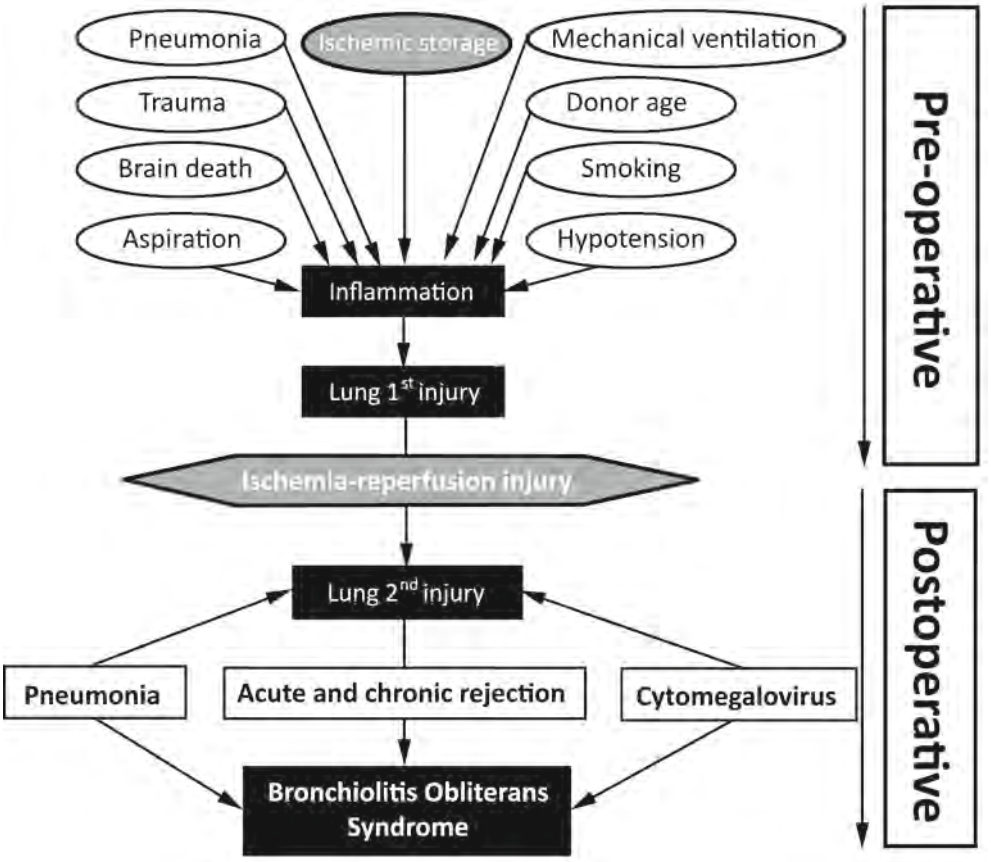

Figure 1. Lung transplantation: A multiple hit theory for the development of bronchiolitis obliterans syndrome.

treatment of LIRI may decrease the severity of PGD, but may also prevent or delay the onset of BOS, thereby influencing late morbidity and mortality of lung transplantation.

2.1 Pathophysiology

Ischemia can be defined by a blood (oxygen) deficiency in the lung caused by a constriction or obstruction of its blood vessels. Reperfusion occurs when the blood flow to the organ is restored. During the transplantation period, the lung is hypothermically stored to reduce the rate of biochemical reactions, which results in a decreased degradation of cellular components. Still, adenosine triphosphate (ATP) is depleted during ischemia, which ultimately causes inactivation of ATP-dependent membrane pumps, accumulation of intracellular calcium, formation of eicosanoids and reactive oxygen species (ROS), inflammation and cell death (Figure 2) [1, 15].

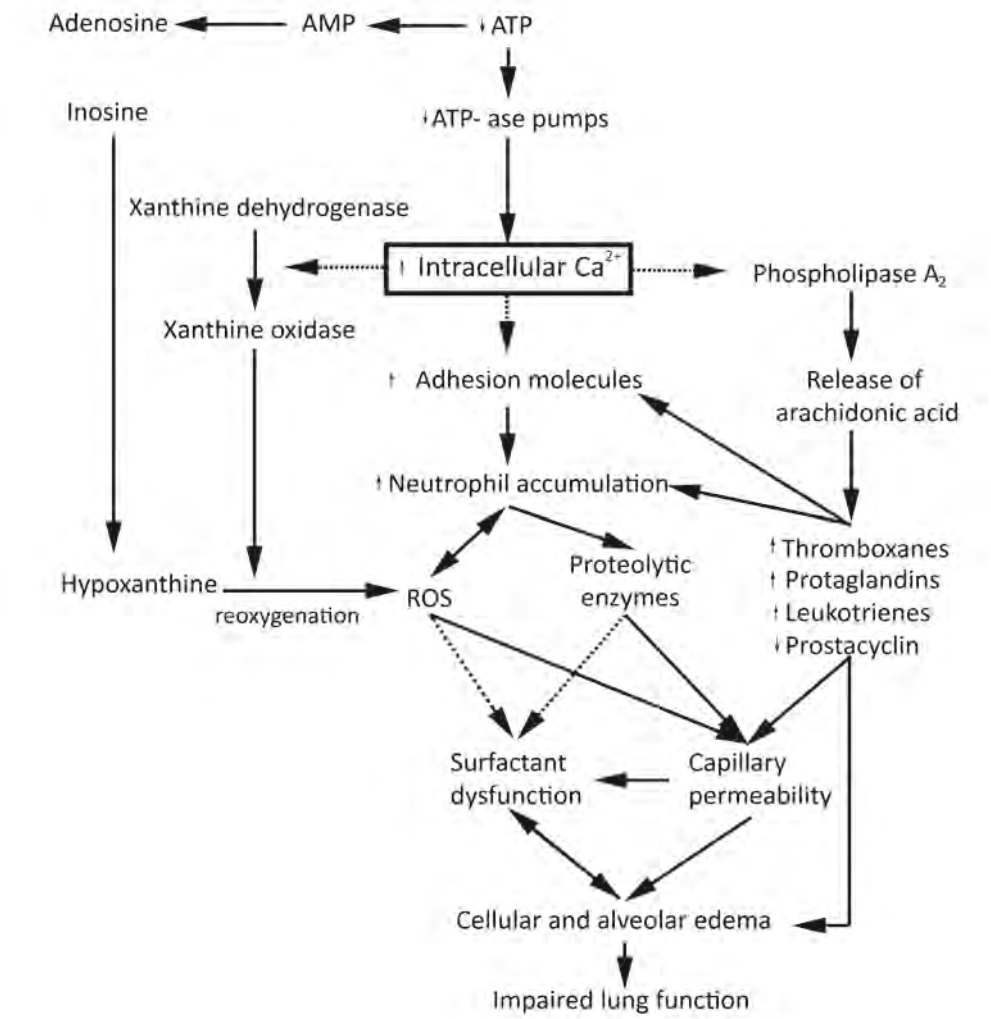

Figure 2. Pathophysiology of lung ischemia-reperfusion injury. See text for explanation. ATP: Adenosine triphosphate; AMP: Adenosine monophosphate; ROS: Reactive oxygen species.

2.2 ATP-dependent membrane pumps and intracellular calcium

Under normal conditions, the action of the Na^+/K^+-ATPase pump sets up a gradient of high extracellular Na^+ relative to intracellular levels, which in turn drives the Na^+/Ca^{2+}-exchanger, so that Ca^{2+} is pumped out of the cell. During ATP depletion, the Na^+/K^+-ATPase pump becomes inactivated, leading to an increase of intracellular Na^+. As a result, the Na^+/Ca^{2+} pump will not function, causing Ca^{2+} to accumulate inside the cell. Other mechanisms contributing to high intracellular Ca^{2+} levels are an inactive plasmalemmal ATP-dependent Ca^{2+}-pump, liberation of stored cytoplasmic Ca^{2+} due to acidosis, and a decreased uptake by the sarcoplasmic/endoplasmic reticulum [1, 15].

Cytosol elevated Ca^{2+} activates phospholipase A_2, which results in the induction of arachidonic acid. Arachidonic acid is normally incorporated in the cell membrane and functions as a precursor for the production of eicosanoids, consisting of thromboxanes, leukotrienes, prostacyclin and prostaglandins. Where thromboxanes are predominantly produced by platelets, leukotrienes are formed by leukocytes, prostacyclin by endothelial cells and prostaglandins by smooth muscle cells. The effects of eicosanoids are various but include vasoconstriction, activation of adhesion molecules, the increase in capillary permeability, and the accumulation and extravasation of neutrophils [1, 15]. Finally, increased intracellular Ca^{2+} causes transformation of xanthine dehydrogenase into xanthine oxidase, thereby facilitating the production of ROS, as described in the next section [1, 15].

2.3 Production of reactive oxygen species

In the aerobic setting ATP is converted to urea and xanthine by the effect of xanthine dehydrogenase. However, due to the formation of xanthine oxidase in LIRI, hypoxanthine is broken down into ROS at the moment of reoxygenation. A second system to generate ROS is by the NADPH oxidase system, which is predominantly present on the membrane surfaces of monocytes, macrophages, neutrophils and endothelial cells and catalyses the reduction of oxygen to superoxide and hydrogen peroxide. The superoxide anion, hydrogen peroxide and hydroxyl radical, which all are part of the family of ROS, are very unstable and damage cellular membranes by lipid peroxidation [1, 15].

2.4 Inflammation

LIRI causes release of proinflammatory cytokines by macrophages. Consequently, neutrophils and lymphocytes are recruited into the lung. Because of expression of adhesion molecules on endothelium and leukocytes, leukocytes roll, adhere, and extravasate into the lung tissue. Macrophages and neutrophils contribute to cellular damage by the production of ROS and several other mediators, such as proteolytic enzymes, lysozyme, and lactoferrin [1, 15].

3 | Studying LIRI

Several experimental models can be used to study treatment modalities and the pathophysiology of LIRI. Although the best model is obviously a lung transplantation model, this is a very time-consuming procedure, especially in small animals, and is technically difficult with often high mortality rates. Therefore, an in situ lung clamp model, in which the bronchus, pulmonary veins and artery of (usually) the left lung are clamped to induce LIRI, has been developed. Clamping time generally ranges from 60 to 150 minutes [10, 16, 17]. Although this model is technically much easier than a transplantation model, there are some disadvantages. Firstly, only warm ischemia can be studied since the lung is kept in situ. Furthermore, no preservation solutions are used and mortality may still be high, because (preferably) the animals are ventilated after reperfusion for as short periods as possible due to the confounding effects of ventilation on LIRI [18].

Although the clamp model does not fully resemble the events of real lung transplantation, the symptoms of LIRI found in this model approximate the symptoms seen with PGD. The

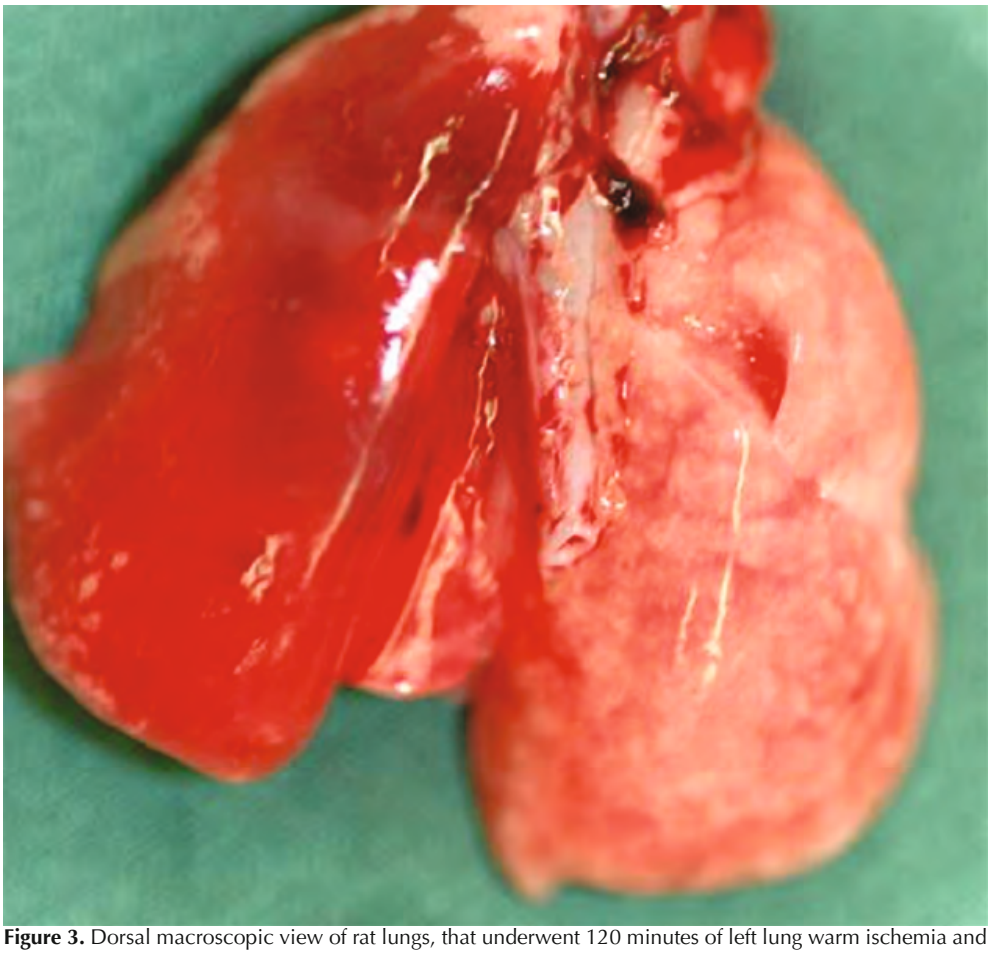

Figure 3. Dorsal macroscopic view of rat lungs, that underwent 120 minutes of left lung warm ischemia and 180 minutes of ventilation after reperfusion. LIRI was not induced in the right lung.

macroscopic result of severe LIRI (120 minutes) is demonstrated in Figure 3. Figure 4 shows the microscopic effect of 120 minutes of warm ischemia, 24 hours after reperfusion as compared to the right non-ischemic lung. LIRI results in intra-alveolar and septal edema, inflammation, atelectasis, and intra-alveolar hemorrhage.

4 | Surfactant

Surfactant is responsible for decreasing the surface tension between air and the alveolo-capillary membrane. It hereby prevents the alveoli of the lung from collapsing at the end of expiration, facilitating inflation of the lung with minimal effort [12]. In addition, lowering the surface tension is important for fluid homeostasis in the lung. Furthermore, surfactant protects the lung against micro-organisms and serves as a functional barrier in the alveolus,

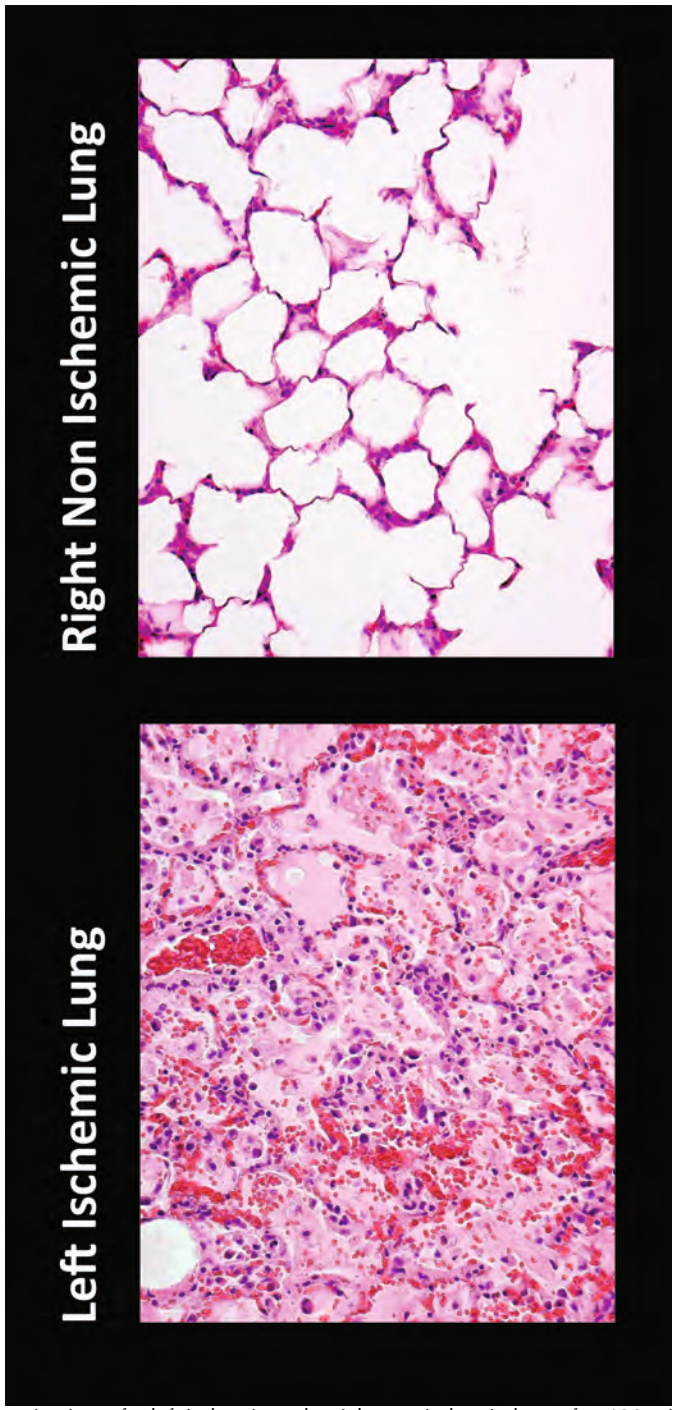

Figure 4. Microscopic view of a left ischemic and a right non ischemic lung after 120 minutes of left lung warm ischemia. Histological assessment was performed 24 hours after reperfusion. LIRI results in intra-alveolar and septal edema, inflammation, atelectasis, and intra-alveolar hemorrhage.

so that the transfer of molecules across the alveolo-capillary membrane is limited. Finally, surfactant is presumed to have immune downregulating effects [12, 19].

Surfactant is composed of lipids (90%) (mainly dipalmitoyl-phosphatidylcholine (DPPC)) and surfactant associated proteins (SPs) (10%). The proteins can be divided into two groups: the hydrophilic SP-A and SP-D, and the hydrophobic SP-B and SP-C [12, 19].

The surface lowering capacity of surfactant is predominantly due to DPPC. SP-B and SP-C have been demonstrated to enhance lipid insertion into the monolayer at the air-liquid interface. In this way they protect the surface film from being contaminated by non-surfactant proteins that degrade surfactant [12 ,19]

SP-A and SP-D are believed to be molecules of the innate immune system through their ability to recognize a broad spectrum of pathogens. SP-A and SP-D interact with a number of viruses, bacteria, fungi, and allergens. SP-A has also been suggested to play an important role in phospholipid secretion and recycling, formation of tubular myelin and blocking surfactant inhibition by serum proteins [12, 19].

Surfactant can be divided by ultra centrifugation in two sub fractions, which differ in morphological appearance and density. The heavy subtype or large aggregate (LA) subform of surfactant is highly surface active, contains a high amount of SPs and is made up of tubular myelin, lamellar bodies and large vesicles. The light subtype or small aggregate (SA) subform has a poor surface lowering capacity and consists of small vesicles. When a lung sustains injury, a conversion of LA into SA occurs, resulting in an increased SA/LA ratio [7, 10, 12, 19].

Surfactant is produced and secreted by alveolar type II (ATII) cells. ATII cells and alveolar macrophages are important for recycling of surfactant lipids and are thus essential for maintaining the composition of the endogenous surfactant pool [19-21].

5 | Surfactant damage

5.1 Surfactant- associated proteins

SPs are damaged due to the formation of proteases and ROS, resulting in impairment of surfactant recycling (SP-A), inability to block surfactant inhibition by serum proteins (SP-A), and decreased lipid insertion (SP-A & B & C). Lower levels or inactivation of SPs can result in a diminished quantity of phospholipids, but also in a changed composition of surfactant, both interfering with its function [22]. A decrease in SP-A & C was measurable after prolonged ischemic storage without reperfusion and decreased further after the start of reperfusion [22]. Moreover, in lung transplant recipients, the level of SP-A is decreased more than one year after transplantation [23]. Also the level of SP-A decreases with increasing severity of LIRI, suggesting that preservation of SP-A is essential for improvement after LIRI [24]. Some studies have reported surfactant dysfunction without an alteration in the overall amount of phospholipid, but with changes in surfactant composition. In this regard, a decrease in DPPC and phosphatidyl-glycerol and an increase in sphingomyelin have been described [7, 8, 22, 25].

5.2 Plasma proteins

The presence of plasma proteins in the alveoli after LIRI has been reported in many studies [7, 8, 22, 24, 26-35]. Both warm and cold ischemic intervals have resulted in increased levels of plasma protein between 1 and 24 hours after reperfusion [7, 8, 24, 26-29]. Due to ROS, proteolytic enzymes and phospholipases, endogenous surfactant and the endothelial and epithelial membranes are damaged [1, 15]. This results in leakage of proteins into the alveolus and surfactant components into the bloodstream. Since surfactant is rate limiting for the transfer of proteins across the alveolo-capillary membrane and is either inactivated or lost due to increased capillary permeability after LIRI, a further influx of proteins is facilitated. Because proteins, once accumulated in the alveolus, dose-dependently inhibit surfactant, a self-triggering mechanism of surfactant inactivation occurs [36]. Under normal conditions SP-A is partly able to counteract the inactivating effects of serum proteins [37]. However, after LIRI, a decrease in SP-A was found in human lung transplant recipients and animal models of LIRI [23, 24]. Figure 5 summarizes the pathways of the damaging effect of LIRI on surfactant.

6 | Surfactant therapy

Because damaged surfactant contributes to PGD symptoms, surfactant replacement therapy has been investigated using experimental ARDS and LIRI models [9, 22, 24, 26-31, 38, 39]. The effect of surfactant therapy has also been investigated in some case studies.

6.1 Clinical (case) studies

Strüber and colleagues reported in 1995 on a 26-year-old woman, who underwent right-sided lung transplantation and developed PGD 5 hours after transplantation [40]. She was subsequently treated with an intrapulmonary nebulized synthetic surfactant. Shortly hereafter, lung compliance, PaO_2, and tidal volume increased. Moreover, 24 hours after therapy, the edematous infiltrate of the transplanted lung had resolved on chest X-ray. Another study in six lung transplant patients also suggested improvement in severity of PGD after surfactant replacement therapy [41]. However, in 1 of 6 recipients, surfactant therapy failed, which could be attributed to the application approach, the type of surfactant used or the severity of the disease.

6.2 Experimental studies

Although surfactant damage could be reduced by shifting the preservation solution from Euro-Collins to low potassium dextran solution, and by flushing the graft retrograde instead of antegrade, there was still some rationale for the use of surfactant replacement therapy in the case of severe LIRI [31-35, 42]. In this regard, surfactant administration improved lung compliance and PaO_2 and prevented the increase in the SA/LA ratio [26, 28]. Most studies investigating the effect of surfactant replacement therapy have only addressed the first hours after reperfusion. Studies on LIRI and surfactant treatment on the longer term are scarce. Erasmus and colleagues demonstrated that surfactant treatment just before reperfusion enhanced recovery from LIRI [38]. We confirm that LIRI resulted in an increased SA/LA ratio, and impaired PaO_2 and lung compliance throughout the first week after reperfusion [10].

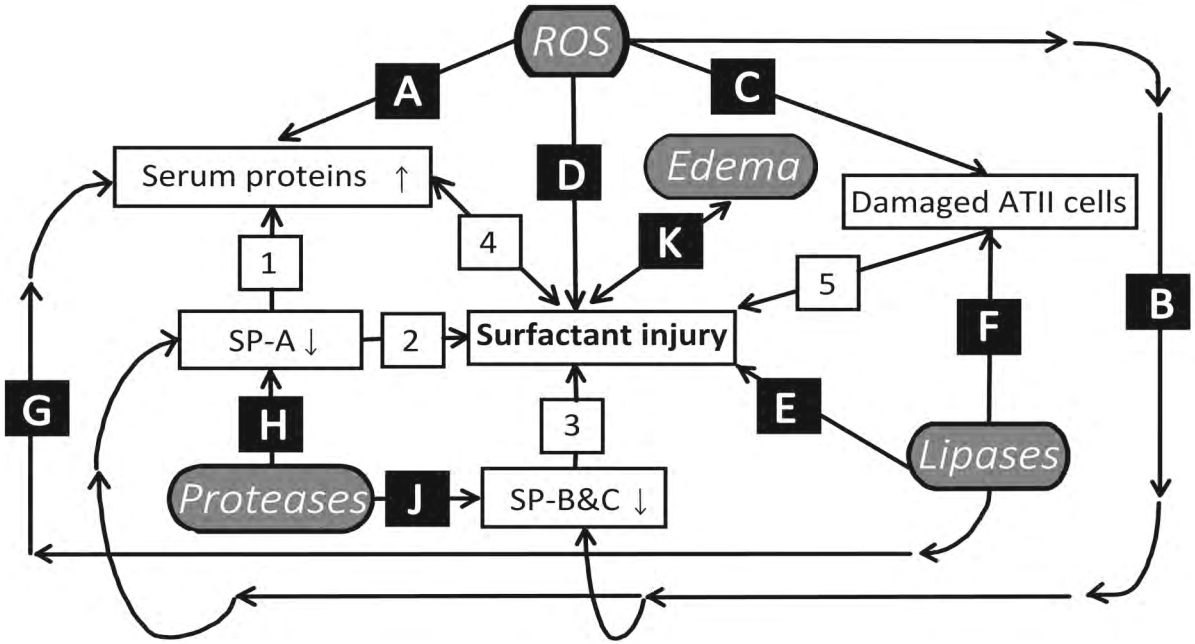

Figure 5. Reactive oxygen species (ROS) directly damage [A] the alveolo-capillary membrane, thereby facilitating the influx of serum proteins into the alveolus, [B] surfactant-associated proteins (SP), [C] alveolar type II (ATII) cells, and [D] the large aggregate (LA) surfactant subtype. Phospholipases cause inactivation of [E] surfactant phospholipids inside the alveolus, [F] the membranes of ATII cells and [G] capillary endothelium, resulting in the influx of serum proteins. Proteinases damage [H] SP-A and [J] SP-B & C. [K] Edema results in dilution of the surfactant phospholipids inside the alveolus, which causes further formation of edema. A decrease in SP-A leads to [1] less inhibition of serum proteins, and [2] impaired phospholipid secretion, recycling, and formation of tubular myelin. [3] SP-B & C injury causes less phospholipids to be inserted into the phospholipid monolayer lining the alveolar epithelium. [4] Once serum proteins have infiltrated the alveolus, they compete for a place at the air-liquid interface, thereby dose-dependently inhibiting surfactant function. Furthermore, once the phospholipid monolayer is damaged, the molecule transfer limiting function of surfactant is also impaired, resulting in further influx of serum proteins, so that a vicious circle has developed. [5] ATII cells, important in the production, recycling and secretion of surfactant phospholipids are injured, so that a reduction in the secretion of large aggregate surfactant subtype occurs and a smaller amount of small aggregate surfactant subtype is being recycled. Due to these factors, a decrease in large aggregate surfactant and an increase in small aggregate surfactant have been recorded after LIRI.

Months after reperfusion, diffuse alveolar damage and decreased lung compliance were still visible [43]. Surfactant pretreatment completely normalized these parameters from day 3 to 90 after reperfusion [10, 44].

6.3 Pretreatment or post treatment

Some studies have suggested that treatment with exogenous surfactant before the onset of ischemia is more beneficial as compared to treatment at or after reperfusion [10, 28, 44-47]. This can be explained by an enlarged surfactant pool before the lung sustains LIRI, thereby preventing deterioration of the entire endogenous surfactant pool due to LIRI. This can be illustrated by the fact that the normal endogenous surfactant pool is about 10-15 mg lipid per kilogram, and that the amount of surfactant used for treatment is in the range of 50-400 mg lipid per kilogram [9, 46]. The surfactant pool is inversely proportional to ischemic time because of the remaining activity of phospholipases during ischemia, so that enlargement

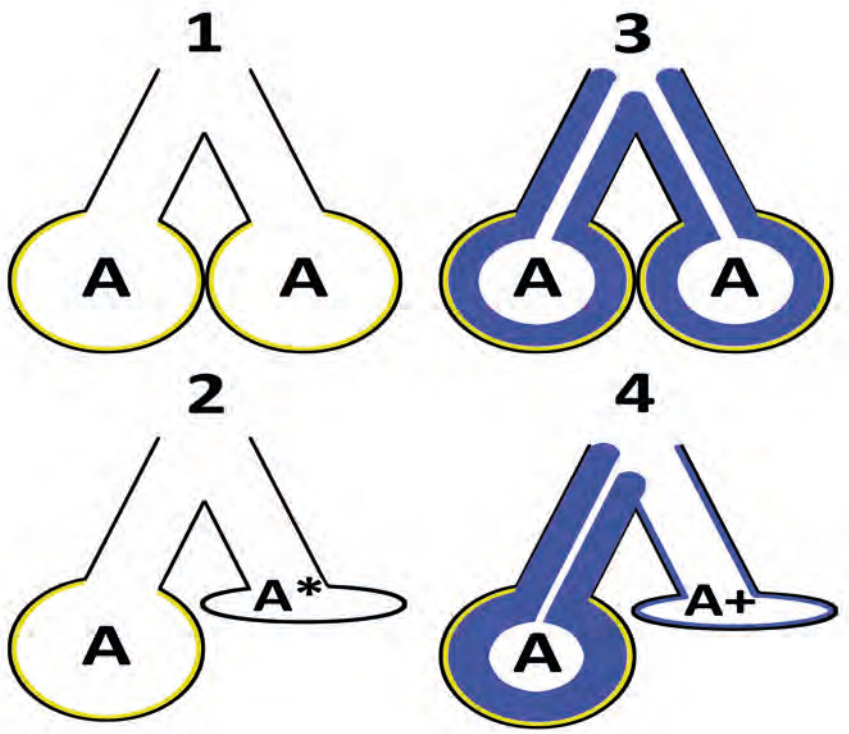

Figure 6. The hypothesis that pretreatment of the lung is more beneficial than treatment after development of lung ischemia-reperfusion injury (LIRI). [1] Normal alveoli (A) of the lung. The normal endogenous surfactant system (yellow) keeps the alveoli open at the end of expiration. [2] Alveoli after the development of LIRI: The endogenous surfactant system is inactivated, resulting in collapse of alveoli (A*). [3] Surfactant pretreatment results in a homogeneous distribution of the exogenous surfactant (blue), so that all alveoli are optimal protected for upcoming injury. [4] Surfactant treatment after LIRI: Exogenous surfactant will predominantly accumulate in the open areas of the lung, whereas it will not arrive in those areas of the lung where it is most needed (A+), resulting in an inhomogeneous distribution of the exogenous surfactant.

of the pool before ischemia reduces the risk of inactivation of the total surfactant pool. Pretreatment also has the advantage of preservation of the endogenous SPs, although some (natural) surfactants may contain SP-B&C. Protecting the endogenous surfactant system by pretreatment seems thus important. Also, if surfactant is given to the donor, this results in a more homogeneous distribution as compared to treatment after reperfusion, when alveolar damage has already occurred (Figure 6) [9]. In the latter case, intratracheally instilled surfactant will predominantly accumulate in open areas of the lung instead of atelectatic areas, where it is most needed. Although surfactant pretreatment may be beneficial in the prevention or downstaging of PGD, the clinical use is troubled by the cost of the material. Therefore, if surfactant (pre)treatment would be considered in the clinical setting, the cost of surfactant must be weighed against the possible improved outcome.

7 | The pathways of surfactant therapy

The rationale behind surfactant replacement therapy is to ameliorate the damage caused by ROS, to preserve the levels of DPPC and SPs, and to decrease the inhibitory effects of serum proteins.

7.1 Anti-inflammatory and antioxidant function

Surfactant inhibits cytokine release from activated monocytes and macrophages [48, 49], while the modulation of lymphocytes has also been suggested [10]. Furthermore, surfactant is known to have antioxidant capacities [50]. Surfactant treatment can thus ameliorate the effects of inflammatory cells, so that endothelial and ATII injury is decreased, normalizing cell permeability and surfactant recycling. Surfactant therapy has been found to preserve the function of ATII cells in LIRI models [23].

7.2 Restoration or preservation of surfactant composition

Surfactant therapy mainly consists of the LA subform (DPPC), which is the active surface tension lowering form of surfactant, so that the level of LA surfactant in the alveolus is restored, which has a major impact on lung function. Surfactant pretreatment preserves the level of LA before the lung sustains ischemia and reperfusion, thereby decreasing lung injury instead of treating lung injury [10]. Surfactant therapy preserves the level of SPs so that their function is maintained [22]. Also, SP-A enriched surfactant improved lung function after prolonged ischemia by an increased recycling capacity, whereas this was not possible to the same extent with SP-A deficient surfactant [24].

7.3 Decreasing the inhibitory effects of serum proteins

When the quantity of surfactant is low, its composition has changed, or capillary permeability has developed, serum proteins leak into the alveolus, where they further interfere with surfactant [51]. Surfactant post treatment may interrupt this vicious circle by restoring the quantity and composition of surfactant phospholipids [26, 36, 52, 53]. Pretreatment with surfactant decreases capillary permeability and preserves surfactant phospholipid composition, quantity, and level of SPs. In this regard, preservation of SP-A is important, due to its inhibiting effect on serum proteins. Cockshutt et al showed a reversed inhibition

of serum proteins when SP-A was administered [37].

8 | Conclusions

In an experimental setting surfactant therapy for severe LIRI has proven to be effective. However, in human lung transplantation it has not yet become standard treatment for PGD. Further studies should investigate whether surfactant (pre)treatment is a realistic option in the field of human lung transplantation.

9 | References

1. de Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia-reperfusion-induced lung injury. Am J Respir Crit Care Med 2003;167:490-511.
2. Hosenpud JD, Bennett LE, Keck BM, Boucek MM, Novick RJ. The Registry of the International Society for Heart and Lung Transplantation: seventeenth official report-2000. J Heart Lung Transplant 2000;19:909-931.
3. Fiser SM, Tribble CG, Long SM, et al. Ischemia-reperfusion injury after lung transplantation increases risk of late bronchiolitis obliterans syndrome. Ann Thorac Surg 2002;73:1041-1047; discussion 1047-1048.
4. Thabut G, Mal H, Cerrina J, et al. Graft ischemic time and outcome of lung transplantation: a multicenter analysis. Am J Respir Crit Care Med 2005;171:786-791.
5. Khan SU, Salloum J, O'Donovan PB, et al. Acute pulmonary edema after lung transplantation: the pulmonary reimplantation response. Chest 1999;116:187-194.
6. Hohlfeld JM, Tiryaki E, Hamm H, et al. Pulmonary surfactant activity is impaired in lung transplant recipients. Am J Respir Crit Care Med 1998;158:706-712.
7. Veldhuizen RA, Lee J, Sandler D, et al. Alterations in pulmonary surfactant composition and activity after experimental lung transplantation. Am Rev Respir Dis 1993;148:208-215.
8. Erasmus ME, Petersen AH, Oetomo SB, Prop J. The function of surfactant is impaired during the reimplantation response in rat lung transplants. J Heart Lung Transplant 1994;13:791-802.
9. Novick RJ, Veldhuizen RA, Possmayer F, Lee J, Sandler D, Lewis JF. Exogenous surfactant therapy in thirty-eight hour lung graft preservation for transplantation. J Thorac Cardiovasc Surg 1994;108:259-268.
10. van der Kaaij NP, Haitsma JJ, Kluin J, et al. Surfactant pretreatment ameliorates ischemia-reperfusion injury of the lung. Eur J Cardiothorac Surg 2005;27:774-782.
11. King RC, Binns OA, Rodriguez F, et al. Reperfusion injury significantly impacts clinical outcome after pulmonary transplantation. Ann Thorac Surg 2000;69:1681-1685.
12. Haitsma JJ, Papadakos PJ, Lachmann B. Surfactant therapy for acute lung injury/acute respiratory distress syndrome. Curr Opin Crit Care 2004;10:18-22.
13. Boehler A, Kesten S, Weder W, Speich R. Bronchiolitis obliterans after lung transplantation: a review. Chest 1998;114:1411-1426.
14. Tullius SG, Tilney NL. Both alloantigen-dependent and -independent factors influence chronic allograft rejection. Transplantation 1995;59:313-318.
15. Grace PA, Mathie RT. Ischemia-Reperfusion-Injury: Blackwell Science Ltd, 1999.
16. Krishnadasan B, Naidu B, Rosengart M, et al. Decreased lung ischemia-reperfusion injury in rats after preoperative administration of cyclosporine and tacrolimus. J Thorac Cardiovasc Surg 2002;123:756-767.
17. Takeyoshi I, Otani Y, Koibuchi Y, et al. Effects of FR167653 on pulmonary ischemia-reperfusion injury: administration timing. Transplant Proc 1999;31:1935-1936.
18. de Perrot M, Imai Y, Volgyesi GA, et al. Effect of ventilator-induced lung injury on the development of reperfusion injury in a rat lung transplant model. J Thorac Cardiovasc Surg 2002;124:1137-1144.
19. Griese M. Pulmonary surfactant in health and human lung diseases: state of the art. Eur Respir J 1999;13:1455-1476.
20. Haitsma JJ, Lachmann RA, Lachmann B. Lung protective ventilation in ARDS: role of mediators, PEEP and surfactant. Monaldi Arch Chest Dis 2003;59:108-118.
21. Ochs M, Nenadic I, Fehrenbach A, et al. Ultrastructural alterations in intraalveolar surfactant subtypes after experimental ischemia and reperfusion. Am J Respir Crit Care Med 1999;160:718-724.
22. Gunther A, Balser M, Schmidt R, et al. Surfactant abnormalities after single lung transplantation in dogs: impact of bronchoscopic surfactant administration. J Thorac Cardiovasc Surg 2004;127:344-354.
23. Semik M, Schnabel R, Bruske T, et al. Ultrastructural studies of acute rejection following single lung transplantation in the rat--histological and immunohistological findings. Thorac Cardiovasc Surg 1994;42:290-297.
24. Erasmus ME, Hofstede GJ, Petersen AH, et al. SP-A-enriched surfactant for treatment of rat lung transplants with SP-A deficiency after storage and reperfusion. Transplantation 2002;73:348-352.
25. Klepetko W, Lohninger A, Wisser W, et al. Pulmonary surfactant in bronchoalveolar lavage after canine lung transplantation: effect of L-carnitine application. J Thorac Cardiovasc Surg 1990;99:1048-1058.
26. Erasmus ME, Petersen AH, Hofstede G, Haagsman HP, Bambang Oetomo S, Prop J. Surfactant treatment before reperfusion improves the immediate function of lung transplants in rats. Am J Respir Crit Care Med 1996;153:665-670.

27. Novick RJ, Gilpin AA, Gehman KE, et al. Mitigation of injury in canine lung grafts by exogenous surfactant therapy. J Thorac Cardiovasc Surg 1997;113:342-353.
28. Friedrich I, Borgermann J, Splittgerber FH, et al. Bronchoscopic surfactant administration preserves gas exchange and pulmonary compliance after single lung transplantation in dogs. J Thorac Cardiovasc Surg 2004;127:335-343.
29. Warnecke G, Struber M, Fraud S, Hohlfeld JM, Haverich A. Combined exogenous surfactant and inhaled nitric oxide therapy for lung ischemia-reperfusion injury in minipigs. Transplantation 2001;71:1238-1244.
30. Andrade RS, Solien EE, Wangensteen OD, Tsai MY, Kshettry VR, Bolman RM, 3rd. Surfactant dysfunction in lung preservation. Transplantation 1995;60:536-541.
31. Andrade RS, Wangensteen OD, Jo JK, Tsai MY, Bolman RM, 3rd. Effect of hypothermic pulmonary artery flushing on capillary filtration coefficient. Transplantation 2000;70:267-271.
32. Struber M, McGregor CG, Locke TJ, Miller VM. Effect of flush-perfusion with Euro-Collins solution on pulmonary arterial function. Transplant Proc 1990;22:2206-2211.
33. Struber M, Ehlers KA, Nilsson FN, Miller VM, McGregor CG, Haverich A. Effects of lung preservation with Euro-Collins and University of Wisconsin solutions on endothelium-dependent relaxations. Ann Thorac Surg 1997;63:1428-1435.
34. Struber M, Hohlfeld JM, Fraund S, Kim P, Warnecke G, Haverich A. Low-potassium dextran solution ameliorates reperfusion injury of the lung and protects surfactant function. J Thorac Cardiovasc Surg 2000;120:566-572.
35. Struber M, Hohlfeld JM, Kofidis T, et al. Surfactant function in lung transplantation after 24 hours of ischemia: advantage of retrograde flush perfusion for preservation. J Thorac Cardiovasc Surg 2002;123:98-103.
36. Lachmann B, Eijking EP, So KL, Gommers D. In vivo evaluation of the inhibitory capacity of human plasma on exogenous surfactant function. Intensive Care Med 1994;20:6-11.
37. Cockshutt AM, Weitz J, Possmayer F. Pulmonary surfactant-associated protein A enhances the surface activity of lipid extract surfactant and reverses inhibition by blood proteins in vitro. Biochemistry 1990;29:8424-8429.
38. Erasmus ME, Hofstede GJ, Petersen AH, Haagsman HP, Oetomo SB, Prop J. Effects of early surfactant treatment persisting for one week after lung transplantation in rats. Am J Respir Crit Care Med 1997;156:567-572.
39. Hohlfeld JM, Struber M, Ahlf K, et al. Exogenous surfactant improves survival and surfactant function in ischemia-reperfusion injury in minipigs. Eur Respir J 1999;13:1037-1043.
40. Struber M, Cremer J, Harringer W, Hirt SW, Costard-Jackle A, Haverich A. Nebulized synthetic surfactant in reperfusion injury after single lung transplantation. J Thorac Cardiovasc Surg 1995;110:563-564.
41. Struber M, Hirt SW, Cremer J, Harringer W, Haverich A. Surfactant replacement in reperfusion injury after clinical lung transplantation. Intensive Care Med 1999;25:862-864.
42. Struber M, Wilhelmi M, Harringer W, et al. Flush perfusion with low potassium dextran solution improves early graft function in clinical lung transplantation. Eur J Cardiothorac Surg 2001;19:190-194.
43. van der Kaaij NP, Kluin J, Haitsma JJ, et al. Ischemia of the lung causes extensive long-term pulmonary injury: an experimental study. Unpublished data.
44. van der Kaaij NP, Kluin J, Haitsma JJ, et al. Surfactant pretreatment decreases long-term damage after ischemia-reperfusion injury of the lung. Unpublished data.
45. Novick RJ, MacDonald J, Veldhuizen RA, et al. Evaluation of surfactant treatment strategies after prolonged graft storage in lung transplantation. Am J Respir Crit Care Med 1996;154:98-104.
46. Hausen B, Rohde R, Hewitt CW, et al. Exogenous surfactant treatment before and after sixteen hours of ischemia in experimental lung transplantation. J Thorac Cardiovasc Surg 1997;113:1050-1058.
47. Koletsis E, Chatzimichalis A, Fotopoulos V, et al. Donor lung pretreatment with surfactant in experimental transplantation preserves graft hemodynamics and alveolar morphology. Exp Biol Med (Maywood) 2003;228:540-545.
48. Speer CP, Gotze B, Curstedt T, Robertson B. Phagocytic functions and tumor necrosis factor secretion of human monocytes exposed to natural porcine surfactant (Curosurf). Pediatr Res 1991;30:69-74.
49. Thomassen MJ, Meeker DP, Antal JM, Connors MJ, Wiedemann HP. Synthetic surfactant (Exosurf) inhibits endotoxin-stimulated cytokine secretion by human alveolar macrophages. Am J Respir Cell Mol Biol 1992;7:257-260.
50. Matalon S, Holm BA, Baker RR, Whitfield MK, Freeman BA. Characterization of antioxidant activities of

pulmonary surfactant mixtures. Biochim Biophys Acta 1990;1035:121-127.
51. Casals C, Varela A, Ruano ML, et al. Increase of C-reactive protein and decrease of surfactant protein A in surfactant after lung transplantation. Am J Respir Crit Care Med 1998;157:43-49.
52. Buchanan SA, Mauney MC, Parekh VI, et al. Intratracheal surfactant administration preserves airway compliance during lung reperfusion. Ann Thorac Surg 1996;62:1617-1621.
53. Novick RJ, Gehman KE, Ali IS, Lee J. Lung preservation: the importance of endothelial and alveolar type II cell integrity. Ann Thorac Surg 1996;62:302-314.

3

Ischemia of the lung causes extensive long-term pulmonary injury: an experimental study

Based on
NP van der Kaaij, J Kluin, JJ Haitsma, MA den Bakker, BN Lambrecht, B Lachmann, RWF de Bruin, AJJC Bogers
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Summary

Background: Lung ischemia-reperfusion injury (LIRI) is suggested to be a major risk factor for development of primary graft dysfunction (PGD) following lung transplantation, although other factors have been found to interplay with LIRI. The question whether LIRI exclusively results in PGD seems difficult to answer, which is partly due to the lack of a long-term experimental LIRI model, in which PGD changes can be studied. In addition, the long-term effects of LIRI are unclear and a detailed description of the immunological changes over time after LIRI is missing. Therefore our purpose was to establish a long-term experimental model of LIRI, and to study the impact of LIRI on the development of PGD, using a broad spectrum of LIRI parameters including leukocyte kinetics. Methods: Male Sprague-Dawley rats (n=135) were subjected to 120 minutes of left lung warm ischemia or were sham-operated. A third group served as unoperated controls. Animals were sacrificed 1, 3, 7, 30 or 90 days after surgery. Blood gas values, lung compliance, surfactant conversion, capillary permeability, and the level of MMP-2 and MMP-9 in bronchoalveolar lavage fluid (BALf) were determined. Infiltration of granulocytes, macrophages and lymphocyte subsets ($CD45RA^+$, $CD3^+CD4^+$, $CD3^+CD8^+$) was measured by flowcytometry in BALf, lung parenchyma, thoracic lymph nodes and spleen. Histological analysis was performed on HE sections. Results: LIRI resulted in hypoxemia, impaired left lung compliance, increased capillary permeability, surfactant conversion, and an increase in MMP-2 and MMP-9. In BALf, most granulocytes were found on day 1 and $CD3^+CD4^+$ and $CD3^+CD8^+$-cells were elevated on day 3. Increased numbers of macrophages were recorded on day 1, 3, 7 and 90. Histology on day 1 showed diffuse alveolar damage, resulting in fibroproliferative changes up to 90 days after LIRI. Conclusions. The short-, and long-term changes after LIRI in this model are similar to the changes found in both PGD and ARDS after clinical lung transplantation. LIRI seems an independent risk factor for the development of PGD and

resulted in progressive deterioration of lung function and architecture, leading to extensive immunopathological and functional abnormalities up to 3 months after reperfusion.

1 | Goals of the study

1) To establish an in vivo model of unilateral severe LIRI and to determine whether symptoms resembling PGD after clinical lung transplantation can be induced.
2) To investigate a broad spectrum of LIRI parameters, including lung function, capillary permeability, inflammatory cells, matrix metalloproteinase (MMP) production, surfactant conversion, and histological changes on the short (days) and long-term (months).
3) To assess changes in non-ischemic right lung in animals undergoing left-sided LIRI.

2 Materials and methods in brief

For a full description of the materials and methods, see the materials and methods section at the end of this thesis.

2.1 Experimental design

Male Sprague-Dawley rats (n= 135, weighing 295 ± 4 grams) (Harlan, the Netherlands) were randomised into the experimental LIRI (n=75), sham-operated (n=50) or unoperated (n=10) group. LIRI (n=15 per time point) and sham-operated (n=10 per time point) animals were euthanized on day 1, 3, 7, 30 or 90 postoperatively. Animals in the LIRI group were subjected to 120 minutes of warm ischemia of the left lung. Sham-operated animals underwent the same protocol as LIRI animals without applying left lung ischemia. Unoperated controls did not undergo any intervention.

2.2 Measurements

Measurements include survival, weight loss, blood gas values, static pulmonary compliance, capillary permeability, matrix metalloproteinase (MMP) activity, surfactant small and large aggregates, flowcytometric analysis of infiltrating cells (in BALf, left and right lung, spleen, and thoracic lymph nodes (TLN)) and histological analysis. The static pulmonary compliance is presented as Vmax and Cmax. Vmax is the maximal lung volume at a pressure of 35 cm H_2O, while Cmax is the maximal compliance of the expiration curve.

2.3 Statistical analysis

The results in text, tables and figures are presented as mean ± standard error of the mean (SEM). Data were analysed using SPSS version 11.1 statistical software (SPSS Inc., Chicago, Illinois, USA). If an overall difference between groups was found by the Kruskal-Wallis test, Mann-Whitney U tests were performed for intergroup comparison. Difference in mortality rate was assessed by the Fisher's exact test. P values <0.05 were considered to be significant.

Table 1. Mean (SEM) PaO_2/FiO_2 ratio and mean (SEM) $PaCO_2$ based on both lungs.

Group	**PaO_2/FiO_2** (mm Hg)	**$PaCO_2$** (mm Hg)
Unoperated	562 (25)	45.4 (4.6)
Sham day 1	559 (17)	39.3 (2.3)
Sham day 3	520 (23)	40.8 (4.5)
Sham day 7	573 (17)	50.4 (7.8)
Sham day 30	561 (12)	41.5 (3.5)
Sham day 90	576 (21)	37.2 (4.0)
LIRI day 1	282 (41) **$U S^{1} L^{7-90}$**	61.1 (6.1) **$U S^{1} L^{7-90}$**
LIRI day 3	241 (38) **$U S^{3} L^{7-90}$**	48.0 (4.8)
LIRI day 7	435 (48) **$U S^{7} L^{90}$**	44.8 (2.2)
LIRI day 30	543 (22)	42.3 (2.0)
LIRI day 90	607 (14)	30.2 (2.4) **$U L^{1-30}$**

FiO_2: Fraction of inspired Oxygen; LIRI: Lung Ischemia-Reperfusion Injury; PaO_2: Arterial Oxygen pressure; $PaCO_2$: Arterial Carbon dioxide pressure; SEM: Standard-Error of the Mean.
U: P<0.05 versus unoperated animals
S^{x-y}: P<0.05 versus sham-operated animals day x to day y
L^{x-y}: P<0.05 versus LIRI animals day x to day y

3 | Results

3.1 Survival and weight loss

All sham-operated animals survived the experimental period. LIRI resulted in a mortality rate of 25% (0/50 in sham-operated animals versus 19/75 after LIRI, P<0.0001). Non-surviving LIRI animals died shortly after weaning due to the development of pulmonary edema. Surviving LIRI animals had lost more weight on day 3 as compared to sham-operated rats (-34.91 ± 3.86g versus -21.10 ± 2.86g, P=0.01). From day 7 on these differences had disappeared.

3.2 PaO_2 & $PaCO_2$

Arterial oxygenation was lower in LIRI animals than in unoperated and sham-operated controls on day 1, 3, and 7 (Table 1). On day 30 and 90, these differences had disappeared. An elevated $PaCO_2$ was found 1 day after LIRI, as compared to unoperated and sham-operated animals.

3.3 Static compliance of the left lung

LIRI had a detrimental effect on both the Cmax and Vmax of the left ischemic lung (Table 2).

Table 2. Mean (SEM) static compliance of the left lung, corrected for body weight.

Group	**Vmax** (ml/kg)	**Cmax** ((ml/kg)/cm H_2O)
Unoperated	13.4 (0.48)	1.12 (0.10)
Sham day 1	15.9 (1.13)	1.32 (0.11)
Sham day 3	15.9 (0.81) **U**	1.26 (0.18)
Sham day 7	14.1 (1.21)	0.95 (0.04) **S^{1}**
Sham day 30	12.3 (0.63) **S^{1-3}**	1.00 (0.08) **S^{1}**
Sham day 90	11.8 (0.58) **S^{1-3}**	1.09 (0.06)
LIRI day 1	4.8 (0.59) **$US^{1}L^{7}$**	0.29 (0.05) **$US^{1}L^{30-90}$**
LIRI day 3	5.0 (0.68) **$US^{3}L^{7}$**	0.32 (0.05) **$US^{3}L^{90}$**
LIRI day 7	9.0 (1.51) **US^{7}**	0.53 (0.12) **US^{7}**
LIRI day 30	6.2 (0.75) **US^{30}**	0.51 (0.06) **US^{30}**
LIRI day 90	6.9 (1.04) **US^{90}**	0.67 (0.11) **US^{90}**

Cmax: Maximal compliance of the expiration curve, corrected for body weight; LIRI: Lung Ischemia-Reperfusion Injury; SEM: Standard-Error of the Mean; Vmax: Maximal lung volume corrected for body weight at a pressure of 35 cm H_2O.
U: P<0.05 versus unoperated animals
S^{x-y}: P<0.05 versus sham-operated animals day x to day y
L^{x-y}: P<0.05 versus LIRI animals day x to day y

Up to 90 days after LIRI, Vmax and Cmax of the left lung was lower than in sham-operated and unoperated rats.

3.4 Capillary permeability

The alveolar serum protein level of the ischemic left lung, as parameter for capillary permeability, was increased 1 day after reperfusion compared to controls (Table 3). On day 3 the amount of alveolar serum protein in left BALf of LIRI animals was still higher than in unoperated rats. From day seven on, no differences were present.

3.5 Matrix metalloproteinase activity

MMP-2 is expressed constitutively in all animals (Figure 1 and 2). However, the total amount of pro- and active MMP-2 and MMP-9 per microliter BALf is increased in LIRI animals on day 1 (Figure 2) (recovered volume did not differ between the groups). MMP activity per microgram protein in the BALf, does not differ between the groups (data not shown), which indicates that the increased activity after LIRI must be due to elevated alveolar serum proteins. After day 3, no differences were demonstrable between the groups.

3.6 Surfactant small and large aggregates

While an increase in SA was recorded in BALf of the left lung of sham-operated animals on

Table 3. Mean (SEM) alveolar serum proteins of the left lung.

Group	**Alveolar proteins** (μg/ml)
Unoperated	226 (51)
Sham day 1	386 (131)
Sham day 3	323 (76)
Sham day 7	154 (51)
Sham day 30	151 (50)
Sham day 90	202 (65)
LIRI day 1	1,663 (202) **$US^{1}L^{3\text{-}90}$**
LIRI day 3	447 (75) **$UL^{7\text{-}90}$**
LIRI day 7	168 (60)
LIRI day 30	79 (25)
LIRI day 90	74 (25)

LIRI: Lung Ischemia-Reperfusion Injury; SEM: Standard-Error of the Mean.
U: $P<0.05$ versus unoperated animals
$S^{x\text{-}y}$ $P<0.05$ versus sham-operated animals day x to day y
$L^{x\text{-}y}$ $P<0.05$ versus LIRI animals day x to day y

day 1, a higher level was measured in LIRI lungs (Figure 3). After LIRI, an elevated amount of SA was also found in the right lung on day 1. The LA level in the left lung dropped from day 3 until day 30 following LIRI, whereafter the LA level returned to normal on day 90.

3.7 Infiltrating cells

3.7.1 Neutrophils

Sham operation resulted in some infiltration of neutrophils in the first days after the operation, as demonstrated by an elevated percentage in left and right BALf and lung tissue (Table 4A, 5A, 6A and 7A supplemental files). However, after LIRI even more neutrophils were measured in predominantly the left, but also the right BALf (Figure 4A this manuscript, Table 4B and 5B supplemental files) and lung tissue (Figure 4C this manuscript, Table 6B and 7B supplemental files). Hereafter the number of neutrophils gradually decreased, and could not be measured anymore on day 30 and 90.

3.7.2 Macrophages

Macrophage occurrence followed similar kinetics in sham-operated and ischemic lungs, but more macrophages were present on day 1 and 3 in ischemic lung tissue and on day 3 and 7 in BALf (Figure 4B and 4D this manuscript, Table 4B and 6B supplemental files). LIRI also led to an increase in macrophages in BALf of the contralateral lung on day 3 and 7 as compared to sham and unoperated animals (Figure 4B this manuscript, Table

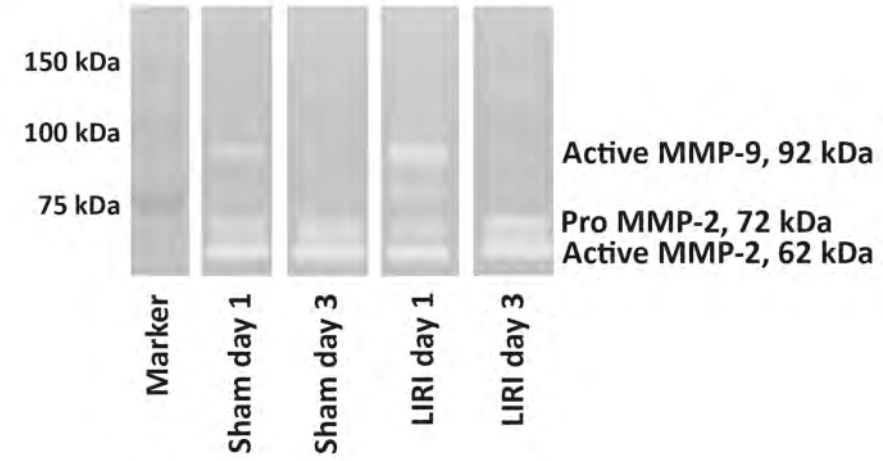

Figure 1. MMP-2 and MMP-9 zymography. Pro MMP-9 could not be measured in any of the samples and active MMP-9 was detectable in the BALf of sham-operated animals on day 1 and in LIRI animals on day 1 and 3. Pro and active MMP-2 is expressed constitutively in all animals. BALf: BronchoAlveolar Lavage Fluid; LIRI: Lung Ischemia-Reperfusion Injury; MMP: Matrix MetalloProteinase.

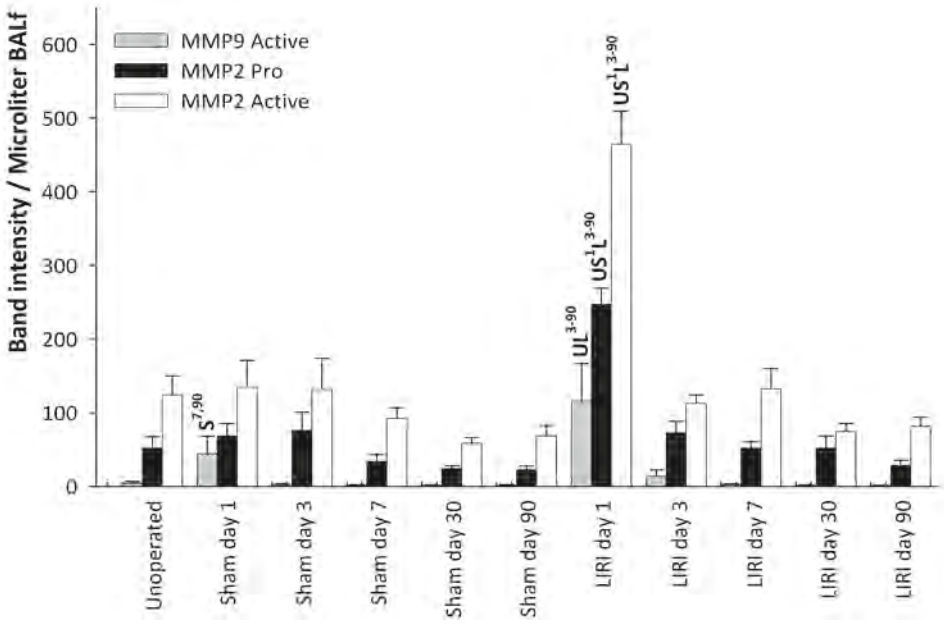

Figure 2. MMP level in BALf by zymography. On day 1, significant more pro -and active MMP-2 and active MMP-9 were found in the BALf of LIRI animals as compared to sham-operated and unoperated controls. BALf: BronchoAlveolar Lavage Fluid; LIRI: Lung Ischemia-Reperfusion Injury; MMP: Matrix MetalloProteinase.

U: P<0.05 versus unoperated animals

$S^{x-y:}$ P<0.05 versus sham-operated animals day x to day y

L^{x-y}: P<0.05 versus LIRI animals day x to day y

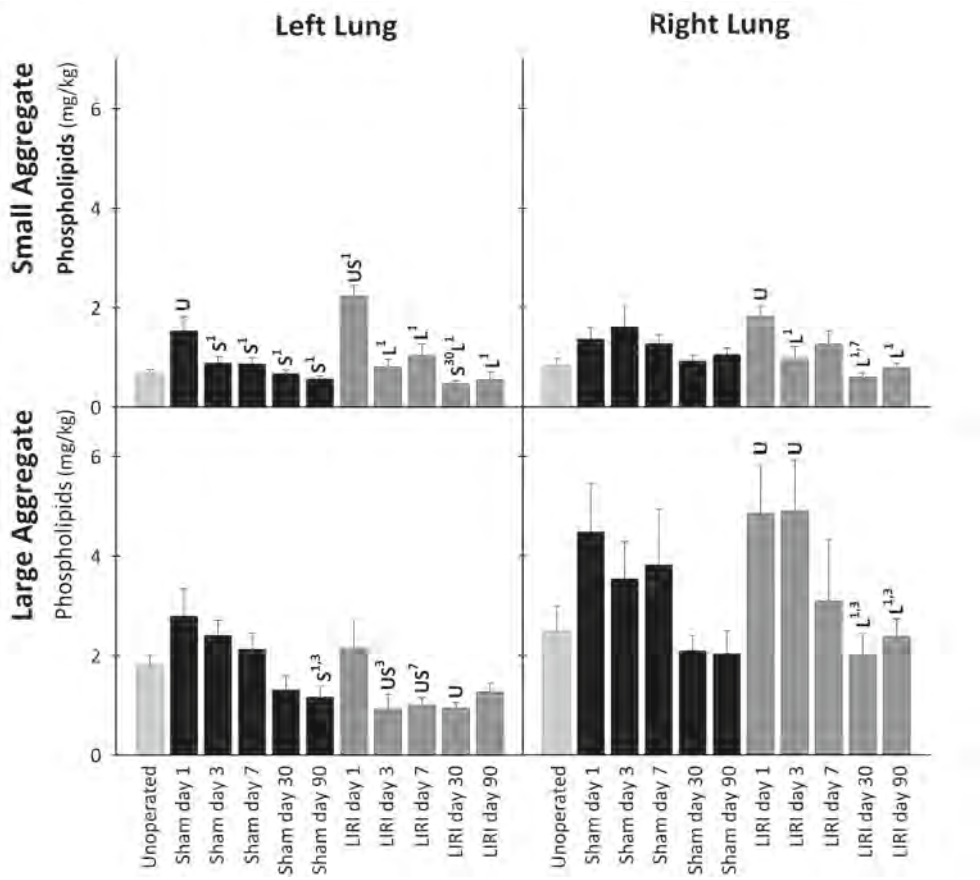

Figure 3. Total amount of SA and LA phospholipids in left and right BALf. SA and LA phospholipids (mg/kg body weight) were measured in left and right BALf of unoperated, sham-operated and LIRI animals on day 1, 3, 7, 30 and 90. Elevated levels of SA were found in both left and right BALf on day 1 and a decreased level of LA was recorded up to day 30 in LIRI animals. BALf: BronchoAlveolar Lavage Fluid; LIRI: Lung Ischemia-Reperfusion Injury; SA: Small Aggregate; LA: Large Aggregate.
U: P<0.05 versus unoperated animals
S^{x-y}: P<0.05 versus sham-operated animals day x to day y
L^{x-y}: P<0.05 versus LIRI animals day x to day y

5B supplemental files). Although the macrophage level returned to normal in left BALf of sham-operated and LIRI animals on day 30, this was again elevated on day 90 (Figure 4B this manuscript, Table 4B supplemental files).

3.7.3 Lymphocytes

Sham operation did not result in infiltration of lymphocytes in BALf (Figure 5A-C this manuscript, Table 4B supplemental files). After LIRI, an infiltration of mainly $CD3^+CD4^+$ and $CD3^+CD8^+$ and to a lesser extent $CD45RA^+$-lymphocytes occurred in mainly the left, but also right BALf. Lymphocyte infiltration peaked on day 3, with levels decreasing thereafter (Figure 5A-C this manuscript, Table 4B and 5B supplemental files).

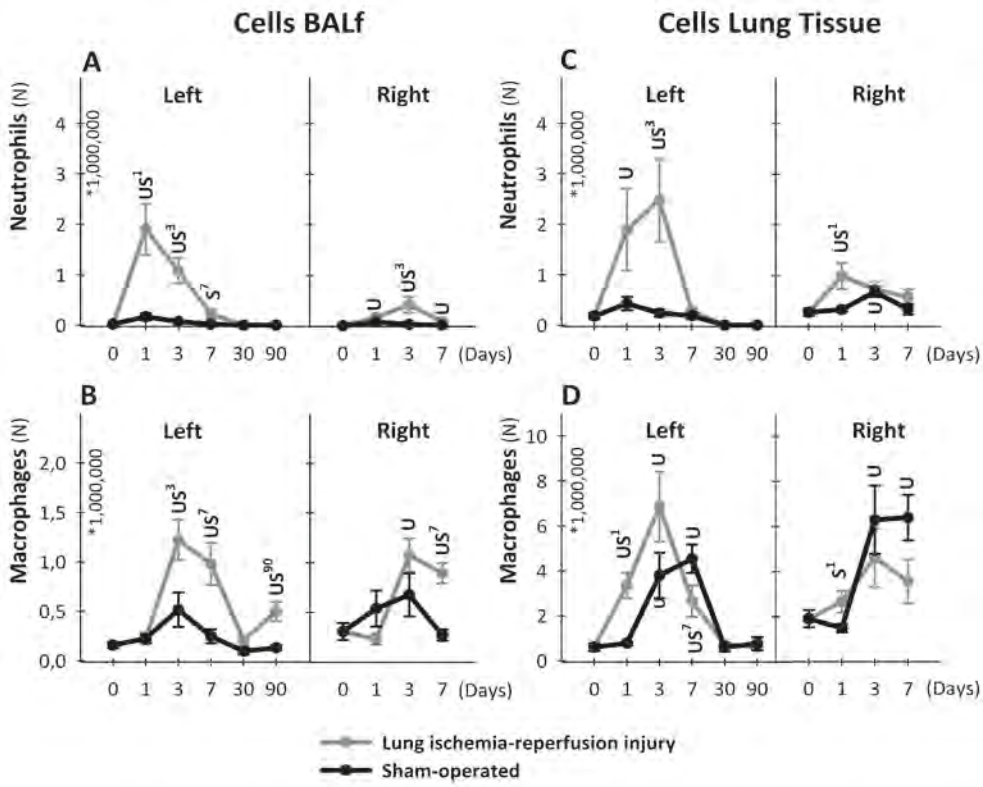

Figure 4. The number of inflammatory cells in BALf and lung tissue of the left (day 0-90) and right lung (day 0-7). Presented are (A) neutrophils, and (B) macrophages in BALf; (C) neutrophils, and (D) macrophages in lung tissue. Day 0 represents the baseline value measured in unoperated animals. BALf: BronchoAlveolar Lavage Fluid; LIRI: Lung Ischemia-Reperfusion Injury; N: Number of cells.
U: P<0.05 versus unoperated animals
S^{x-y}: P<0.05 versus sham-operated animals day x to day y
L^{x-y}: P<0.05 versus LIRI animals day x to day y

Although lymphocytes in right lung tissue of LIRI animals followed the same kinetics as in sham-operated animals, demonstrated by a decreased number on day 1 (Figure 5D-F this manuscript, Table 7B supplemental files), more $CD3^+CD4^+$ and $CD3^+CD8^+$-cells were found in left lung tissue on day 1 and 3 as compared to sham-operated and unoperated animals (Figure 5D-E this manuscript, Table 6B supplemental files). On day 1 also more $CD45RA^+$-cells were present in the left lung of LIRI animals (Figure 5F this manuscript, Table 6B supplemental files). On day 90, the level of $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD45RA^+$ lymphocytes in left lung tissue of LIRI animals had decreased as compared to controls (Figure 5D-F this manuscript, Table 6B supplemental files).
No differences were found between groups in percentage or total number of cells within the spleen (data not shown). However, more $CD3^+CD4^+$, and $CD3^+CD8^+$-cells were measured in TLN on day 3 (Figure 6A-C this manuscript, Table 8B supplemental files). Whereas

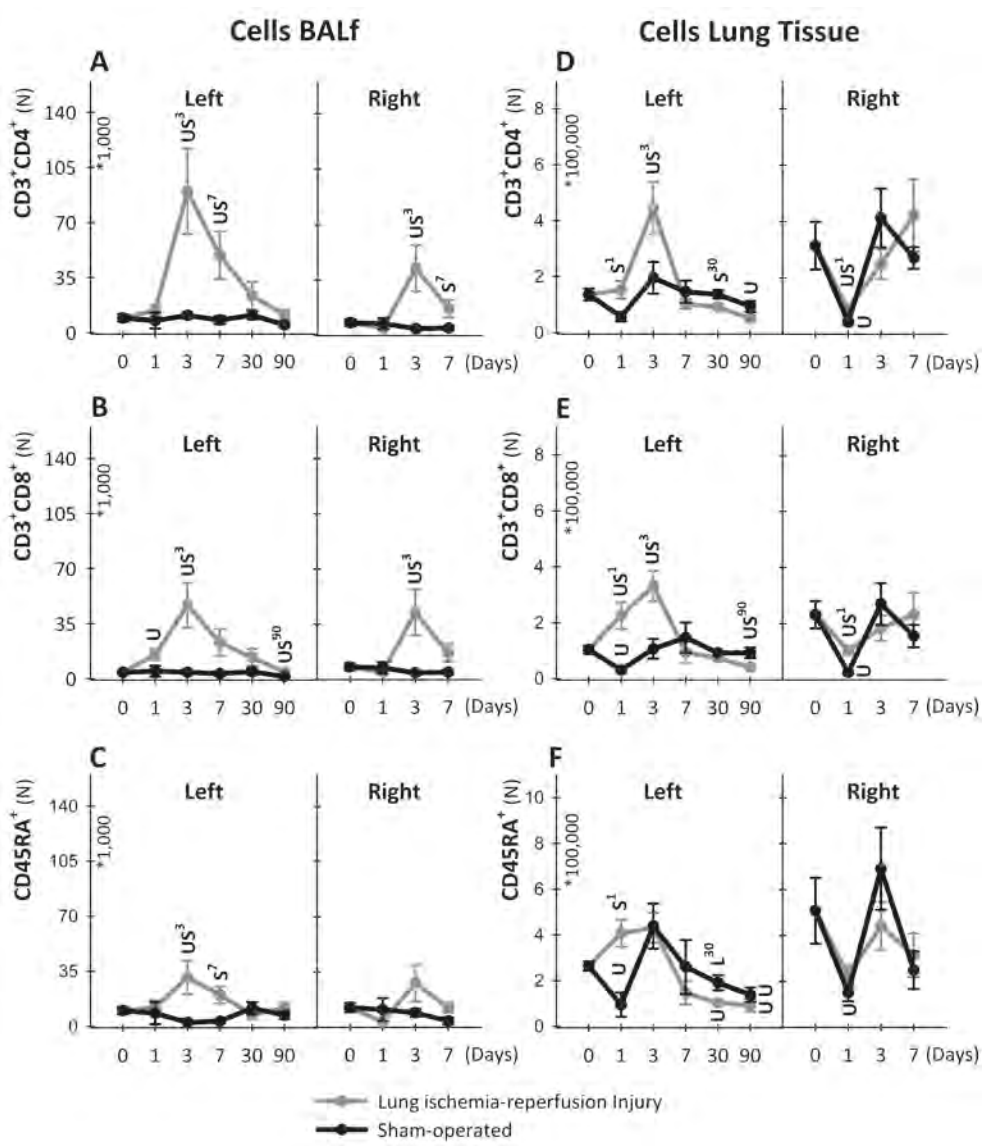

Figure 5. The number of inflammatory cells in BALf and lung tissue of the left (day 0-90) and right lung (day 0-7). Presented are (A) helper T-lymphocytes ($CD3^+CD4^+$), (B) cytotoxic T-lymphocytes ($CD3^+CD8^+$), and (C) B-lymphocytes ($CD45RA^{+}$) in BALf; (D) helper T-lymphocytes, (E) cytotoxic T-lymphocytes, and (F) B-lymphocytes in lung tissue. Day 0 represents the baseline value measured in unoperated animals. BALf: BronchoAlveolar Lavage Fluid; LIRI: Lung Ischemia-Reperfusion Injury; N: Number of cells.
U: P<0.05 versus unoperated animals
S^{x-y}: P<0.05 versus sham-operated animals day x to day y
L^{x-y}: P<0.05 versus LIRI animals day x to day y

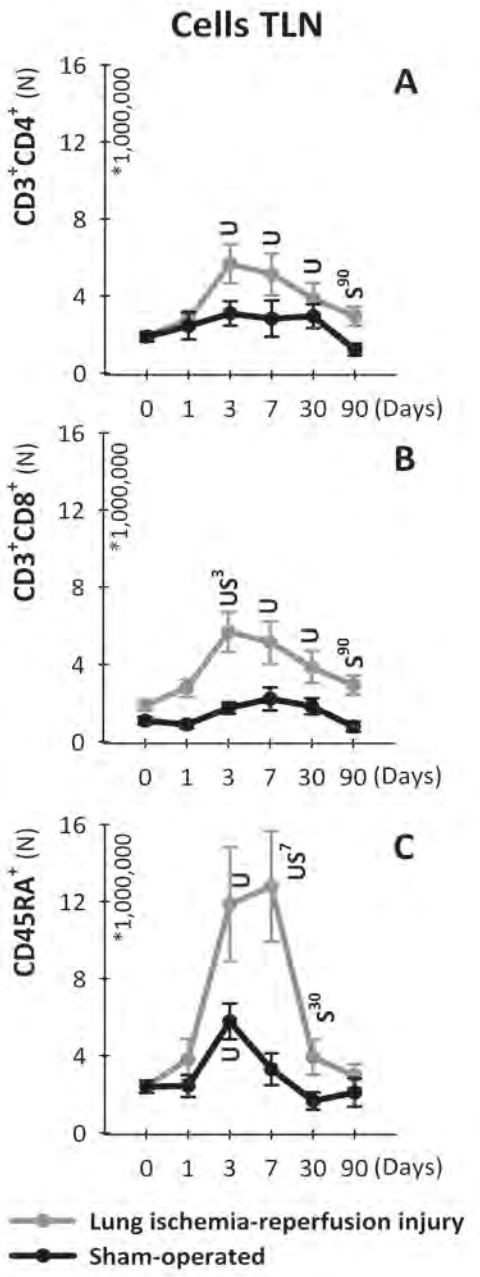

Figure 6. The number of inflammatory cells in thoracic lymph nodes (day 0-90). Shown are (A) helper T-lymphocytes ($CD3^+CD4^+$), (B) cytotoxic T-lymphocytes ($CD3^+CD8^+$), and (C) B-lymphocytes ($CD45RA^+$) in TLN. Day 0 represents the baseline value measured in unoperated animals. N: Number of cells; TLN: Thoracic Lymph Nodes.
U: $P<0.05$ versus unoperated animals
S^{x-y}: $P<0.05$ versus sham-operated animals day x to day y

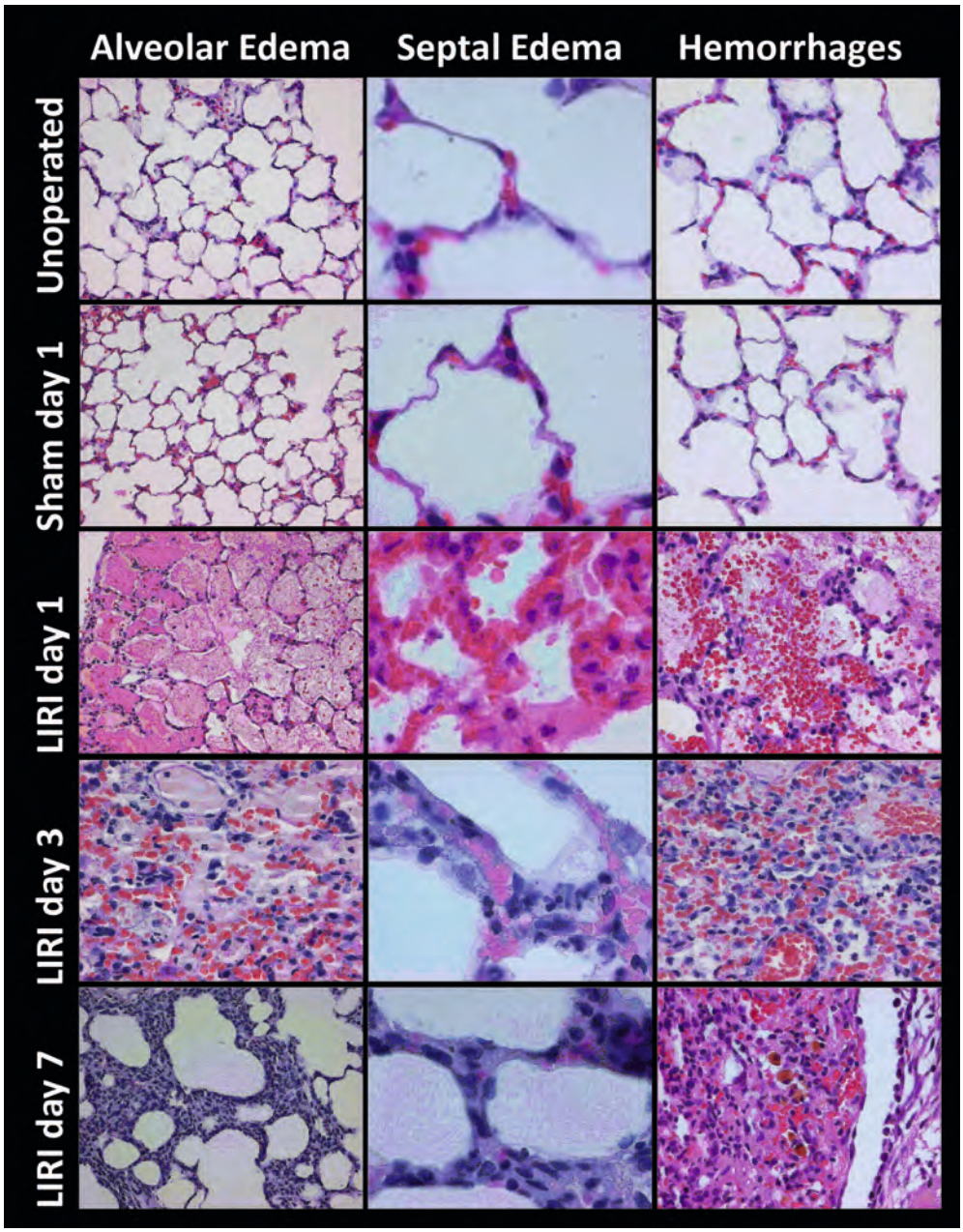

Figure 7. Histologic examples of alveolar edema (25*), septal edema (100*) and intra-alveolar hemorrhage (40*) on HE slides. LIRI causes alveolar and septal edema and alveolar hemorrhages, which were most severe on day 1 and 3 after LIRI and resolved thereafter. On day 7 brownish macrophages were found after clearance of erythrocytes in the alveolus. HE: Hematoxylin and Eosin staining; LIRI: Lung Ischemia-Reperfusion Injury.

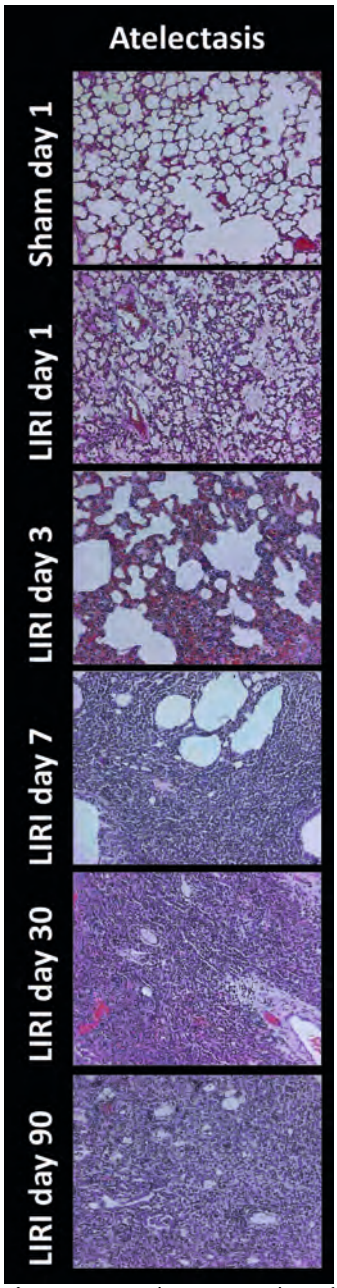

Figure 8. Histologic examples of atelectasis (10*) on HE slides. Severe atelectasis was demonstrated up to day 90 after LIRI. HE: Hematoxylin and Eosin staining; LIRI: Lung Ischemia-Reperfusion Injury

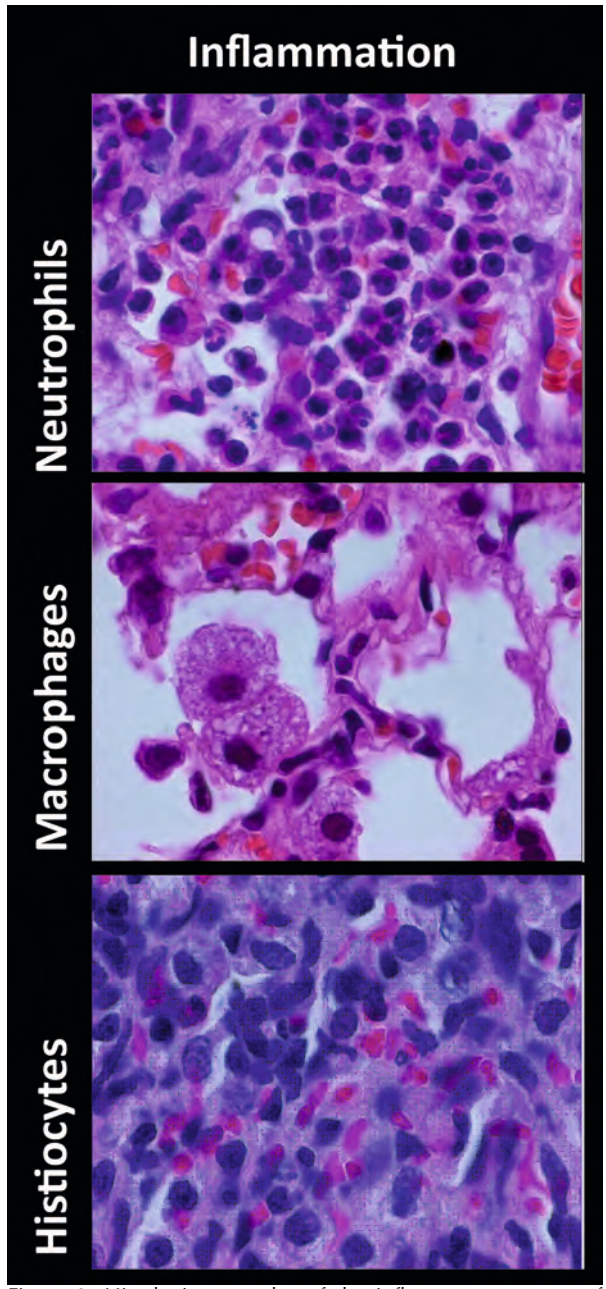

Figure 9. Histologic examples of the inflammatory pattern of LIRI on HE slides (100*). Analysis confirms the flowcytometric analysis with the presence of predominantly neutrophils on day 1, alveolar macrophages on day 3, and histiocytes on day 30 following LIRI. HE: Hematoxylin and Eosin staining; LIRI: Lung Ischemia-Reperfusion Injury.

$CD3^+CD4^+$ and $CD3^+CD8^+$-cells remained higher in LIRI animals than in unoperated animals up to day 90, $CD45RA^+$-cells had returned to pre-operative values on day 90.

3.8 Histology

LIRI resulted in diffuse alveolar damage consisting of severe intra-alveolar edema up to day 3, septal edema, which was mild on day 1 and increased to moderate on day 3, and intra-alveolar hemorrhages (Figure 7 this manuscript, Table 9 supplemental files). The overall classification of LIRI animals changed from exudative on day 1 to proliferative from day 3 to day 90. Although no atelectasis and fibrosis were seen on day 1 following LIRI, mild fibrosis and mild to severe atelectasis were seen from day 3 up to day 90 after LIRI (Figure 8 this manuscript, Table 9 supplemental files). Identification of infiltrating cells confirmed the flowcytometry measurements. A mild inflammatory pattern consisting of histiocytes was found on day 3 and 7 in sham-operated animals. LIRI caused moderate to severe inflammation, which changed from mixed (granulocytic, lymphocytic, and histiocytic) inflammation on day 1 to a histiocytic and lymphocytic pattern from day 3 to 90 (Figure 9 this manuscript, Table 10 supplemental files). No major differences between unoperated, sham-operated and LIRI animals were found in the right lung (data not shown).

4 | Discussion

This study describes the effect of warm LIRI on a broad spectrum of LIRI parameters, such as lung function, capillary permeability, MMP production, surfactant conversion, and histology on the short and long term after LIRI. Furthermore, a detailed description of the subset of leukocytes and the time course of infiltration on both short and long term after LIRI is given.

LIRI has been suggested to be a major risk factor for PGD. The clinical course of PGD symptomatically resembles the acute respiratory distress syndrome (ARDS) and can be characterized by different stages, each with their specific clinical, histological and immunological changes [1]. The acute, exudative phase is featured by a sudden onset of hypoxemia, decreased lung compliance, increased pulmonary artery pressure, and development of non-cardiogenic pulmonary edema [1, 2]. Experimental studies have shown that abnormalities in, and depletion of pulmonary surfactant contribute to these symptoms of LIRI [3-8]. Histological analysis of LIRI shows diffuse alveolar damage with atelectasis, inflammation, intra-alveolar hemorrhage, formation of hyaline membranes and protein-rich edema [1, 9]. Finally, production of MMPs is thought to be important in the acute phase of PGD since MMPs increase the microvascular permeability and thereby enable extravasation of inflammatory cells [10-13].

We found hypoxemia, impaired left lung compliance, a mortality rate of 25% due to development of severe pulmonary edema, and an increase in SA subtype surfactant as early effects of LIRI. Conversion of highly surface active LA into poor surface active SA occurs shortly after reperfusion and is partly due to increased capillary permeability, resulting in influx of serum proteins into the alveolus, as confirmed in our study [5, 14]. Serum proteins inhibit surfactant in a dose-dependent manner by competing with surfactant components at the alveolo-capillary membrane [15]. HE slides confirmed extensive alveolar and septal

edema, intra-alveolar bleeding, atelectasis and inflammation, which are all indicative of diffuse alveolar damage. The increase in alveolar proteins and neutrophils on day 1 occurred simultaneously with an increased MMP activity. MMP-2 is usually constitutively expressed by endothelial cells, vascular smooth muscle cells and fibroblasts, fitting with the observation in our study of high levels of both pro- and active MMP-2 found in unoperated animals [10-13, 16]. Yano et al demonstrated that LIRI resulted in MMP-9 induction, but not MMP-2 expression 24 hours after LIRI [13]. A higher concentration of MMP-9 was also found in lung edema fluid of ARDS patients [17]. Nevertheless, we found that levels of both pro- and active MMP-9 and MMP-2 per microliter BALf are elevated 24 hours after LIRI, which correlates well with the presence of neutrophils in left lung BALf. Thus 120 minutes of warm ischemia resulted in our experimental study in a mortality rate of 25%, hypoxemia, early impaired left lung compliance, surfactant conversion, diffuse alveolar damage on HE slides and MMP production, which are all features of the exudative phase of PGD.

Human lung transplant patients surviving the acute phase of LIRI may either recover from injury or enter a 'chronic' fibroproliferative state, which develops within 4-7 days after the onset of symptoms [1, 2]. The progression from an exudative phase to a 'chronic' fibroproliferative state within one week after LIRI is supported in our study by the presence of fibroproliferative changes on HE slides in the first week after LIRI and an increased number of macrophages, which are important mediators in the regulation of fibroblast function. Importantly, LIRI induced progressive changes resulting in extensive pulmonary injury up to 3 months after reperfusion. This is supported by a decreased number of lymphocytes found in lung tissue on day 30 and 90, impaired left lung compliance up to day 90, extensive atelectasis on HE slides, and a decreased surfactant recycling and secreting capacity of alveolar type II cells reflected by the decreased LA surfactant subtype [18]. Although extensive left pulmonary injury was found on the long-term, hypoxemia was demonstrated up to day 7. Thereafter, no differences in PaO_2 were measured between LIRI animals and controls. This discrepancy may be explained by the fact that PaO_2 depended on both lungs, so that the loss of left lung function was compensated by the right lung. Furthermore, even though MMPs are important mediators of pulmonary remodelling, no changes in activity on the long-term were found. It is questionable however whether MMPs are present in the BALf of severe atelectatic and fibrotic lungs. Therefore, in future studies, measurement of MMP activity should also be performed in homogenized lung tissue.

Thus, 120 minutes of warm ischemia in this model induces injury comparable to PGD and ARDS in clinical lung transplantation on the short, but also on the long-term. Nevertheless, this experimental model has several shortcomings we wish to address. First of all, we used warm ischemia to induce LIRI, whereas in the clinical setting cold ischemic time is associated with PGD. However, it has been demonstrated that there are no major differences between short periods of warm and longer periods of cold ischemia and warm ischemia has been used extensively in IRI models of liver and kidney as an accelerated model of clinically relevant cold IRI [19-23]. Another disadvantage is that a rather long period of 120 minutes warm ischemia has been used. Shorter periods of warm ischemia have been investigated in a pilot study to setup our model (data not shown) and we found that 120 minutes of warm ischemia is necessary in our hands to induce symptoms comparable to PGD grade 3. This finding is supported by a clinical study by Thabut et al, which shows that the relationship

between cold graft ischemic time and survival appears to be of cubic form with a cut-off value of 330 minutes [24]. Thereafter short-term mortality increases rapidly mainly due to development of PGD [24].

Another goal of this study was to describe leukocyte kinetics following LIRI. The immunological effects of LIRI have only been studied up to hours after reperfusion, whereas we investigated leukocyte kinetics after LIRI up to 90 days after reperfusion. Several studies have shown that macrophages are activated during ischemia, followed by the recruitment of neutrophils within hours after the start of reperfusion [20, 23, 25-31]. We now add that neutrophil infiltration lasted for 3 days after reperfusion, thereby strengthening the theory that neutrophils are important in perpetuating LIRI. The extended presence of neutrophils after LIRI may be also explained by the fact that phagocytes are important elements of the repair process after LIRI by clearing apoptotic cells and necrotic debris [29]. Nevertheless, since ischemia-reperfusion injury still develops in neutropenic models, it is questionable whether neutrophils are pivotal in LIRI [32, 33].

In this regard, other studies suggest an early role for T-cells as important mediators of ischemia induced injury. An infiltration of $CD4^+$-T-cells occurred in these studies from 1 until 12 hours after reperfusion and disappeared hereafter [20, 23, 25, 30], whereas we found elevated numbers of mainly $CD3^+CD4^+$ and $CD3^+CD8^+$ T-lymphocytes 3 and 7 days after reperfusion in the left lung and TLN. Infiltration and activation of T-cells has been classically attributed to the presence of antigen. Our findings in an autologous setting may be explained by different mechanisms. First, LIRI induced upregulation of adhesion molecules may attract T-cells, such as effector and memory T-cells, which are able to proliferate in an antigen independent fashion by cytokines produced locally (bystander effect), in contrast with naïve T-cells which require antigen presentation by antigen presenting cells [34]. Moreover, antigen specific T-cells may be attracted by released self-antigens [35, 36]. Myosin, heat shock proteins and type V collagen, which is released after LIRI in BALf comparable to the level observed in allografted lungs, have been shown to be capable of inducing a helper T-cell reaction within days after reperfusion [35, 36]. The latter is supported by the finding of Waddell and colleagues, who demonstrated upregulation of major histocompatibility II complex after LIRI [37]. Finally, the elevated levels of $CD3^+CD4^+$ and $CD3^+CD8^+$ may be explained by their possible role in the pathogenesis of lung fibrosis, which is also supported by the presence of macrophages in the BALf and lung parenchyma of ischemic animals 3, 7, and 90 days after reperfusion, since they are also thought to be important mediators in the regulation of fibroblast function [1, 2, 38, 39].

Our study furthermore demonstrates an immunosuppressive effect of operation, as measured by the decreased number of lymphocytes in lung tissue on day 1. Although it is very well known that major surgery may cause a short-lasting decrease in blood circulating lymphocytes [40], we now additionally report that thoracotomy causes a one day decrease in the number of lymphocyte subset in lung parenchyma, while the number of lymphocytes in the BALf of sham-operated animals is close to normal. The immunosuppressive effect of surgery may be due to reduced T-cell proliferation and reduced secretion of interleukin-2 (IL-2), IL-4, and gamma interferon by T-lymphocytes, which may be the effect of inhibitory factors secreted by mononuclear phagocytic cells as a result of injury [41]. Moreover, altered migration of memory and activated effector T-cells to injury sites may have also contributed

to the decreased level of measured cells [42].
Finally, another interesting point arising from this study is the effect of left LIRI on right-sided pulmonary injury. While no major changes were seen on HE slides of the right lung, the inflammatory profile of the right BALf resembled that of the left, although it was less severe. Also, an increased amount of SA was measured in these parts of the lung, demonstrating that the right lung has sustained injury. Since the right lung did not sustain ischemia, induction of systemic components, similar to that seen after mesenteric artery ischemia, may have caused right-sided lung injury. In this regard, induction of high-mobility group-1 protein, a downstream proinflammatory cytokine produced by necrotic cells [43, 44], and production of uric acid could explain this phenomenon [45]. Furthermore, activated neutrophils lose their ability to deform, so that they might have plugged the capillaries of the right lung and may have subsequently caused lung injury [46]. However, LIRI of the left lung did not result in long-term damage in the right lung.

5 | Conclusions

The short and long-term changes after LIRI in this model resemble those found in both PGD and ARDS. Thus, LIRI seems a major risk factor for PGD in the absence of other influencing factors, such as alloimmunity. Importantly, LIRI resulted in progressive deterioration of lung function and architecture, leading to extensive immunopathological and functional abnormalities up to 3 months after reperfusion. Immunologically, LIRI caused neutrophil infiltration early after reperfusion, followed by T lymphocytes and macrophages. There were also signs of inflammation in the non-ischemic lung on the short-term, but to a lesser extent. Long-term changes were not found in the right lung.

6 | References

1. Ware LB, Matthay MA. The acute respiratory distress syndrome. N Engl J Med 2000;342:1334-1349.
2. Ingbar DH. Mechanisms of repair and remodelling following acute lung injury. Clin Chest Med 2000;21:589-616.
3. Friedrich I, Borgermann J, Splittgerber FH, et al. Bronchoscopic surfactant administration preserves gas exchange and pulmonary compliance after single lung transplantation in dogs. J Thorac Cardiovasc Surg 2004;127:335-343.
4. Gunther A, Balser M, Schmidt R, et al. Surfactant abnormalities after single lung transplantation in dogs: Impact of bronchoscopic surfactant administration. J Thorac Cardiovasc Surg 2004;127:344-354.
5. Maitra G, Inchley K, Novick RJ, et al. Acute lung injury and lung transplantation influence in vitro subtype conversion of pulmonary surfactant. Am J Physiol Lung Cell Mol Physiol 2002;282:L67-74.
6. Erasmus ME, Petersen AH, Oetomo SB, et al. The function of surfactant is impaired during the reimplantation response in rat lung transplants. J Heart Lung Transplant 1994;13:791-802.
7. Erasmus ME, Veldhuizen RA, Novick RJ, et al. The effect of lung preservation on alveolar surfactant. Transplantation 1996;62:143-144.
8. Veldhuizen RA, Lee J, Sandler D, et al. Alterations in pulmonary surfactant composition and activity after experimental lung transplantation. Am Rev Respir Dis 1993;148:208-215.
9. de Perrot M, Liu M, Waddell TK, et al. Ischemia-reperfusion-induced lung injury. Am J Respir Crit Care Med 2003;167:490-511.
10. Shapiro SD, Senior RM. Matrix metalloproteinases. Matrix degradation and more. Am J Respir Cell Mol Biol 1999;20:1100-1102.
11. Soccal PM, Gasche Y, Miniati DN, et al. Matrix metalloproteinase inhibition decreases ischemia-reperfusion injury after lung transplantation. Am J Transplant 2004;4:41-50.
12. Soccal PM, Gasche Y, Pache JC, et al. Matrix metalloproteinases correlate with alveolar-capillary permeability alteration in lung ischemia-reperfusion injury. Transplantation 2000;70:998-1005.
13. Yano M, Omoto Y, Yamakawa Y, et al. Increased matrix metalloproteinase 9 activity and mRNA expression in lung ischemia-reperfusion injury. J Heart Lung Transplant 2001;20:679-686.
14. Struber M, Hohlfeld JM, Fraund S, et al. Low-potassium dextran solution ameliorates reperfusion injury of the lung and protects surfactant function. J Thorac Cardiovasc Surg 2000;120:566-572.
15. Lachmann B, Eijking EP, So KL, et al. In vivo evaluation of the inhibitory capacity of human plasma on exogenous surfactant function. Intensive Care Med 1994;20:6-11.
16. O'Connor CM, FitzGerald MX. Matrix metalloproteases and lung disease. Thorax 1994;49:602-609.
17. Torii K, Iida K, Miyazaki Y, et al. Higher concentrations of matrix metalloproteinases in bronchoalveolar lavage fluid of patients with adult respiratory distress syndrome. Am J Respir Crit Care Med 1997;155:43-46.
18. Ochs M, Nenadic I, Fehrenbach A, et al. Ultrastructural alterations in intraalveolar surfactant subtypes after experimental ischemia and reperfusion. Am J Respir Crit Care Med 1999;160:718-724.
19. Warnecke G, Sommer SP, Gohrbandt B, et al. Warm or cold ischemia in animal models of lung ischemia-reperfusion injury: is there a difference? Thorac Cardiovasc Surg 2004;52:174-179.
20. Zwacka RM, Zhang Y, Halldorson J, et al. CD4(+) T-lymphocytes mediate ischemia/reperfusion-induced inflammatory responses in mouse liver. J Clin Invest 1997;100:279-289.
21. Katsumi H, Nishikawa M, Yamashita F, et al. Prevention of hepatic ischemia/reperfusion injury by prolonged delivery of nitric oxide to the circulating blood in mice. Transplantation 2008;85:264-269.
22. Zhai Y, Qiao B, Gao F, et al. Type I, but not type II, interferon is critical in liver injury induced after ischemia and reperfusion. Hepatology 2008;47:199-206.
23. Burne MJ, Daniels F, El Ghandour A, et al. Identification of the CD4(+) T cell as a major pathogenic factor in ischemic acute renal failure.J Clin Invest 2001;108:1283-1290.
24. Thabut G, Mal H, Cerrina J, et al. Graft ischemic time and outcome of lung transplantation: a multicenter analysis. Am J Respir Crit Care Med 2005;171:786-791.
25. de Perrot M, Young K, Imai Y, et al. Recipient T cells mediate reperfusion injury after lung transplantation in the rat. J Immunol 2003;171:4995-5002.
26. Eppinger MJ, Deeb GM, Bolling SF, et al. Mediators of ischemia-reperfusion injury of rat lung. Am J Pathol 1997;150:1773-1784.
27. Fiser SM, Tribble CG, Long SM, et al. Lung transplant reperfusion injury involves pulmonary macrophages and circulating leukocytes in a biphasic response. J Thorac Cardiovasc Surg 2001;121:1069-1075.

28. Fiser SM, Tribble CG, Long SM, et al. Pulmonary macrophages are involved in reperfusion injury after lung transplantation. Ann Thorac Surg 2001;71:1134-1138; discussion 1138-1139.
29. Nakamura T, Abu-Dahab R, Menger MD, et al. Depletion of alveolar macrophages by clodronate-liposomes aggravates ischemia-reperfusion injury of the lung. J Heart Lung Transplant 2005;24:38-45.
30. Van Putte BP, Kesecioglu J, Hendriks JM, et al. Cellular infiltrates and injury evaluation in a rat model of warm pulmonary ischemia-reperfusion. Crit Care 2005;9:R1-8.
31. Zhao M, Fernandez LG, Doctor A, et al. Alveolar macrophage activation is a key initiation signal for acute lung ischemia-reperfusion injury. Am J Physiol Lung Cell Mol Physiol 2006;291:L1018-1026.
32. Deeb GM, Grum CM, Lynch MJ, et al. Neutrophils are not necessary for induction of ischemia-reperfusion lung injury. J Appl Physiol 1990;68:374-381.
33. Steimle CN, Guynn TP, Morganroth ML, et al. Neutrophils are not necessary for ischemia-reperfusion lung injury. Ann Thorac Surg 1992;53:64-72; discussion 72
34. Eberl G, Brawand P, MacDonald HR. Selective bystander proliferation of memory CD4+ and CD8+ T cells upon NK T or T cell activation. J Immunol 2000;165:4305-4311.
35. Haque MA, Mizobuchi T, Yasufuku K, et al. Evidence for immune responses to a self-antigen in lung transplantation: role of type V collagen-specific T cells in the pathogenesis of lung allograft rejection. J Immunol 2002;169:1542-1549.
36. Sumpter TL, Wilkes DS. Role of autoimmunity in organ allograft rejection: a focus on immunity to type V collagen in the pathogenesis of lung transplant rejection. Am J Physiol Lung Cell Mol Physiol 2004;286:L1129-1139.
37. Waddell TK, Gorczynski RM, DeCampos KN, et al. Major histocompatibility complex expression and lung ischemia-reperfusion in rats. Ann Thorac Surg 1996;62:866-872.
38. Crimi E, Slutsky AS. Inflammation and the acute respiratory distress syndrome. Best Pract Res Clin Anaesthesiol 2004;18:477-492.
39. Crystal RG, Bitterman PB, Mossman B, et al. Future research directions in idiopathic pulmonary fibrosis: summary of a National Heart, Lung, and Blood Institute working group. Am J Respir Crit Care Med 2002;166:236-246.
40. Isitmangil G, Isitmangil T, Balkanli K, et al. Detection of thoracotomy-induced alterations in cell- and humoral-mediated immune response. Eur J Cardiothorac Surg 2002;21:497-501.
41. Hensler T, Hecker H, Heeg K, et al. Distinct mechanisms of immunosuppression as a consequence of major surgery. Infect Immun 1997;65:2283-2291.
42. Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. Science 1996;272:60-66.
43. Tsung A, Sahai R, Tanaka H, et al. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J Exp Med 2005;201:1135-1143.
44. Watanabe T, Kubota S, Nagaya M, et al. The role of HMGB-1 on the development of necrosis during hepatic ischemia and hepatic ischemia/reperfusion injury in mice. J Surg Res 2005;124:59-66.
45. Weinbroum AA, Hochhauser E, Rudick V, et al. Direct induction of acute lung and myocardial dysfunction by liver ischemia and reperfusion. J Trauma 1997;43:627-633; discussion 633.
46. Nanobashvili J, Neumayer C, Fuegl A, et al. Development of 'no-reflow' phenomenon in ischemia/reperfusion injury: failure of active vasomotility and not simply passive vasoconstriction. Eur Surg Res 2003;35:417-424.

7 | Supplemental files

Table 4A. Mean Percentage (SEM) of inflammatory cells in left BALf.

Group	Neutrophils	Macrophages	$CD3^+CD4^+$	$CD3^+CD8^+$	$CD45RA^+$
Unoperated	3.5 (0.9)	31 (5.2)	2.0 (0.4)	1.0 (0.2)	2.1 (0.4)
Sham day 1	24 **US^{30-90}** (8.4)	53 (9.3)	0.4 **US^{7-90}** (0.1)	0.2 **US^{7-30}** (0.1)	0.3 **US^{30-90}** (0.1)
Sham day 3	11 **S^{30-90}** (2.7)	47 (7.1)	1.1 (0.4)	0.4 (0.2)	0.5 **US^{30-90}** (0.1)
Sham day 7	6.1 **S^{30-90}** (1.9)	52 **US^{30-90}** (4.4)	1.8 (0.3)	0.9 (0.2)	1.2 (0.4)
Sham day 30	ND	23 (7.5)	2.9 (0.7)	1.1 (0.5)	3.0 (0.7)
Sham day 90	ND	34 (4.2)	1.7 (0.4)	0.4 **U** (0.0)	2.0 (0.7)
LIRI day 1	45 **UL^{7-90}** (8.3)	13 **US^1L^{3-90}** (3.4)	0.7 **UL^{7-90}** (0.2)	0.4 **UL^{30}** (0.1)	0.6 **UL^{30-90}** (0.2)
LIRI day 3	32 **US^3L^{7-90}** (3.4)	34 (4.2)	1.2 (0.3)	1.0 (0.5)	0.6 **UL^{30-90}** (0.2)
LIRI day 7	13 **L^{30-90}** (5.0)	60 **$UL^{1-3,30}$** (8.7)	2.3 (0.4)	1.0 (0.4)	0.8 **U** (0.2)
LIRI day 30	ND	35 (4.3)	4.1 (1.2)	2.3 (0.7)	2.4 (0.8)
LIRI day 90	ND	56 **$US^{90}L^{1-3,30}$** (5.3)	1.4 (0.3)	0.6 **S^{90}** (0.1)	1.3 (0.3)

Neutrophils, macrophages, helper T-lymphocytes ($CD3^+CD4^+$), cytotoxic T-lymphocytes ($CD3^+CD8^+$), and B-lymphocytes ($CD45RA^+$) in left BALf, presented as a percentage of total measured cells. BALf: BronchoAlveolar Lavage Fluid; LIRI: Lung Ischemia-Reperfusion Injury; SEM: Standard Error of the Mean; ND: Not Detectable

U: $P<0.05$ versus unoperated animals

S^{x-y}: $P<0.05$ versus sham-operated animals day x to day y

L^{x-y}: $P<0.05$ versus LIRI animals day x to day y

Table 4B. Mean (SEM) total number of inflammatory cells (*1,000) in left BALf.

Group	Neutrophils	Macrophages	$CD3^+CD4^+$	$CD3^+CD8^+$	$CD45RA^+$
Unoperated	24 (9.9)	156 (32)	9.2 (2.3)	4.0 (1.0)	10.0 (2.3)
Sham day 1	164 (70)	223 (44)	7.6 (5.1)	4.8 (3.6)	8.4 (7.0)
Sham day 3	70 (18)	515 $\mathbf{S^{90}}$ (173)	11.0 $\mathbf{S^{90}}$ (2.0)	4.1 (1.6)	2.5 $\mathbf{US^{30}}$ (0.5)
Sham day 7	18 (4.6)	246 (70)	7.9 (2.6)	3.2 (1.1)	3.3 $\mathbf{US^{30}}$ (0.9)
Sham day 30	ND	95 (34)	11.0 (2.6)	4.5 (2.2)	11.7 (3.8)
Sham day 90	ND	129 (25)	4.9 (1.4)	1.4 **U** (0.3)	7.1 (2.4)
LIRI day 1	1,906 $\mathbf{US^1L^{7\text{-}90}}$ (505)	226 (58)	14.0 (3.8)	15.3 $\mathbf{UL^{90}}$ (3.6)	11.9 (4.7)
LIRI day 3	1,079 $\mathbf{US^3L^{7\text{-}90}}$ (251)	1,221 $\mathbf{S^3L^{1,30\text{-}90}}$ (205)	90.0 $\mathbf{US^3L^{1,30\text{-}90}}$ (27.1).	46.6 $\mathbf{US^3L^{30\text{-}90}}$ (14.2)	31.2 $\mathbf{US^3L^{30}}$ (10.6)
LIRI day 7	222 $\mathbf{S^7L^{30\text{-}90}}$ (90)	978 $\mathbf{US^7L^{1,30}}$ (212)	50.0 $\mathbf{US^7L^{1,90}}$ (15.1)	23.0 (8.7)	20.0 $\mathbf{S^7L^{30}}$ (5.3)
LIRI day 30	ND	204 (50)	23.4 (8.9)	13.6 (5.4)	7.5 (2.6)
LIRI day 90	ND	497 $\mathbf{US^{90}L^{1,30}}$ (96)	11.3 (3.1)	4.2 $\mathbf{S^{90}}$ (0.9)	11.7 (3.3)

Neutrophils, macrophages, helper T-lymphocytes ($CD3^+CD4^+$), cytotoxic T-lymphocytes ($CD3^+CD8^+$), and B-lymphocytes ($CD45RA^+$) in left BALf, presented as total number of cells. BALf: BronchoAlveolar Lavage Fluid; LIRI: Lung Ischemia-Reperfusion Injury; SEM: Standard Error of the Mean; ND: Not Detectable
U: P<0.05 versus unoperated animals
$S^{x\text{-}y}$: P<0.05 versus sham-operated animals day x to day y
$L^{x\text{-}y}$: P<0.05 versus LIRI animals day x to day y

Table 5A. Mean (SEM) percentage of inflammatory cells in right BALf.

Group	Neutrophils	Macrophages	$CD3^+CD4^+$	$CD3^+CD8^+$	$CD45RA^+$
Unoperated	1.4 (0.7)	58 (7.3)	1.1 (0.3)	0.6 (0.2)	2.2 (0.6)
Sham day 1	20 **U** (8.0)	53 (9.0)	0.7 (0.2)	0.3 (0.1)	0.8 (0.3)
Sham day 3	11 **U** (4.1)	68 (7.3)	0.9 (0.5)	0.6 (0.4)	0.7 (0.2)
Sham day 7	11 **U** (4.6)	74 (4.1)	1.3 (0.3)	0.4 (0.1)	0.6 (0.2)
LIRI day 1	25 **UL^7** (5.2)	39 (4.6)	0.5 (0.2)	0.3 (0.1)	0.4 (0.1)
LIRI day 3	25 **US^3L^7** (3.1)	49 **S^3** 5.2)	1.0 (0.3)	0.6 (0.3)	0.8 (0.2)
LIRI day 7	5.9 (2.3)	67 **L^{1-3}** (5.1)	1.1 (0.3)	0.2 (0.1)	0.8 (0.2)

Neutrophils, macrophages, helper T-lymphocytes ($CD3^+CD4^+$), cytotoxic T-lymphocytes ($CD3^+CD8^+$), and B-lymphocytes ($CD45RA^+$) in right BALf, presented as a percentage of total measured cells. BALf: BronchoAlveolar Lavage Fluid; LIRI: Lung Ischemia-Reperfusion Injury; SEM: Standard Error of the Mean.
U: $P<0.05$ versus unoperated animals
S^{x-y}: $P<0.05$ versus sham-operated animals day x to day y
L^{x-y}: $P<0.05$ versus LIRI animals day x to day y

Table 5B. Mean (SEM) total number of inflammatory cells (*1,000) in right BALf.

Group	Neutrophils	Macrophages	$CD3^+CD4^+$	$CD3^+CD8^+$	$CD45RA^+$
Unoperated	13 (5)	1,917 (384)	8.1 (2.1)	4.5 (1.3)	12.5 (2.9)
Sham day 1	90 (41)	1,500 (188)	7.5 (3.5)	3.3 (1.6)	11.3 (7.3)
Sham day 3	40 (18)	6,299 (1,510)	4.3 (1.1)	2.5 (1.4)	9.2 (2.5)
Sham day 7	28 (8)	6,394 (1,011)	4.7 (1.3)	1.2 (0.4)	4.0 (2.2)
LIRI day 1	178 **U** (41)	1,663 (479)	4.8 (1.0)	3.6 (0.9)	3.6 (0.6)
LIRI day 3	428 **US^3L^7** (156)	4,612 **UL^1** (1,293)	42.5 **US^3L^1** (14.5)	34.1 **$US^3L^{1,7}$** (19.3)	28.0 (11.7)
LIRI day 7	96 **U** (40)	3,575 **US^7L^{1-3}** (970)	16.9 **S^7** (5.4)	3.9 (1.6)	12.5 (3.1)

Neutrophils, macrophages, helper T-lymphocytes ($CD3^+CD4^+$), cytotoxic T-lymphocytes ($CD3^+CD8^+$), and B-lymphocytes ($CD45RA^+$) in right BALf, presented as total number of cells. BALf: BronchoAlveolar Lavage Fluid; LIRI: Lung Ischemia-Reperfusion Injury; SEM: Standard Error of the Mean.
U: $P<0.05$ versus unoperated animals
S^{x-y}: $P<0.05$ versus sham-operated animals day x to day y
L^{x-y}: $P<0.05$ versus LIRI animals day x to day y

Table 6A. Mean (SEM) Percentage of inflammatory in left lung tissue.

Group	Neutrophils	Macrophages	$CD3^+CD4^+$	$CD3^+CD8^+$	$CD45RA^+$
Unoperated	2.4 (0.8)	10 (2.8)	2.5 (0.4)	1,9 (0.2)	4.9 (0.4)
Sham day 1	11 **$US^{3,30}$** (4.2)	29 **$US^{30\text{-}90}$** (3.9)	1.0 **$US^{3,30\text{-}90}$** (0.2)	0.7 **$US^{30\text{-}90}$** (0.2)	1.8 **$US^{3,30}$** (0.5)
Sham day 3	3.0 (1.1)	30 **$US^{30\text{-}90}$** (4.1)	2.5 (0.4)	1.6 (0.4)	4.6 (1.2)
Sham day 7	10 (6.0)	41 **$US^{30\text{-}90}$** (6.9)	1.7 (0.5)	1.0 **$US^{30\text{-}90}$** (0.3)	1.4 **$US^{3,30}$** (0.5)
Sham day 30	1.7 (0.5)	13 (3.6)	3.2 **S^{90}** (0.3)	2.2 (0.3)	4.1 (0.5)
Sham day 90	3.9 (1.2)	13 (3.9)	1.8 (0.2)	1.8 (0.2)	2.8 **US^{30}** (0.6)
LIRI day 1	19 **$UL^{7\text{-}90}$** (4.9)	29 **UL^{30}** (3.1)	1.0 **$UL^{30\text{-}90}$** (0.1)	1.2 (0.3)	3.2 (0.6)
LIRI day 3	18 **$US^3L^{7\text{-}90}$** (4.4)	37 **UL^{30}** (5.9)	1.4 **U** (0.3)	0.9 **$UL^{30\text{-}90}$** (0.2)	2.0 **US^3** (0.3)
LIRI day 7	3.5 (0.6)	36 **$UL^{30\text{-}90}$** (5.5)	1.3 **$UL^{30\text{-}90}$** (0.2)	0.6 **$UL^{30\text{-}90}$** (0.2)	1.2 **$UL^{1\text{-}90}$** (0.2)
LIRI day 30	1.5 (0.3)	16 (1.6)	2.1 **S^{30}** (0.3)	1.6 (0.2)	2.4 **US^{30}** (0.1)
LIRI day 90	2.8 (0.8)	20 **U** (3.0)	2.1 (0.2)	1.7 (0.2)	3.2 (0.6)

Neutrophils, macrophages, helper T-lymphocytes ($CD3^+CD4^+$), cytotoxic T-lymphocytes ($CD3^+CD8^+$), and B-lymphocytes ($CD45RA^+$) in left lung tissue, presented as a percentage of total measured cells. LIRI: Lung Ischemia-Reperfusion Injury; SEM: Standard Error of the Mean.
U: $P<0.05$ versus unoperated animals
$S^{x\text{-}y}$: $P<0.05$ versus sham-operated animals day x to day y
$L^{x\text{-}y}$: $P<0.05$ versus LIRI animals day x to day y

Table 6B. Mean (SEM) Total number of inflammatory cells (*1,000) in left lung tissue.

Group	Neutrophils	Macrophages	$CD3^+CD4^+$	$CD3^+CD8^+$	$CD45RA^+$
Unoperated	596 (166)	596 (166)	136 (22)	104 (14)	263 (20)
Sham day 1	774 (77)	774 (77)	57 **$US^{3,30}$** (17)	32 **$US^{30\text{-}90}$** (9)	95 **U** (53)
Sham day 3	3,796 (1,006)	3,796 **$US^{1,30\text{-}90}$** (1,006)	196 (56)	107 (35)	438 **$S^{1,90}$** (99)
Sham day 7	4,531 (633)	4,531 **$US^{1,30\text{-}90}$** (633)	146 (40)	147 (54)	260 (118)
Sham day 30	595 (198)	595 (198)	137 (15)	92 (6)	191 (33)
Sham day 90	756 (299)	756 (299)	93 (20)	93 (19)	138 **U** (31)
LIRI day 1	3,353 **$UL^{7\text{-}90}$** (568)	3,353 **$US^1L^{30\text{-}90}$** (568)	153 **$S^1L^{3,90}$** (32)	225 **$US^1L^{30\text{-}90}$** (47)	407 **$S^1L^{7\text{-}90}$** (59)
LIRI day 3	6,862 **$US^3L^{7\text{-}90}$** (1,555)	6,862 **$UL^{7\text{-}90}$** (1,555)	445 **$UL^{7\text{-}90}$** (94)	331 **US^3 $L^{7\text{-}90}$** (54)	430 **$L^{7\text{-}90}$** (66)
LIRI day 7	2,649 (705)	2,649 **$US^7L^{30\text{-}90}$** (705)	102 **L^{90}** (17)	95 (39)	147 (51)
LIRI day 30	701 (73)	701 (73)	91 **$S^{30}L^{90}$** (12)	74 **L^{90}** (10)	103 **US^{30}** (10)
LIRI day 90	633 (148)	633 (148)	50 **U** (8)	41 **US^{90}** (7)	93 **U** (28)

Neutrophils, macrophages, helper T-lymphocytes ($CD3^+CD4^+$), cytotoxic T-lymphocytes ($CD3^+CD8^+$), and B-lymphocytes ($CD45RA^+$) in left lung tissue, presented as total number of cells. LIRI: Lung Ischemia-Reperfusion Injury; SEM: Standard Error of the Mean.
U: $P<0.05$ versus unoperated animals
$S^{x\text{-}y}$: $P<0.05$ versus sham-operated animals day x to day y
$L^{x\text{-}y}$: $P<0.05$ versus LIRI animals day x to day y

Table 7A. Mean (SEM) percentage of inflammatory cells in right lung tissue.

Group	Neutrophils	Macrophages	$CD3^+CD4^+$	$CD3^+CD8^+$	$CD45RA^+$
Unoperated	2.2 (0.4)	19 (1.9)	3.5 (0.7)	2.4 (0.4)	4.7 (1.1)
Sham day 1	6.1 **U** (0.8)	31 (3.8)	0.8 **U** (0.1)	0.5 **U** (0.1)	2.9 (0.6)
Sham day 3	5.5 **U** (1.6)	29 (5.7)	1.8 (0.5)	1.1 **U** (0.4)	3.6 (0.7)
Sham day 7	9.1 (4.5)	36 (4.2)	2.0 (0.5)	1.0 (0.3)	1.7 (0.6)
LIRI day 1	8.4 **U** (1.7)	29 (2.9)	0.7 **UL^{3-7}** (0.1)	0.8 **U** (0.2)	1.8 (0.4)
LIRI day 3	9.5 **U** (4.8)	31 (4.6)	1.3 **U** (0.2)	0.8 **U** (0.2)	2.4 (0.5)
LIRI day 7	3.7 (1.3)	32 (5.2)	1.8 **U** (0.3)	0.9 **U** (0.2)	1.9 (0.4)

Neutrophils, macrophages, helper T-lymphocytes ($CD3^+CD4^+$), cytotoxic T-lymphocytes ($CD3^+CD8^+$), and B-lymphocytes ($CD45RA^+$) in right lung tissue, presented as a percentage of total measured cells. LIRI: Lung Ischemia-Reperfusion Injury; SEM: Standard Error of the Mean.
U: $P<0.05$ versus unoperated animals
S^{x-y}: $P<0.05$ versus sham-operated animals day x to day y
L^{x-y}: $P<0.05$ versus LIRI animals day x to day y

Table 7B. Mean (SEM) total number of inflammatory cells (*1,000) in right lung tissue.

Group	Neutrophils	Macrophages	$CD3^+CD4^+$	$CD3^+CD8^+$	$CD45RA^+$
Unoperated	280 (76)	1,917 (384)	314 (84)	232 (49)	508 (144)
Sham day 1	334 (43)	1,500 (188)	43 **US^{3-7}** (10)	27 **US^{3-7}** (6)	152 **US^{3}** (37)
Sham day 3	684 **U** (101)	6,299 **US^{1}** (1,510)	413 (104)	271 (74)	691 (180)
Sham day 7	340 (106)	6,394 **US^{1}** (1,011)	271 (38)	156 (40)	249 (83)
LIRI day 1	993 **US^{1}** (256)	2,663 **S^{1}** (479)	89 **$US^{1}L^{3-7}$** (15)	104 **US^{1}** (15)	224 (47)
LIRI day 3	735 (147)	4,612 (1,293)	249 (53)	183 (46)	442 (103)
LIRI day 7	581 (153)	3,575 (970)	422 (127)	233 (77)	314 (95)

Neutrophils, macrophages, helper T-lymphocytes ($CD3^+CD4^+$), cytotoxic T-lymphocytes ($CD3^+CD8^+$), and B-lymphocytes ($CD45RA^+$) in right lung tissue, presented as total number of cells. LIRI: Lung Ischemia-Reperfusion Injury; SEM: Standard Error of the Mean.
U: P<0.05 versus unoperated animals
S^{x-y}: P<0.05 versus sham-operated animals day x to day y
L^{x-y}: P<0.05 versus LIRI animals day x to day y

Table 8A. Mean (SEM) percentage of inflammatory cells in thoracic lymph nodes.

Group	$CD3^+CD4^+$	$CD3^+CD8^+$	$CD45RA^+$
Unoperated	21 (2.2)	11 (1.2)	28 (2.2)
Sham day 1	27 $\mathbf{S^{3,90}}$ (1.6)	10 (0.9)	31 (2.3)
Sham day 3	19 (2.4)	11 (0.9)	33 $\mathbf{S^{90}}$ (3.7)
Sham day 7	20 (2.9)	12 (2.0)	26 (2.8)
Sham day 30	27 $\mathbf{S^{3\text{-}7,90}}$ (1.1)	17 $\mathbf{US^{1,3}}$ (1.0)	26 (3.1)
Sham day 90	21 (1.7)	15 $\mathbf{S^{1}}$ (1.9)	26 (1.8)
LIRI day 1	24 (1.5)	13 (1.1)	31 (2.1)
LIRI day 3	19 (1.7)	13 (1.1)	40 $\mathbf{UL^{1,7\text{-}90}}$ (2.6)
LIRI day 7	19 (1.8)	10 (1.3)	34 $\mathbf{S^{7}L^{30\text{-}90}}$ (1.9)
LIRI day 30	25 (2.6)	14 (1.4)	27 (1.9)
LIRI day 90	28 $\mathbf{US^{90}L^{3\text{-}7}}$ (1.9)	14 (0.7)	25 (1.9)

Helper T-lymphocytes ($CD3^+CD4^+$), cytotoxic T-lymphocytes ($CD3^+CD8^+$), and B-lymphocytes ($CD45RA^+$) in thoracic lymph nodes, presented as a percentage of total measured cells. LIRI: Lung Ischemia-Reperfusion Injury; SEM: Standard Error of the Mean; TLN: Thoracic Lymph Nodes.
U: $P<0.05$ versus unoperated animals
$S^{x\text{-}y}$: $P<0.05$ versus sham-operated animals day x to day y
$L^{x\text{-}y}$: $P<0.05$ versus LIRI animals day x to day y

Table 8B. Mean (SEM) total number of inflammatory cells (*1,000) in thoracic lymph nodes.

Group	$CD3^+CD4^+$	$CD3^+CD8^+$	$CD45RA^+$
Unoperated	1,886 (248)	1,087 (200)	2,391 (323)
Sham day 1	2,444 (696)	891 (204)	2,430 (577)
Sham day 3	3,093 (637)	1,743 **$S^{1,90}$** (301)	5,783 **$US^{1,30-90}$** (925)
Sham day 7	2,825 (938)	2,221 (597)	3,304 (831)
Sham day 30	2,944 (617)	1,838 (416)	1,653 (444)
Sham day 90	1,215 **$S^{3,30}$** (308)	776 (272)	2,094 (738)
LIRI day 1	2,784 (461)	1,440 (244)	3,826 (1,057)
LIRI day 3	5,670 **$UL^{1,90}$** (1,025)	4,281 **$US^3L^{1,30,90}$** (975)	11,868 **$UL^{1,30-90}$** (2,950)
LIRI day 7	5,130 **U** (1,085)	3,055 **U** (798)	12,793 **$US^7L^{1,30-90}$** (2,883)
LIRI day 30	3,860 **U** (824)	1,954 **U** (329)	3,930 **S^{30}** (910)
LIRI day 90	2,934 **S^{90}** (488)	1,747 **S^{90}** (272)	2,956 (618)

Helper T-lymphocytes ($CD3^+CD4^+$), cytotoxic T-lymphocytes ($CD3^+CD8^+$), and B-lymphocytes ($CD45RA^+$) in thoracic lymph nodes, presented as total number of cells. LIRI: Lung Ischemia-Reperfusion Injury; SEM: Standard Error of the Mean; TLN: Thoracic Lymph Nodes.
U: $P<0.05$ versus unoperated animals
S^{x-y}: $P<0.05$ versus sham-operated animals day x to day y
L^{x-y}: $P<0.05$ versus LIRI animals day x to day y

Table 9. Histologic score of the left lung.

Group	IAE				SE				IAH				FIB				ATL				OC			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	N	E	F	R
Unoperated	3				3				3				3				3				3			
Sham day 1	3				3				3				3				2	1			3			
Sham day 3	1	1	1		2	1			3				3				3				1	1		1
Sham day 7	3				3				3				3				3				3			
Sham day 30	3				2	1			3				3				3				2			1
Sham day 90	3				3				3				3				3				3			
LIRI day 1				3		3				3			3				3					3		
LIRI day 3		2		1		1	2		1	1	1		1	2				3				3	2	
LIRI day 7	3				3				3				1	1	1				2	1			3	
LIRI day 30	3				3				3					3					3				3	
LIRI day 90	3				2	1			3				1	2			1		2		1		2	

Scoring		Classification	
A	None	N	Normal
B	Mild/scattered	E	Exudative
C	Moderate/occasional	F	Fibroproliferative
D	Severe/frequent	R	Resolving

HE sections of 3 animals per group were scored for intra-alveolar edema (IAE), septal edema (SE), intra-alveolar hemorrhage (IAH), fibrosis (FIB), atelectasis (ATL), and overall classification (OC). Presented is the number of animals within each group with a particular parameter score. Lungs were classified as N) normal if no abnormalities were seen, E) exudative if pulmonary edema and/or hyaline membranes were present, F) fibroproliferative, if activated fibroblasts and/or proliferating alveolar type II cells were found, and R) resolving if injury was on return to normal. LIRI: Lung ischemia-reperfusion injury.

Table 10. Histologic score of the left lung.

Group	INF				INF CLS			
	A	B	C	D	L	H	G	M
Unoperated		3			3			
Sham day 1	2	1			1			
Sham day 3		3			1	2		
Sham day 7		3			1	2		
Sham day 30	2	1			1			
Sham day 90	1	2			2			
LIRI day 1		2	1				1	2
LIRI day 3			2	1		3	1	
LIRI day 7			2	1	2	3		
LIRI day 30			3			3		
LIRI day 90		1	2		1	2		

Scoring		Inflammation	
A	None	L	Lymhocytic
B	Mild/scattered	H	Histiocytic
C	Moderate/occasional	G	Granulocytic
D	Severe/frequent	M	Mixed

HE sections of 3 animals per group were scored for inflammation severity (INF), and type of inflammatory cells (INF CLS). Presented is the number of animals within each group with a particular parameter score. Some groups may contain more than 3 scores, since some animals had mixed inflammatory patterns. LIRI: Lung ischemia-reperfusion injury.

4

Invited commentary on: Alveolar macrophage secretory products effect type 2 pneumocytes undergoing hypoxia and reoxygenation

by AS McCourtie, AS Farivar, SM Woolley, HE Merry, PS Wolf, B Mackinnon-Patterson, JC Keech, E Fitzsullivan, MS Mulligan

NP van der Kaaij, AJJC Bogers

Annals of Thoracic Surgery 2008;86:1779-1780

1 | Summary McCourtie et al

Background. Activation of the alveolar macrophage is centrally important in the development of lung ischemia-reperfusion injury. Alveolar macrophages and type 2 pneumocytes secrete a variety of proinflammatory mediators in response to oxidative stress. The manner in which they interact and how the macrophage may influence pneumocyte responses in lung ischemia-reperfusion injury is unknown. Utilizing an in vitro model of hypoxia and reoxygenation, we sought to determine if the proinflammatory response of type 2 pneumocytes to oxidative stress would be amplified by alveolar macrophage secretory products. Methods. Cultured pneumocytes were exposed to control media or media from cultured macrophages exposed to hypoxia and reoxygenation. Pneumocytes were subsequently subjected to hypoxia and reoxygenation and assessed for both nuclear translocation of nuclear factor kappa beta and inflammatory cytokine and chemokine secretion. To examine for any reciprocal interactions, we reversed the experiment, exposing macrophages to conditioned pneumocyte media. Results. In the presence of media from stimulated macrophages, production of proinflammatory mediators by type 2 pneumocytes was dramatically enhanced. In contrast, exposure of the macrophage to conditioned pneumocyte media had an inhibitory effect on macrophage responses subsequently exposed to hypoxia and reoxygenation. Conclusions. The alveolar macrophage drives the development of lung reperfusion injury in part through amplification of the inflammatory response of type 2 pneumocytes subjected to hypoxia and reoxygenation. (Annals of Thoracic Surgery, 2008;86:1774–1780)

2 | Commentary

The study by McCourtie and colleagues investigates whether the inflammatory response of type 2 pneumocytes to oxidative stress is influenced by mediators produced by alveolar macrophages exposed to hypoxia and reoxygenation and the other way around [1]. The authors demonstrate by the use of an in vitro cell culture model, that the production of proinflammatory mediators by type 2 pneumocytes was increased after incubation with media from hypoxia and reoxygenation stimulated alveolar macrophages. Incubating alveolar macrophages with type 2 pneumocytes media exposed to hypoxia and reoxygenation resulted in inhibition of cytokine production. The methodology of this paper closely resembles an earlier report by this group, which showed that mediators produced by alveolar macrophages following hypoxia and reoxygenation augmented the cytokine production of pulmonary artery endothelial cells exposed to hypoxia and reoxygenation [2].
In vivo experiments have provided evidence that alveolar macrophages play a key role in the pathogenesis of lung ischemia-reperfusion injury (LIRI), although still some injury is found in LIRI studies after depletion of alveolar macrophages [3-5]. It has been suggested that the alveolar macrophages become activated during ischemia, resulting in production and secretion of inflammatory mediators, which cause recruitment of other inflammatory cells like neutrophils within hours after the start of reperfusion. Several mediators have been identified that may be important in the overall inflammatory reaction after LIRI, but the exact role of these mediators in the crosstalk between individual cells is hard to determine in in vivo experiments. Therefore, a cell culture system like the one used in the study by McCourtie is an essential tool to help unravel the inflammatory mechanism of LIRI.
McCourtie et al nicely demonstrate that mediators produced by alveolar macrophages and type 2 pneumocytes respectively augment or inhibit the production of CINC and MCP-1 by type 2 pneumocytes or alveolar macrophages. However, the specific mediators coordinating the effect of alveolar macrophages on type 2 pneumocytes, and maybe more important, the mediators responsible for the negative feedback loop of type 2 pneumocytes media on alveolar macrophages have not been investigated. In addition, the question why early and late media had a different effect on cytokine production and nuclear translocation of NFκß cannot be answered, since the components of the media have not been investigated. In our opinion, the study by McCourtie nicely demonstrates that mediators produced by alveolar macrophages and type 2 pneumocytes affect the level of cytokine production, while the exact messengers responsible for the crosstalk between these cells remain unclear. Therefore, future in vitro studies should be performed to identify the mediators produced by cells exposed to hypoxia and reoxygenation and to investigate their pro- or anti-inflammatory impact on cells. Following identification of important messengers in in vitro studies, their role in the pathophysiology of LIRI should be confirmed in in vivo experiments. Thereafter, new treatment modalities may be developed.
In conclusion, in our opinion, treatment or prevention of LIRI can only be achieved if the most important mediators in the pathophysiology of LIRI are identified and if the magnitude

of the role they play is recognized. Only then, new possible interventions can be established. The authors do have an important tool which, if used properly, may give us new information on how to minimize LIRI.

3 | References

1. McCourtie AS, Farivar AS, Woolley SM, et al. Alveolar macrophage secretory products effect type 2 pneumocytes undergoing hypoxia-reoxygenation. Ann Thorac Surg 2008;86:1774-1779.
2. McCourtie AS, Merry HE, Farivar AS, et al. Alveolar macrophage secretory products augment the response of rat pulmonary artery endothelial cells to hypoxia and reoxygenation. Ann Thorac Surg 2008;85:1056-1060.
3. de Perrot M, Liu M, Waddell TK, et al. Ischemia-reperfusion-induced lung injury. Am J Respir Crit Care Med 2003;167:490-511.
4. van der Kaaij NP, Kluin J, Haitsma JJ, et al. Ischemia of the lung causes extensive long-term pulmonary injury: an experimental study. Respir Res 2008;9:28.
5. Fiser SM, Tribble CG, Long SM, et al. Lung transplant reperfusion injury involves pulmonary macrophages and circulating leukocytes in a biphasic response. J Thorac Cardiovasc Surg 2001;121:1069-1075.

5

Invited commentary on: Respiratory viral infection in obliterative airway disease after orthotopic tracheal transplantation

by E Kuo, A Bharat, T Goers, W Chapman, L Yan, T Street, W Lu, M Walter, A Patterson, T Mohanakumar

NP van der Kaaij, AJJC Bogers

Annals of Thoracic Surgery 2006;82:1050-1051

1 | Summary manuscript Kuo et al

Background. The long-term survival after human lung transplantation is limited by development of the bronchiolitis obliterans syndrome (BOS). Clinically, community-acquired respiratory viral infections have been correlated with an increased incidence of BOS. The goal of this study was to investigate the role of respiratory viral infections in chronic lung allograft rejection using the murine orthotopic tracheal transplantation model. Methods. Eighty orthotopic tracheal transplants were performed using BALB/c and C57BL/6 mice. Recipient mice were infected intranasally with Sendai virus (SdV), a murine parainfluenza type I virus. Experiments altering the infectious dose, infection time, harvest time, allogeneic response, and viral response were performed. Tracheal allograft rejection was monitored using fibrosis and lamina propria to cartilage ratio measurements. Interferon-γ ELISPOT analysis against irradiated donor (BALB/c) splenocytes was used as immunological indicator of alloreactivity after transplantation. Results. Sendai virus infection revealed a dose-dependent transient suppression of alloreactivity with a decrease in tracheal allograft fibrosis and frequency of alloreactive T-cells at 30 days. This immunosuppression was reversed by day 60, leading to increased tracheal allograft fibrosis with a concomitant increase in the frequency of interferon-γ producing alloreactive T-cells. Pretransplant sensitization with donor antigens prevented the initial suppression of alloreactivity due to SdV infection. Furthermore, pretransplant immunization against SdV infection resulted in rapid clearing of the infection and reduced the immunopathology of rejection. Conclusions. Respiratory viral infections can cause enhanced tracheal allograft rejection despite the initial phase of transient immunosuppression. Early treatment or vaccination against the respiratory infections may represent a viable intervention to reduce the risk of chronic rejection. (Annals of Thoracic Surgery 2006;82:1043–1051)

2 | Commentary

The outcome following human lung transplantation remains limited, mainly due to development of the bronchiolitis obliterans syndrome (BOS). Development of BOS may be considered as a multiple injury hit model, in which lung injury-causing factors (e.g. ischemia-reperfusion injury, pneumonia, rejection, donor ventilation and cytomegalovirus (CMV)) seem to have a (major or minor) role in BOS development. All these factors may interplay in BOS initiation and progression. However, pathological pathways by which (acute) lung injury factors may contribute to and interplay in BOS development and progression are mostly unclear.

The hypothesis of the experimental study by Kuo et al was to determine whether a relation between respiratory virus infection and chronic rejection exists [1]. The authors clearly demonstrated enhanced allograft rejection after viral infection in a mouse tracheal transplant model. Nevertheless, in a clinical setting, several studies have investigated the effect of respiratory viruses on the outcome of human lung transplantation. In this regard, Kumar et al have shown that community acquired respiratory viruses (CARV) are associated with development of acute rejection and BOS [2]. In previous reports from the group of Kuo et al, it was proven that CARV infections are linked to an increased risk of BOS development, progression and death, independent of other risk factors like acute rejection [3,4]. Furthermore, Billings et al have found a link between lower respiratory tract infections and high grade BOS development and other clinical studies have also demonstrated a correlation between CMV and BOS [5-7]. Although some clinical studies did not find an association between CMV infection and BOS [8-10], a fall in CMV incidence after the introduction of ganciclovir prophylaxis resulted in reduced incidence of BOS [11]. Moreover, ganciclovir resistance causes earlier BOS onset [12]. Finally in a study by Westall et al, a strong association between BOS and early HCMV DNAaemia was demonstrated [13].

In an experimental setting of chronic airway rejection, viral and bacterial infection resulted in enhanced development of chronic airway and vascular rejection in a rat lung transplantation model [14]. Moreover, a synergistic role between viral infections and chronic rejection in the development of BOS has been shown before in an experimental lung transplantation study by Winter et al [15].

With the aforementioned literature in mind, this well-executed study by Kuo and associates provides us with an experimental model rather than new insights in the contribution of respiratory viruses to human BOS development, since several experimental and clinical studies already suggested a relation. The challenge is to use this model and the experimental setting to study underlying pathological mechanisms by which these factors may contribute to and interplay in BOS. In our opinion, the question whether respiratory viruses are a risk factor for BOS development should be answered affirmatively and studied quantitatively at a clinical level of human lung transplantation, while the laboratory could be better used to unravel underlying pathways and investigate treatment modalities of BOS onset and progression.

3 | References

1. Kuo E, Bharat A, Goers T, et al. Respiratory viral infection in obliterative airway disease after orthotopic tracheal transplantation. Ann Thorac Surg 2006;82:1043-1050.
2. Kumar D, Erdman D, Keshavjee S, et al. Clinical impact of community-acquired respiratory viruses on bronchiolitis obliterans after lung transplant. Am J Transplant 2005;5:2031-2036.
3. Khalifah AP, Hachem RR, Chakinala MM, et al. Respiratory viral infections are a distinct risk for bronchiolitis obliterans syndrome and death. Am J Respir Crit Care Med 2004;170:181-187.
4. Khalifah AP, Hachem RR, Chakinala MM, et al. Minimal acute rejection after lung transplantation: a risk for bronchiolitis obliterans syndrome. Am J Transplant 2005;5:2022-2030.
5. Billings JL, Hertz MI, Savik K, et al. Respiratory viruses and chronic rejection in lung transplant recipients. J Heart Lung Transplant 2002;21:559-566.
6. Girgis RE, Tu I, Berry GJ, et al. Risk factors for the development of obliterative bronchiolitis after lung transplantation. J Heart Lung Transplant 1996;15:1200-1208.
7. Fiser SM, Tribble CG, Long SM, et al. Ischemia-reperfusion injury after lung transplantation increases risk of late bronchiolitis obliterans syndrome. Ann Thorac Surg 2002;73:1041-1047; discussion 1047-1048.
8. Bando K, Paradis IL, Similo S, et al. Obliterative bronchiolitis after lung and heart-lung transplantation. An analysis of risk factors and management. J Thorac Cardiovasc Surg 1995;110:4-13; discussion 13-14.
9. Ettinger NA, Bailey TC, Trulock EP, et al. Cytomegalovirus infection and pneumonitis. Impact after isolated lung transplantation. Washington University Lung Transplant Group. Am Rev Respir Dis 1993;147:1017-1023.
10. Keenan RJ, Lega ME, Dummer JS, et al. Cytomegalovirus serologic status and postoperative infection correlated with risk of developing chronic rejection after pulmonary transplantation. Transplantation 1991;51:433-438.
11. Soghikian MV, Valentine VG, Berry GJ, et al. Impact of ganciclovir prophylaxis on heart-lung and lung transplant recipients. J Heart Lung Transplant 1996;15:881-887.
12. Kruger RM, Shannon WD, Arens MQ, et al. The impact of ganciclovir-resistant cytomegalovirus infection after lung transplantation. Transplantation 1999;68:1272-1279.
13. Westall GP, Michaelides A, Williams TJ, et al. Bronchiolitis obliterans syndrome and early human cytomegalovirus DNAaemia dynamics after lung transplantation. Transplantation 2003;75:2064-2068.
14. Wiebe K, Fraund S, Steinmuller C, et al. Rat cytomegalovirus and Listeria monocytogenes infection enhance chronic rejection after allogenic rat lung transplantation. Transpl Int 2005;18:1166-1174.
15. Winter JB, Gouw AS, Groen M, et al. Respiratory viral infections aggravate airway damage caused by chronic rejection in rat lung allografts. Transplantation 1994;57:418-422.

6

Surfactant pretreatment ameliorates ischemia-reperfusion injury of the lung

Based on
NP van der Kaaij, JJ Haitsma, J Kluin, BN Lambrecht, B Lachmann, RWF de Bruin, AJJC Bogers
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Summary

Background. Lung ischemia-reperfusion injury (LIRI) is suggested to be a major risk factor for development of primary graft dysfunction following lung transplantation. LIRI hereby contributes profoundly to both morbidity and mortality after lung transplantation. It is unknown what the effect of surfactant treatment before ischemia is on LIRI. The goal of this study is to investigate whether surfactant pretreatment provides lung protection in an animal model of lung ischemia-reperfusion injury (LIRI). Methods. Male Sprague Dawley rats (n=100) were randomised to receive intratracheally administered surfactant or no pretreatment. One hour thereafter, animals underwent 120 minutes of warm ischemia of the left lung, or were sham-operated. A third group served as unoperated controls. Measurements were performed on day 1, 3 or 7. Blood gas values were determined and lung compliance was recorded. Bronchoalveolar lavage fluid (BALf) was obtained to assess the amount of alveolar protein, the ratio of small to large aggregate surfactant phospholipids (SA/LA ratio), and leukocyte infiltration (granulocytes, macrophages and lymphocytes, measured by flowcytometry). Results. LIRI resulted in a mortality rate of 17% and significantly decreased lung compliance and PaO_2 (day 1 and 3 $P<0.001$, day 7 $P<0.05$) as compared to sham-operated and unoperated controls. On day 1 more protein was present in the alveoli of ischemic lungs ($P<0.001$) than in sham-operated and unoperated controls. Furthermore, LIRI resulted in an increased SA/LA ratio in the left lung on day 1 ($P<0.05$) and caused infiltration of granulocytes (day 1, 3 and 7 ($P<0.01$)), macrophages (day 3 ($P<0.05$) and 7 ($P<0.01$) and lymphocytes (day 3 and 7 ($P<0.01$)) in the BALf as compared to sham and unoperated controls. Surfactant pretreatment improved survival, lung compliance (day 3 $P<0.001$) and PaO_2 (day 1, 3 ($P<0.01$ and 7 ($P<0.05$)). It also reduced protein leakage ($P<0.05$) and prevented an increase in SA/LA ratio ($P<0.01$). Although the number of macrophages and granulocytes in BALF was increased on day 1 and 3 ($P<0.01$) after surfactant pretreatment as compared to all other groups, the number of lymphocytes was reduced on day 3 ($P<0.05$). Conclusions. This study demonstrates that surfactant pretreatment enhances recovery of

lung function and lung mechanics after LIRI, resulting in normal parameters from day 3 onwards. Surfactant pretreatment in this LIRI model may provide useful information to improve donor lung function after lung transplantation.

1 | Goals of the study

1) To investigate whether surfactant treatment before the induction of ischemia ameliorates LIRI up to one week after LIRI.
2) To determine if surfactant therapy mitigates the inflammatory response after LIRI.

2 | Materials and methods in brief

For a full description of the materials and methods, see the materials and methods section at the end of this thesis.

2.1 Experimental design

One hundred male Sprague-Dawley rats (Harlan, Horst, the Netherlands), weighing 301 ± 40 gram, were randomised into four groups: Surfactant pretreated LIRI (n=30), untreated LIRI (n=30), sham-operated (n=30), and unoperated controls (n=10). Whereas unoperated animals did not receive any treatment, sham-operated animals underwent a thoracotomy, dissection of the left lung hilus and were ventilated and anesthetized during the same period as LIRI animals, but did not receive ischemia. Untreated and surfactant pretreated animals underwent 120 minutes of warm ischemia of the left lung.
Exogenous natural porcine surfactant, HL-10™ (Leo Pharmaceutical Products, Ballerup, Denmark and Halas Pharma, Oldenburg, Germany), dissolved in 50 mg/ml saline, was administered in the surfactant pretreated group intratracheally in three dosages (total dose 400 mg/kg bodyweight) over 1 hour after the animals were briefly anesthetized (65% nitrous oxide/33% oxygen/2% isoflurane) and intubated. After each dose, animals recovered from anaesthesia and breathed spontaneously to allow the instilled surfactant to be adsorbed. One hour after the first dose, animals were operated. Measurements were performed 1, 3 and 7 days after the operation.

2.2 Measurements

Measurements include survival, blood gas values, static pulmonary compliance, alveolar protein level, surfactant small and large aggregates and flowcytometric analysis of infiltrating cells in BALf (Figure 1). The static pulmonary compliance is presented as Vmax and Cmax. Vmax is the maximal lung volume at a pressure of 35 cm H_2O, while Cmax is the maximal compliance of the expiration curve.

2.3 Statistical analysis

The results in text and tables are presented as mean ± standard deviation (SD) and data were analysed using SPSS version 11.1 statistical software (SPSS Inc., Chicago, Illinois, USA). In the figures the data are displayed as mean ± standard error of the mean (SEM). If the distribution within a group was normal, as assessed by the Kolmogorov-Smirnov test, and if the condition of equal variances was met by the Levene's test, differences between groups were tested for significance by one-way ANOVA. If the overall level of ANOVA was significant, intergroup comparisons were made by the Bonferroni post hoc test. In the case of unequal variances or an abnormal distribution, a non-parametric Kruskal-Wallis test was

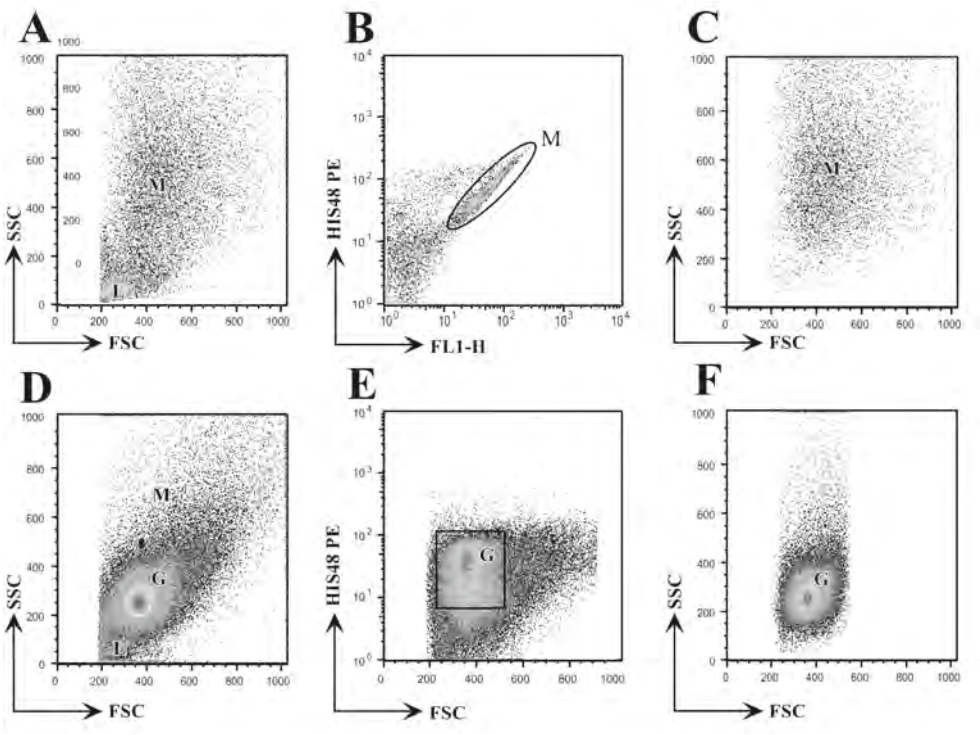

Figure 1. Flowcytometry dot plots of cells acquired from BALf of an unoperated animal (A-C) and an untreated LIRI animal (D-F). Macrophages (M) are high on FSC (large size) and on SSC (high granularity), (B) autofluorescent and are the most frequently occurring cells in the BALf of unoperated rats. Lymphocytes (L) are low on FSC and SSC. LIRI caused infiltration of granulocytes (G), which are intermediate on FSC and SSC, and (E) HIS48PE positive. Plot C shows that the cells in the gate on plot B are predominantly macrophages and plot F proves that the HIS48 PE positive cells on plot E are granulocytes. BALf: BronchoAlveolar Lavage fluid; FSC: Forward SCatter; SSC: Side SCatter; LIRI: Lung Ischemia-Reperfusion Injury.

performed, followed by Mann-Whitney U tests for intergroup comparisons. The difference in mortality rate between untreated and surfactant pretreated groups was assessed by the Fisher exact test. P values <0.05 were considered to be significant.

3 | Results

3.1 Survival

In the untreated LIRI group, 5 out of 30 (17%) operated animals died shortly after weaning from the ventilator, due to respiratory failure. After surfactant pretreatment, only 1 out of 30 animals (3%) died. However, the difference in mortality between the untreated and surfactant pretreated LIRI groups was not significant (P=0.19). All sham-operated animals survived the experimental period.

Table 1. Mean (SD) maximal compliance of the expiration curve and mean (SD) maximal volume of the left lung.

Group	**Cmax** ((ml/kg)/cm H_2O)	**Vmax** (ml/kg)
Unoperated	1.07 (0.29)	13.03 (1.82)
Sham day 1	1.24 (0.37)	15.28 (3.14)
Sham day 3	1.06 (0.23)	15.49 (2.25)
Sham day 7	0.95 (0.11)	14.21 (3.00)
LIRI day 1	0.27 (0.17) **US¹**	4.60 (1.90) **US¹**
LIRI day 3	0.32 (0.17) **US³**	4.91 (2.25) **US³**
LIRI day 7	0.60 (0.37)	8.09 (3.62) **US⁷**
LIRI & Su day 1	0.62 (0.33) **S¹**	8.24 (2.56) **US¹**
LIRI & Su day 3	1.18 (0.23) **L³**	13.73 (1.33) **L³**
LIRI & Su day 7	0.93 (0.15)	11.34 (1.70)

Cmax and Vmax can be deducted from the pressure volume curves presented in Figure 3. The data are presented as mean ± standard deviation (SD). LIRI: Lung Ischemia-Reperfusion Injury; Cmax: Maximal compliance of the expiration curve; Su: Surfactant pretreatment; Vmax: Maximal volume at a pressure of 35 cm H_2O.
U: P<0.05 versus unoperated animals,
S^x: P<0.05 versus sham-operated animals on day x
L^x: P<0.05 versus LIRI animals on day x

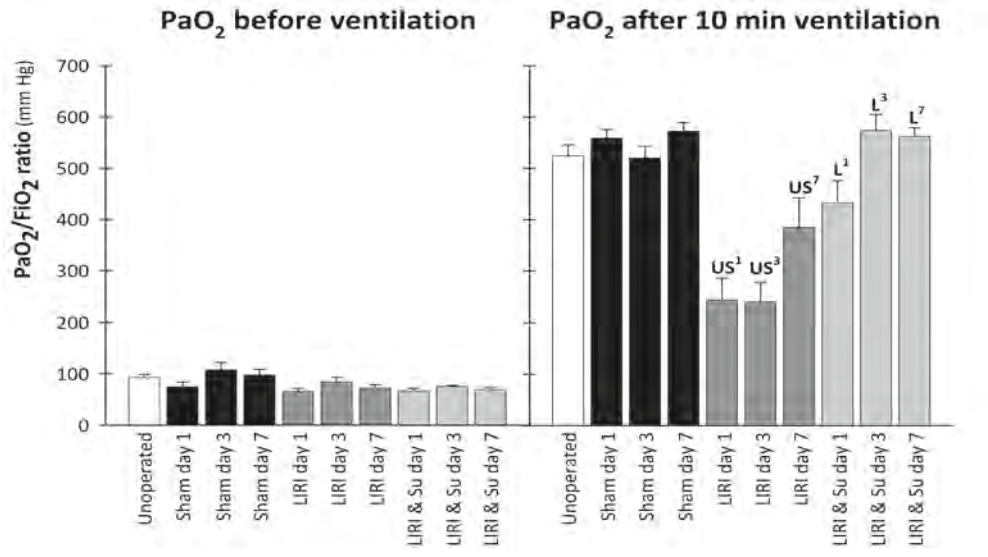

Figure 2. PaO_2 values before and after 10 minutes ventilation with a FiO_2 of 1 on day 1, 3 and 7 after LIRI. LIRI resulted in significantly lower PaO_2 values on all days compared to sham-operated and unoperated controls. In surfactant pretreated animals, normal PaO_2 values were recorded on day 3 and 7. LIRI: Lung Ischemia-Reperfusion Injury; Su: Surfactant pretreatment.
U: P<0.05 versus unoperated animals,
S^x: P<0.05 versus sham-operated animals on day x
L^x: P<0.05 versus LIRI animals on day x

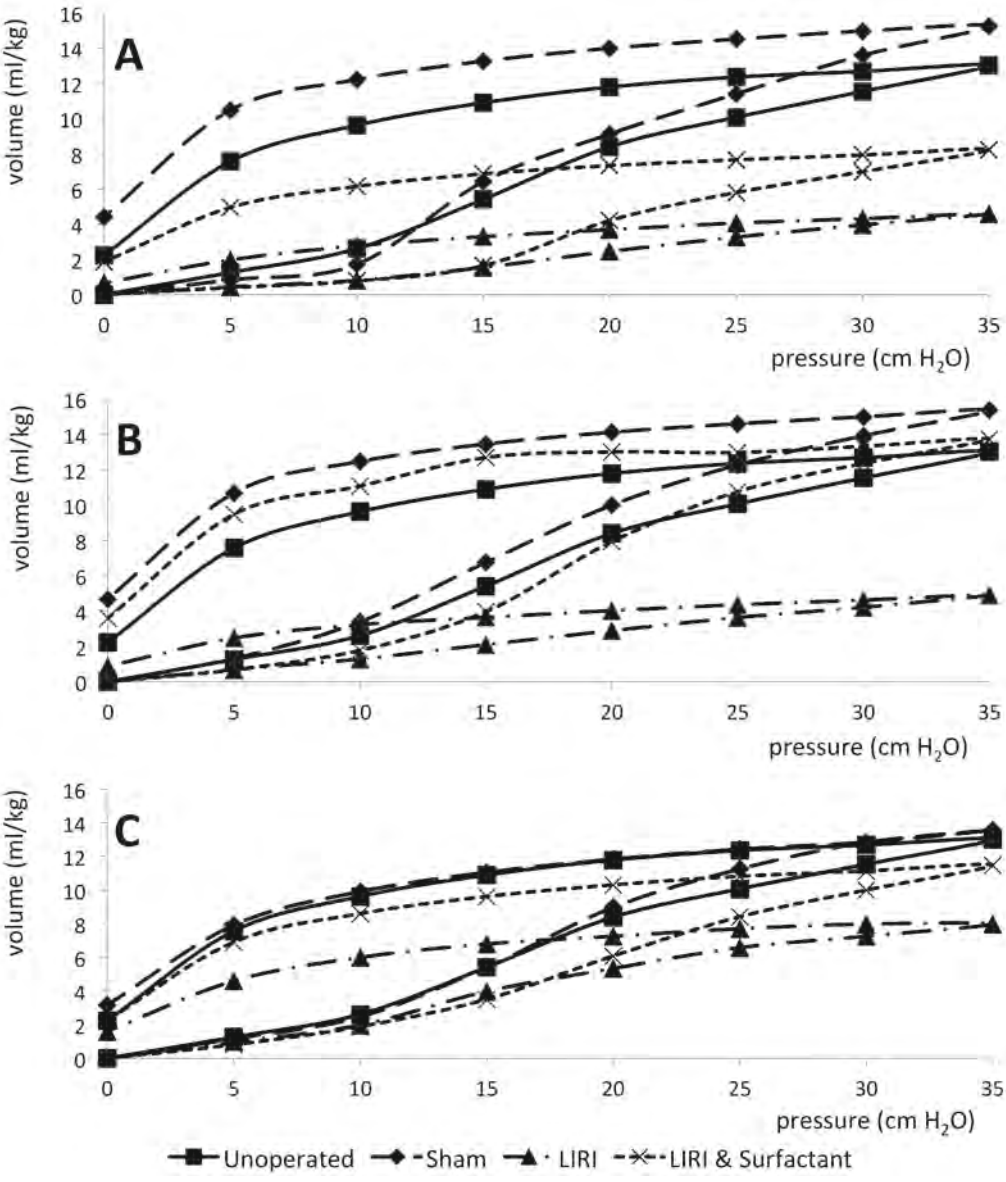

Figure 3. Pressure-volume curves (PVC) of unoperated, sham-operated, LIRI untreated, and surfactant pretreated groups on day 1 (A), 3 (B) and 7 (C). LIRI had detrimental effects on the PVC: On day 1 and 3, Cmax and Vmax of untreated LIRI animals were lower than the values found in sham-operated and unoperated controls. On day 7, the Vmax was still significantly decreased compared to sham-operated and unoperated controls. Pretreatment with surfactant restored lung compliance to the levels observed in sham-operated and unoperated animals on day 3 and 7 as compared to untreated LIRI animals. For mean and SD see Table 1. Cmax: Maximal compliance of the expiration curve, corrected for body weight; LIRI: Lung Ischemia-Reperfusion Injury; Vmax: Maximal lung volume at a pressure of 35 cm H_2O, corrected for body weight.

3.2 PaO_2 and $PaCO_2$

PaO_2 was similar in all groups before the start of ventilation (Figure 2). After 10 minutes of ventilation with a FiO_2 of 1, PaO_2 increased five-fold in unoperated and sham-operated animals. However, LIRI resulted in a significantly lower PaO_2 on day 1, 3 and 7 after reperfusion compared to sham-operated and unoperated controls. After surfactant pretreatment, PaO_2 had improved on day 1 compared to untreated LIRI animals. Normal, pre-operative values were found in surfactant pretreated animals on day 3 and 7 in contrast to untreated LIRI animals. No significant differences were seen in $PaCO_2$ on any day (data not shown).

3.3 Pressure-volume curve

Cmax and Vmax did not differ between sham-operated and unoperated controls (Figure 3, Table 1). However, LIRI had a detrimental effect on the static PVC on both day 1 and 3. Cmax and Vmax of untreated LIRI animals were lower than the values found in sham-operated and unoperated controls (Figure 3A&B, Table 1). On day 7, the Vmax was still significantly impaired compared to the values found in unoperated and sham-operated animals (Figure 3C, Table 1). After surfactant pretreatment, Vmax and Cmax on day 1 were still significantly lower than in sham-operated and unoperated controls (Figure 3A, Table 1). However, from day 3 on, lung compliance returned to the level observed in sham-operated and unoperated animals (Figure 3B&C, Table 1).

3.4 Alveolar protein

The total amount of alveolar protein measured in BALf of the left and right lung was not influenced by sham operation (Figure 4). However, 120 minutes of warm ischemia and reperfusion resulted in a significant increase in the amount of alveolar protein in the BALf of the left and right lung as compared to sham-operated and unoperated controls on day 1. When animals were pretreated with surfactant, the total protein content of the left BALf was lower compared to the untreated LIRI animals. However, significantly more proteins were still found in the alveolar spaces than in sham-operated and unoperated controls. On day 3 and day 7 no differences in the amount of alveolar protein were found between all groups.

3.5 SA/LA ratio

Whereas sham operation had no effect on the SA/LA ratio (Figure 5), an increased ratio was seen after LIRI on day 1 compared to sham-operated and unoperated controls. This was predominantly due to an increased total amount of SA (Table 2). Pretreatment with surfactant prevented an increase in the SA/LA ratio on day 1. The instilled exogenous surfactant consisted predominantly of the LA subform, thereby inducing an increase in the amount of LA in both the left and right BALf on day 1 and 3 as compared to all other groups (Table 2). In surfactant pretreated animals, the conversion of LA to SA after LIRI also occurred, causing a significantly higher level of SA in both lungs on day 1, 3 and 7 as compared to all other groups.

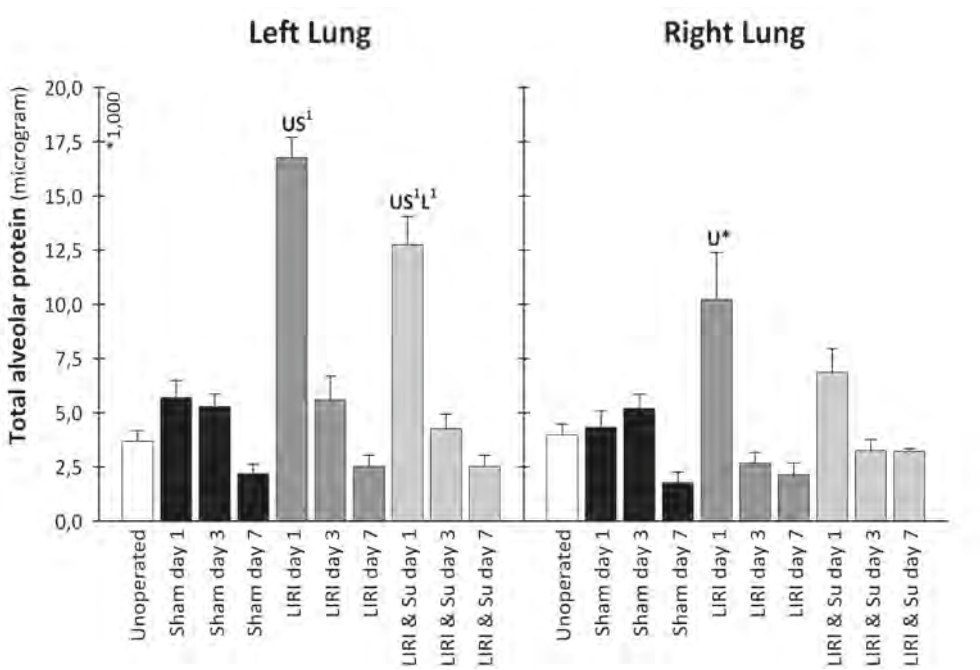

Figure 4. Total amount of alveolar protein (micrograms) in both left and right BALf of unoperated, sham-operated, LIRI untreated, and surfactant pretreated groups on day 1, 3 and 7. LIRI induced a significant increase in the amount of alveolar protein in the BALf of the left and right lung on day 1. If animals were pretreated with surfactant, the total protein content of the left BALf decreased on day 1. On day 3 and 7 no changes in the amount of alveolar protein were found in either the ischemic or non-ischemic lungs. BALf: BronchoAlveolar Lavage fluid; LIRI: Lung Ischemia-Reperfusion Injury; Su: Surfactant pretreatment.
U: $P<0.05$ versus unoperated animals
S^x: $P<0.05$ versus sham-operated animals on day x
L^x: $P<0.05$ versus LIRI animals on day x
*: $P=0.065$ versus sham-operated group on day 1

3.6 Flowcytometry

The macrophage was the most frequently occurring cell in BALf of unoperated and sham-operated rats on all days (Figure 1 & 6). No significant changes were seen in the total number of macrophages, granulocytes and lymphocytes after sham operation (Figure 6). If animals were subjected to ischemia and reperfusion without surfactant pretreatment, granulocytes infiltrated the alveolar spaces and became the most abundant cell on day 1. Over time, the total amount of granulocytes in BALf of untreated LIRI animals decreased, whereas the fraction of macrophages increased with a peak on day 3. Also, three days after reperfusion, the total number of lymphocytes increased 15-fold and was also higher on day 7 as compared to sham-operated and unoperated controls (Figure 6). In surfactant pretreated animals, a significant increase in the total number of macrophages and granulocytes was found on day 1 and 3 as compared to all other groups. On day 3, the number of lymphocytes in BALf of surfactant pretreated animals was lower than in untreated LIRI animals.

Table 2. Mean (SD) total amount of SA and LA surfactant phospholipids.

Group	Left BALf		Right BALf	
	SA (mg)	**LA** (mg)	**SA** (mg)	**LA** (mg)
Unoperated	214 (73)	470 (142)	175 (107)	677 (321)
Sham day 1	412 (154) **U**	724 (245) **U**	363 (150)	1,237 (584)
Sham day 3	340 (107) **U**	553 (277)	565 (288) **U**	828 (556)
Sham day 7	230 (54)	543 (302)	352 (149)	717 (675)
LIRI day 1	640 (77) **US^{1}**	347 (261) **S^{1}**	427 (111) **U**	764 (403)
LIRI day 3	184 (104)	242 (258)	217 (136)	872 (320)
LIRI day 7	194 (114)	223 (62) **U**	241 (93)	436 (274)
LIRI & su day 1	1,008 (406) **US^{1}**	4,864 (1797) **US^{1}L^{1}**	1823 (396) **US^{1}L^{1}**	4,824 (2,182) **US^{1}L^{1}**
LIRI & su day 3	1,251 (1265) **UL^{3}**	2,763 (822) **US^{3}L^{3}**	1140 (793) **UL^{3}**	10,226 (2,336) **US^{3}L^{3}**
LIRI & su day 7	459 (172) **L^{7}**	555 (110) **L^{7}**	783 (336) **US^{7}L^{7}**	3,642 (815) **US^{1}L^{7}**

SA and LA phospholipids were measured in the BALf of unoperated, sham-operated, LIRI untreated, and surfactant pretreated LIRI groups on day 1, 3 and 7. The data are presented as mean ± standard deviation (SD). BALf: BronchoAlveolar Lavage Fluid; LIRI: Lung Ischemia-Reperfusion Injury; Su: Surfactant pretreatment; SA: Small aggregate; LA: Large aggregate.
U: $P<0.05$ versus unoperated animals
S^{x}: $P<0.05$ versus sham-operated animals on day x
L^{x}: $P<0.05$ versus LIRI animals on day x

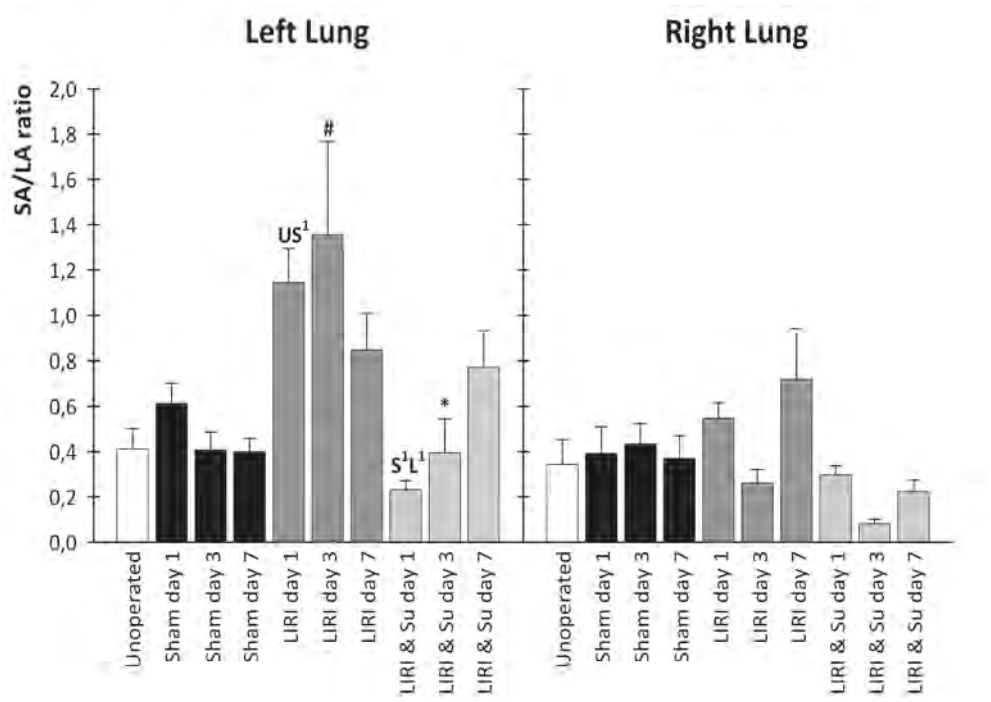

Figure 5. SA/LA ratio in both left and right BALf of unoperated, sham-operated, LIRI untreated and surfactant pretreated groups on day 1, 3 and 7. LIRI induced a significant increase in the SA/LA ratio in the left lung on day 1. When animals were pretreated with surfactant, the increase in the SA/LA ratio was prevented. BALf: BronchoAlveolar Lavage Fluid; LA: Large Aggregate; LIRI: Lung Ischemia-Reperfusion Injury; SA: Small Aggregate; Su: Surfactant pretreatment
U: P<0.05 versus unoperated animals
S^x: P<0.05 versus sham-operated animals on day x
L^x: P<0.05 versus LIRI animals on day x
*: P=0.067 versus untreated LIRI on day 3
#: P=0.093 versus unoperated and sham-operated animals on day 3

4 | Discussion

LIRI resulted in a mortality rate of 17%, a decreased PaO_2, and impaired lung compliance. It also caused an increase in the amount of alveolar protein, an increase in the SA/LA ratio, and an influx of granulocytes, macrophages and lymphocytes in BALf. Surfactant pretreatment reduced mortality to 3%, and resulted in a normal lung function already on day 3 postoperatively. It further decreased alveolar protein leakage in the ischemic lung and resulted in a normal SA/LA ratio. Finally, although more macrophages and granulocytes were found on day 1 and 3 after surfactant pretreatment, the number of lymphocytes had decreased.

Both experimental and clinical studies have demonstrated the beneficial effect of surfactant treatment to lung transplant recipients within several hours after reperfusion [1-5]. Surfactant therapy before ischemia is thought to be more beneficial than treatment at the time of

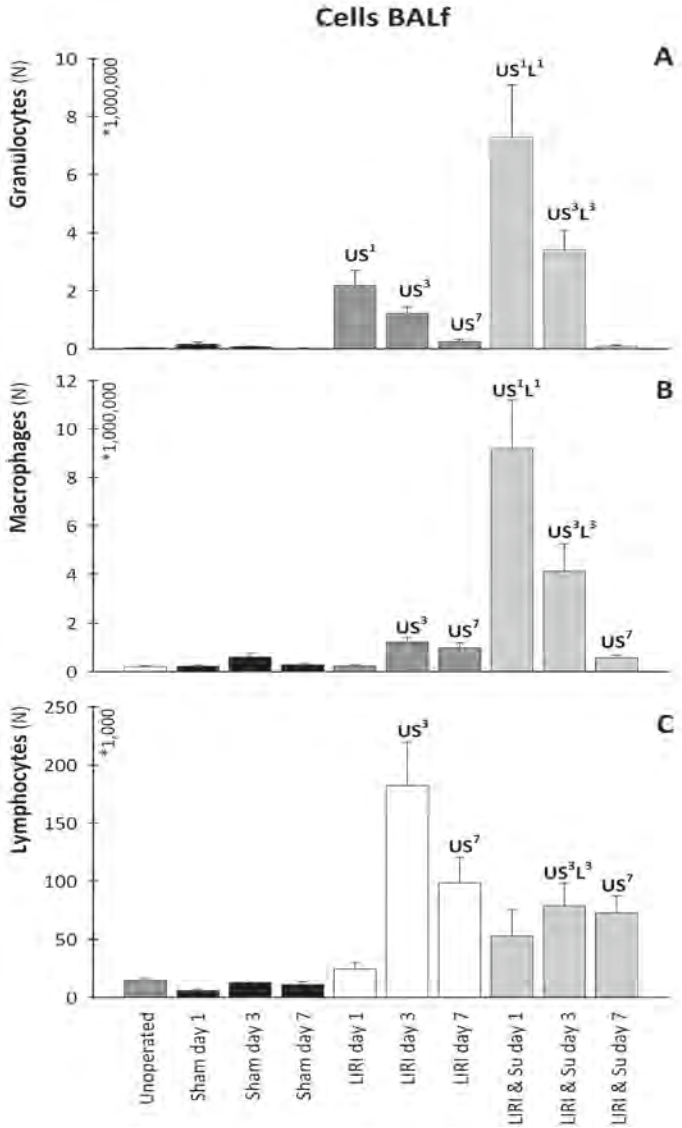

Figure 6. Total number of A) granulocytes, B) macrophages and C) lymphocytes in the BALf of the left lung of unoperated, sham-operated, LIRI untreated, and surfactant pretreated groups on day 1, 3 and 7. LIRI caused an influx of granulocytes (day 1, 3 and 7), macrophages (day 3 and 7), and lymphocytes (day 3 and 7). In surfactant pretreated animals, very high levels of macrophages and granulocytes were present on day 1 and 3. On day 3, the number of lymphocytes in the BALf was lower in surfactant pretreated animals than in untreated LIRI animals. BALf: BronchoAlveolar Lavage Fluid; LIRI: Lung Ischemia-Reperfusion Injury; N: Number of cells; Su: Surfactant pretreatment.
U: $P<0.05$ versus unoperated animals,
S^x: $P<0.05$ versus sham-operated animals on day x
L^x: $P<0.05$ versus LIRI animals on day x

reperfusion or after reperfusion [2]. This may be explained by the fact that surfactant given to the donor results in a more homogenous distribution in the lung as compared to treatment at reperfusion, when alveolar damage has already occurred [6]. In the latter case, intratracheally instilled surfactant will predominantly accumulate in open areas of the lung instead of atelectatic areas, where it is most needed. Moreover, Erasmus and colleagues showed that the endogenous surfactant pool is inversely proportional to the time of ischemia because of the remaining activity of phospholipases during ischemia [7]. This results in influx of serum proteins, which further inhibit surfactant function. Because the normal endogenous surfactant pool contains approximately 10-15 mg lipid per kg, pretreatment with 400 mg lipid per kg exogenous surfactant, predominantly in the LA subform, substantially enlarges the surface-active surfactant pool. The high level of the LA subform found on day 1, 3 and 7 after pretreatment illustrates this. Although after surfactant pretreatment conversion of LA into SA still occurred as indicated by the increased levels of SA in the pretreated group on all days, probably sufficient surface-active phospholipids remained after pretreatment and LIRI to result in normal lung function.

All mortality in our experimental study was due to development of severe pulmonary edema. The accumulation of fluid in the alveolus is predominantly due to the damaged surfactant system, illustrated by an increase in the SA/LA ratio. As a result surfactant cannot maintain its surface lowering function inside the alveolus causing further development of pulmonary edema and subsequently decreased lung compliance and gas exchange, contributing to early morbidity and mortality [1-4, 6]. Although prolonged ventilation strategies could probably reduce mortality in this experimental setting, animals were ventilated postoperatively for as short periods as possible because of the confounding effect of ventilation on LIRI [8].

The conversion of LA into SA and the decrease in lung compliance and PaO_2/FiO_2 ratio within hours after reperfusion has been described in previous experimental studies [1-4, 7 9]. We confirm that LIRI resulted in the conversion of the highly surface active LA subtype into the poor surface active SA subtype on day 1, and impaired PaO_2 and lung compliance throughout the experimental period. We furthermore demonstrate that surfactant treatment before the induction of warm ischemia completely normalised lung compliance and PaO_2 from day 3 onwards and prevented the increase in the SA/LA ratio. Thus, surfactant pretreatment enhances the recovery of lung function but, even more importantly, may prevent in part the damage caused by LIRI.

The high level of alveolar protein in the LIRI animals illustrates the loss of fluid homeostasis, resulting in development of high permeability edema. It is known that when serum proteins accumulate in the alveoli, they are able to further dose-dependently inhibit surfactant function, probably by competing with surfactant phospholipids for a place at the air-water interface, resulting in additional deterioration of lung function [6, 10]. Surfactant replacement therapy at the time of reperfusion interrupts this vicious circle by restoring the fluid homeostasis across the alveolo-capillary membrane [10]. Our results indicate that administration of surfactant before ischemia also decreases the amount of alveolar protein on day 1 following LIRI, which may be an important mechanism in the observed amelioration of LIRI.

In the non-ischemic right lung, an increase in the amount of alveolar protein on day 1 was noticed. Whether this is due to spillover from the injured left lung or a direct injury of the

right lung as a result of left-sided LIRI or ventilator settings cannot be determined. Other studies also showed increased levels of alveolar protein in ischemic and non-ischemic lungs in experimental transplantation models [7, 9]. Friedrich and colleagues described that separate ventilation of the transplanted and non-transplanted lung reduced the level of alveolar protein in the non-transplanted lung suggesting that injury of the native lung occurs due to hyperinflation, which arises when both transplanted and non-transplanted lungs are simultaneously ventilated [4].

Besides the protective effect of surfactant pretreatment on lung architecture after LIRI, surfactant therapy may have suppressive effects on the inflammatory process that is part of LIRI [11]. We therefore examined the influx of macrophages, granulocytes and lymphocytes in BALf of the left lung. Reduction of the inflammatory reaction in the context of ischemia and reperfusion is important and has proven to be successful in amelioration of injury [12-14]. LIRI led to a local inflammatory reaction, characterised by an influx of granulocytes on day 1, followed by infiltration of macrophages and lymphocytes on day 3. We observed a significant increase in the number of macrophages and granulocytes in the surfactant pretreated group, most prominent on day 1 with levels returning to normal at day 7. This increase in macrophages and granulocytes may be partly explained by their surfactant recycling capacity, which mainly depends on alveolar macrophages, but also on alveolar type II cells and to a lesser extent granulocytes [15]. Alveolar macrophages can more easily be recruited to the lung compared to alveolar type II cells. This suggests that macrophages are recruited to the lung in response to the increased demand for surfactant recycling cells. The increased number of granulocytes in the pretreated group can also be explained as follows. In the untreated LIRI group, the endogenous surfactant is impaired following LIRI, thus increasing surface tension. As a result, the alveolus collapses, leading to alveolar shunting and ventilation-perfusion mismatch. To correct for this, hypoxic vasoconstriction occurs. Surfactant pretreatment keeps the alveolus open, thereby preventing shunting and constriction of arterioles, thus facilitating infiltration of granulocytes. Another reason for the higher influx of granulocytes might be that surfactant components are chemotactic for granulocytes [16]. Also, the instillation of saline, in which the surfactant phospholipids were dissolved, may have contributed to the influx of granulocytes. Saline without surfactant phospholipids was not instilled in untreated LIRI animals, because this would lead to high mortality in this group.

The infiltration of macrophages and granulocytes, the leakage of alveolar proteins and the decreased lung compliance in the pretreated group on day 1 suggest that some lung damage still occurred as a result of lung ischemia and reperfusion. However, although the number of the infiltrating cells was higher than in untreated groups, PaO_2 and lung compliance had improved. Thus, surfactant pretreatment did not have a downregulating effect on the number of infiltrating macrophages and granulocytes, but possibly did modulate the effect of the infiltrating cells. Surfactant may have functioned as an anti-oxidant agent or as a physical barrier between lung epithelial, endothelial cells and ROS producing cells so that surfactant itself, but not lung tissue, was damaged after LIRI. The latter can be illustrated by the increased levels of SA in pretreated groups [6, 17].

Surfactant was able to reduce the infiltration of lymphocytes on day 3. Whether this reduction is due to a targeted response of surfactant components, less activation of antigen

presenting cells or preserved lung architecture, resulting in less presentation of self-antigens, remains unanswered. However, the reduction in infiltration of lymphocytes after surfactant pretreatment likely contributes to the improved function observed in this study.

5 | Conclusions

This study shows that surfactant pretreatment improves animal survival and decreases LIRI. Whereas the number of infiltrating macrophages and granulocytes was increased after surfactant pretreatment, the level of lymphocytes decreased. The timing of surfactant administration used in this study would permit donor pretreatment in the clinical setting.

6 | References

1. Erasmus ME, Hofstede GJ, Petersen AH, et al. Effects of early surfactant treatment persisting for one week after lung transplantation in rats. Am J Respir Crit Care Med 1997;156:567-572.
2. Hausen B, Rohde R, Hewitt CW, et al. Exogenous surfactant treatment before and after sixteen hours of ischemia in experimental lung transplantation. J Thorac Cardiovasc Surg 1997;113:1050-1058.
3. Erasmus ME, Petersen AH, Hofstede G, et al. Surfactant treatment before reperfusion improves the immediate function of lung transplants in rats. Am J Respir Crit Care Med 1996;153:665-670.
4. Friedrich I, Borgermann J, Splittgerber FH, et al. Bronchoscopic surfactant administration preserves gas exchange and pulmonary compliance after single lung transplantation in dogs. J Thorac Cardiovasc Surg 2004;127:335-343.
5. Struber M, Hirt SW, Cremer J, et al. Surfactant replacement in reperfusion injury after clinical lung transplantation. Intensive Care Med 1999;25:862-864.
6. van der Kaaij NP, Lachmann RA, Bogers AJJC, et al. Surfactant alterations and treatment of lung transplant ischemia–reperfusion injury. Journal of organ dysfunction 2006;2:221-229.
7. Erasmus ME, Petersen AH, Oetomo SB, et al. The function of surfactant is impaired during the reimplantation response in rat lung transplants. J Heart Lung Transplant 1994;13:791-802.
8. de Perrot M, Imai Y, Volgyesi GA, et al. Effect of ventilator-induced lung injury on the development of reperfusion injury in a rat lung transplant model. J Thorac Cardiovasc Surg 2002;124:1137-1144.
9. Veldhuizen RA, Lee J, Sandler D, et al. Alterations in pulmonary surfactant composition and activity after experimental lung transplantation. Am Rev Respir Dis 1993;148:208-215.
10. Lachmann B, Eijking EP, So KL, et al. In vivo evaluation of the inhibitory capacity of human plasma on exogenous surfactant function. Intensive Care Med 1994;20:6-11.
11. Wright JR. Pulmonary surfactant: a front line of lung host defense. J Clin Invest 2003;111:1453-1455.
12. de Perrot M, Young K, Imai Y, et al. Recipient T cells mediate reperfusion injury after lung transplantation in the rat. J Immunol 2003;171:4995-5002.
13. Krishnadasan B, Naidu B, Rosengart M, et al. Decreased lung ischemia-reperfusion injury in rats after preoperative administration of cyclosporine and tacrolimus. J Thorac Cardiovasc Surg 2002;123:756-767.
14. Uthoff K, Zehr KJ, Lee PC, et al. Neutrophil modulation results in improved pulmonary function after 12 and 24 hours of preservation. Ann Thorac Surg 1995;59:7-12; discussion 12-13.
15. Quintero OA, Wright JR. Clearance of surfactant lipids by neutrophils and macrophages isolated from the acutely inflamed lung. Am J Physiol Lung Cell Mol Physiol 2002;282:L330-339.
16. Kramer BW, Jobe AH, Bachurski CJ, et al. Surfactant protein A recruits neutrophils into the lungs of ventilated preterm lambs. Am J Respir Crit Care Med 2001;163:158-165.
17. Crowther JE, Kutala VK, Kuppusamy P, et al. Pulmonary surfactant protein a inhibits macrophage reactive oxygen intermediate production in response to stimuli by reducing NADPH oxidase activity. J Immunol 2004;172:6866-6874.

7

Surfactant pretreatment decreases long-term damage after ischemia-reperfusion injury of the lung

Based on
NP van der Kaaij, J Kluin, JJ Haitsma, MA den Bakker, BN Lambrecht, B Lachmann, RWF de Bruin, AJJC Bogers
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Summary

Background. Lung ischemia-reperfusion injury (LIRI) is a risk factor for primary graft dysfunction following lung transplantation. LIRI hereby contributes to morbidity and mortality after lung transplantation. We have previously shown that surfactant pretreatment ameliorates LIRI up to one week after reperfusion. However, the impact of surfactant pretreatment on long-term outcome following LIRI is unknown. Therefore, the objective of this study was to investigate the effect of surfactant pretreatment on long-term outcome following LIRI. Methods. Male Sprague-Dawley rats (n=63) were randomized to receive intratracheally administered porcine surfactant (400 mg/kg) or no pretreatment. One hour thereafter, animals underwent 120 minutes of warm ischemia by clamping the bronchus, pulmonary artery and veins of the left lung. A third group was sham-operated; a fourth group served as unoperated controls. Measurements were performed on day 30 or 90 after surgery. Arterial oxygenation and lung compliance were determined. Bronchoalveolar lavage fluid (BALf) was collected to assess surfactant function and alveolar protein. Leukocyte infiltration was determined by flowcytometry in BALf, lung tissue and thoracic lymph nodes. Lungs of 3 animals per group were used for histological assessment. Results: Lung compliance was lower on day 30 and day 90 after LIRI than in sham-operated controls. Furthermore, the number of $CD45RA^+$-lymphocytes in left lung tissue was decreased on day 90 compared to unoperated animals and the number of macrophages elevated in left BALf on day 90. HE slides of LIRI animals were scored as fibroproliferative with moderate atelectasis. Surfactant pretreatment improved lung compliance and normalised the number of $CD45RA^+$-lymphocytes in left lung tissue. Furthermore lung architecture on HE slides was on return to normal. However, more $CD3^+CD4^+$-lymphocytes on day 30 and more macrophages on day 90 were found in pretreated lung tissue compared to LIRI animals. Conclusions. Severe LIRI caused extensive pulmonary injury up to 90 days postoperatively. Surfactant pretreatment normalized pulmonary function, but resulted in an increased number of $CD3^+CD4^+$-cells

and macrophages in lung tissue.

1 | Goal of the study

To investigate the effect of surfactant pretreatment on long-term outcome after LIRI, as assessed by lung function, histology and leukocyte infiltration.

2 | Materials and methods in brief

For a full description of the materials and methods, see the materials and methods section at the end of this thesis.

2.1 Experimental design

Male Sprague-Dawley rats (n=63, Harlan, Horst, the Netherlands), weighing 286 ± 31 gram, were randomized into four groups: Surfactant pretreated LIRI (n=18), untreated LIRI (n=18), sham-operated (n=18), and unoperated controls (n=9). Exogenous porcine surfactant (HL-10, Leo Pharmaceutical Products, Ballerup, Denmark and Halas Pharma, Oldenburg, Germany), dissolved in 50 mg/ml saline, was administered intratracheally in three gifts (total dose 400 mg/kg bodyweight) within 1 hour before operation, as described previously [1]. Surfactant prereated and untreated LIRI animals underwent 120 minutes of warm lung ischemia. Measurements were performed 30 or 90 days after the operation.

2.2 Measurements

Measurements include survival, blood gas values, static pulmonary compliance, alveolar protein level, surfactant small and large aggregates, flowcytometric analysis of infiltrating cells in (BALf, lung tissue and thoracic lymph nodes (TLN)) and histological analysis (in 3 additional animals per group). The static pulmonary compliance is presented as Vmax and Cmax. Vmax is the maximal lung volume at a pressure of 35 cm H_2O, while Cmax is the maximal compliance of the expiration curve.

2.3 Statistical analysis

The results in text, tables and figures are presented as mean ± standard deviation (SD). Data were analysed using SPSS version 11.1 statistical software (SPSS Inc., Chicago, Illinois, USA). If the distribution within a group was normal, as assessed by the Kolmogorov-Smirnov test, and if the condition of equal variances was met by the Levene's test, differences between groups were tested for significance by one-way ANOVA. If the overall level of the ANOVA was significant, intergroup comparisons were made by the Bonferroni post hoc test. In the case of unequal variances or an abnormal distribution, a non-parametric Kruskal-Wallis test was performed, followed by Mann-Whitney U tests for intergroup comparisons. P values <0.05 were considered to be significant

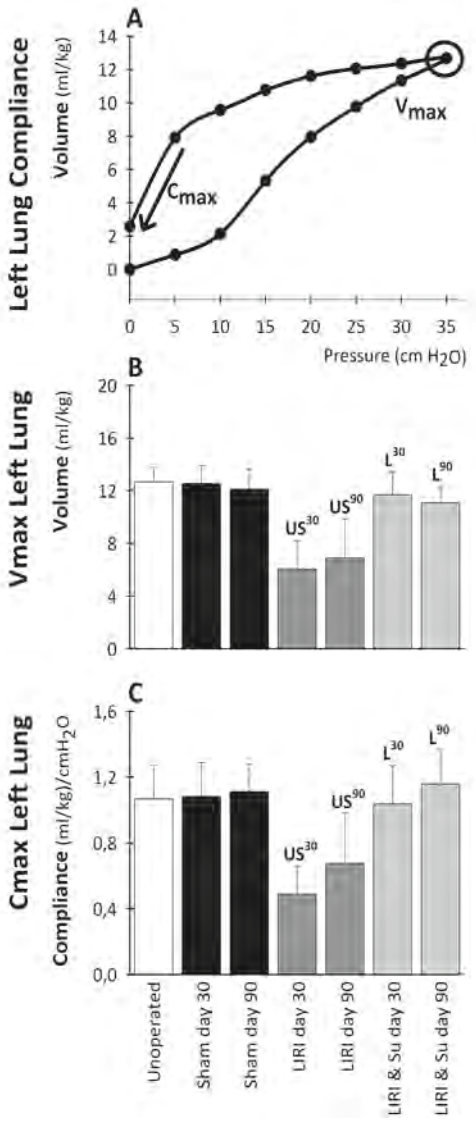

Figure 1. Pressure-volume curves (PVC) of unoperated, sham-operated, untreated LIRI, and surfactant pretreated groups on day 30 and 90 postoperatively. (A) Pressure volume curve of an unoperated animal, denominating Vmax and Cmax. (B) Mean maximal lung volume (Vmax) ± SD, corrected for body weight, was recorded at a pressure of 35 cm H_2O. (C) Mean maximal compliance (Cmax) ± SD was determined as the steepest part of the lung deflation curve. LIRI resulted in decreased Vmax and Cmax on day 30 and 90 after reperfusion. Surfactant pretreatment normalized lung compliance. LIRI: Lung Ischemia-Reperfusion Injury; SD: Standard Deviation; Su: Surfactant pretreatment.
U: $P<0.05$ versus unoperated animals
S^x: $P<0.05$ versus sham-operated animals on day x
L^x: $P<0.05$ versus LIRI animals on day x

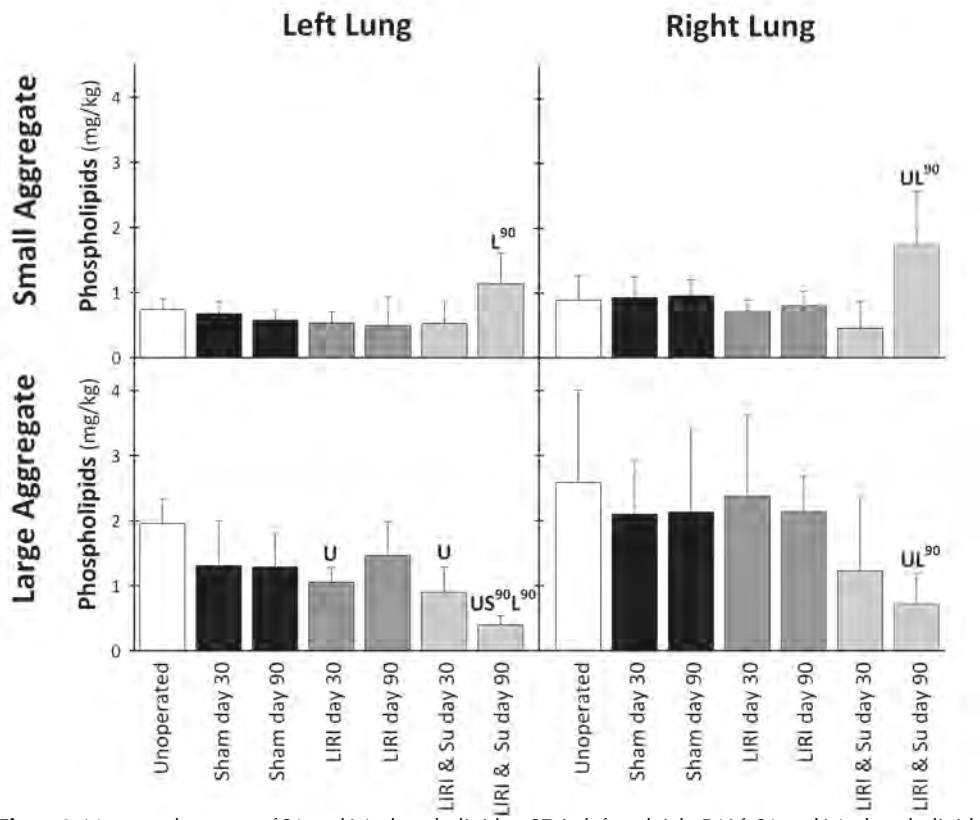

Figure 2. Mean total amount of SA and LA phospholipids ± SD in left and right BALf. SA and LA phospholipids (mg/kg body weight) were measured in left and right BALf of unoperated, sham-operated, untreated LIRI, and surfactant pretreated LIRI animals on day 30 and 90. A reduced level of LA was found in left BALf on day 30 after untreated and surfactant pretreated LIRI. Interestingly, the level of LA had further decreased on day 90 in the ischemic left, but also in the non-ischemic right lung of surfactant pretreated LIRI animals. Also, the amount of SA in the right lung of the surfactant pretreated group was significantly higher on day 90 than in all control groups. BALf: BronchoAlveolar Lavage Fluid; LIRI: Lung Ischemia-Reperfusion Injury; SA: Small Aggregate; SD: Standard Deviation; Su: Surfactant pretreatment; LA: Large Aggregate.
U: P<0.05 versus unoperated animals
S^x: P<0.05 versus sham-operated animals on day x
L^x: P<0.05 versus LIRI animals on day x

3 | Results

3.1 Survival

The mortality in the surfactant pretreated LIRI and the untreated LIRI group was 11% (2 of 18 in both groups). All sham-operated animals survived the experimental period.

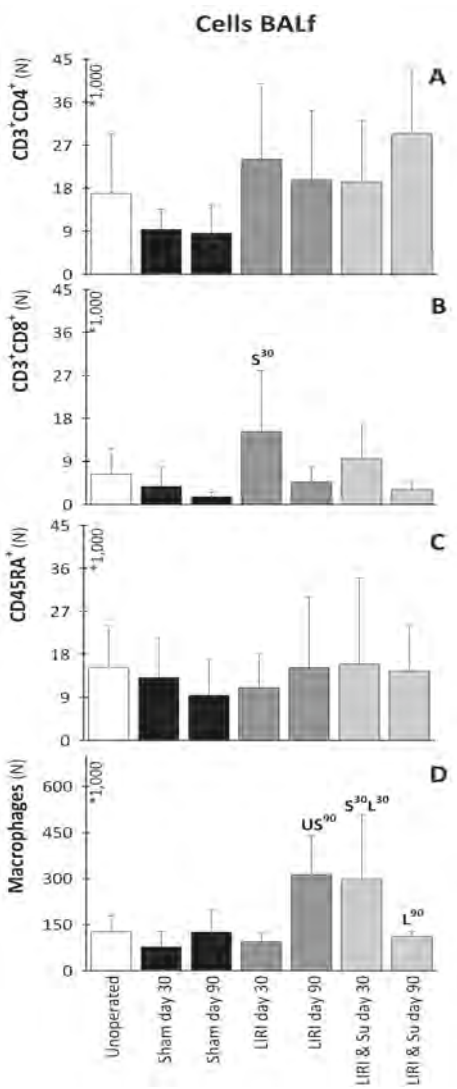

Figure 3. Mean number of inflammatory cells ± SD in BALf of the left lung of unoperated, sham-operated, untreated LIRI, and surfactant pretreated groups on day 30 and 90 postoperatively. Presented are: (A) $CD3^+CD4^+$cells (helper T-lymphocytes), (B) $CD3^+CD8^+$-cells (cytotoxic T-lymphocytes), (C) $CD45RA^+$-cells (B-lymphocytes), and D) macrophages. BALf: BronchoAlveolar Lavage Fluid; LIRI: Lung Ischemia-Reperfusion Injury; N: Number of cells; SD: Standard Deviation; Su: Surfactant pretreatment.
U: P<0.05 versus unoperated animals
Sx: P<0.05 versus sham-operated animals on day x
Lx: P<0.05 versus LIRI animals on day x

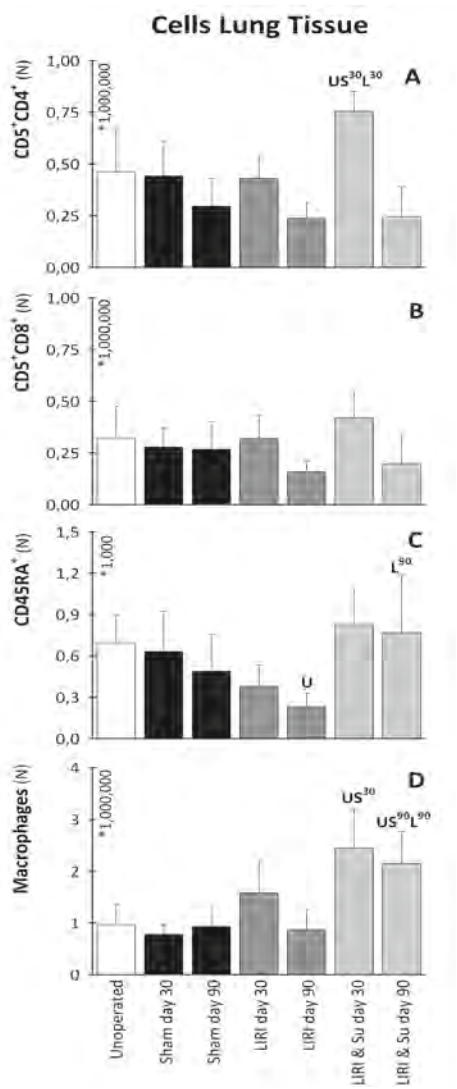

Figure 4. Mean number of inflammatory cells ± SD in left lung tissue of unoperated, sham-operated, untreated LIRI, and surfactant pretreated groups on day 30 and 90 postoperatively. Presented are: (A) $CD3^+CD4^+$-cells (helper T-lymphocytes), (B) $CD3^+CD8^+$-cells (cytotoxic T-lymphocytes), (C) $CD45RA^+$-cells (B-lymphocytes), and D) macrophages. LIRI: Lung Ischemia-Reperfusion Injury; N: Number of cells; SD: Standard Deviation; Su: Surfactant pretreatment.
U: P<0.05 versus unoperated animals
Sx: P<0.05 versus sham-operated animals on day x
Lx: P<0.05 versus LIRI animals on day x

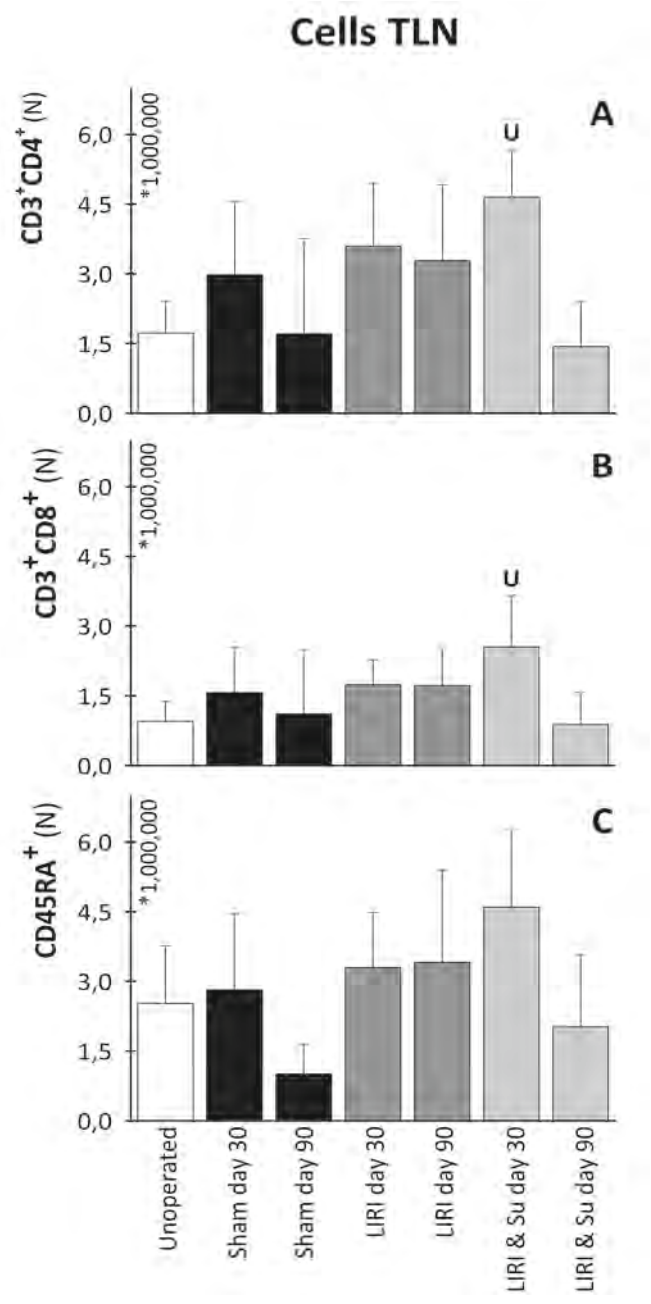

Figure 5. Mean number of inflammatory cells ± SD in thoracic lymph nodes of unoperated, sham-operated, untreated LIRI, and surfactant pretreated groups on day 30 and 90 postoperatively. Shown are: (A) $CD3^+CD4^+$-cells (helper T-lymphocytes), (B) $CD3^+CD8^+$-cells (cytotoxic T-lymphocytes), and (C) $CD45RA^+$-cells (B-lymphocytes). LIRI: Lung Ischemia-Reperfusion Injury; N: Number of cells; SD: Standard Deviation; Su: Surfactant pretreatment; TLN: Thoracic Lymph Nodes.
U: $P<0.05$ versus unoperated animals

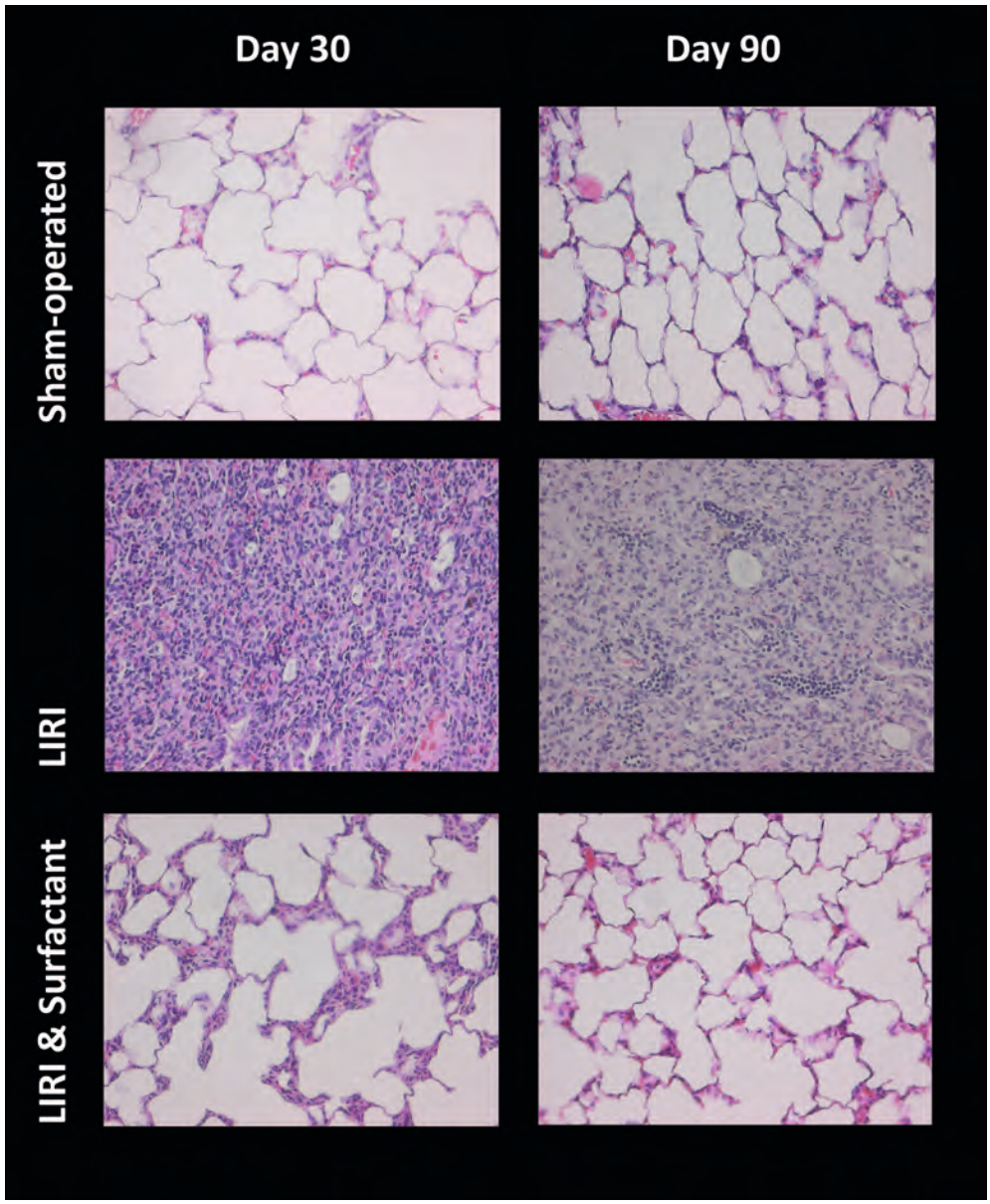

Figure 6. Histologic examples (HE) of sham-operated, untreated LIRI, and surfactant pretreated groups on day 30 and 90 postoperatively. LIRI resulted in atelectasis, fibrosis and a moderate inflammatory pattern, consisting of mainly histiocytes. Although surfactant pretreatment resulted in mild atelectasis and prevented fibrosis, mild inflammation with predominantly histiocytes and lymphocytes was still noticed. However, the overall classification was resolving, since injury was on return to normal. HE: Hematoxylin and Eosin staining; LIRI: Lung Ischemia-Reperfusion Injury; Su: Surfactant pretreatment.

Table 2. Mean histologic score of the left lung; inflammation

Group	Inflammation	Inflammatory cells		
		Lymphocytic	Histiocytic	Neutrophilic
Unoperated	1	3		
Sham day 30	0,33	1		
Sham day 90	0,67	2		
LIRI day 30	2		3	
LIRI day 90	1,67	1	2	
LIRI & Su day 30	0,67		2	
LIRI & Su day 90	1	2	3	

Scoring	
0	None
0-1	Mild/scattered
1-2	Moderate/occasional
2-3	Severe/frequent

Mean score of inflammation and type of inflammatory cells, scored on HE sections of 3 animals per group. Presented in the inflammatory cell column is the number of animals within each group with a particular parameter score. Some groups may contain more than 3 scores, since some animals had mixed inflammatory patterns. LIRI: Lung ischemia-reperfusion injury; Su: Surfactant pretreatment.

3.2 PaO_2/FiO_2 and $PaCO_2$

The PaO_2/FiO_2 ratio of LIRI animals on day 30 was slightly lower than the ratio of sham-operated controls (505±12 mm Hg versus 572±12 mm Hg, P=0.006). This difference had disappeared on day 90. Surfactant pretreatment resulted in a significantly better PaO_2/FiO_2 ratio as compared to untreated LIRI animals on day 30 (559±11 mm Hg versus 505±12 mm Hg, P=0.003). All groups had normal $PaCO_2$ values 30 and 90 days postoperatively (data not shown).

3.3 Static lung compliance

After sham-operation, left lung compliance was completely normal (Figure 1). However, LIRI resulted in significantly decreased Cmax and Vmax on day 30 and 90 postoperatively as compared to sham-operated and unoperated controls. After surfactant pretreatment, Cmax and Vmax of pretreated animals were comparable to the values found in sham-operated and unoperated animals.

3.4 Surfactant and alveolar protein

No differences were found in the amount of alveolar protein between the groups on both time points. However, 30 days after LIRI a decrease in the level of LA was found in the left lung of both untreated LIRI (P=0.008 versus unoperated) and surfactant pretreated animals (P=0.008 versus unoperated), but not in the sham group (Figure 2). Interestingly, the level of

LA had further decreased on day 90 in the ischemic left, but also in the non-ischemic right lung of surfactant pretreated LIRI animals. Also, the level of SA found in the left and right lung on day 90 was significantly higher than in unoperated, and untreated LIRI controls.

3.5 Lymphocytes in BALf and lung tissue

Although no significant differences in the number of $CD45RA^+$ and $CD3^+CD4^+$-cells were measured in BALf between groups on both time points (Figure 3A & 3C), LIRI resulted in infiltration of $CD3^+CD8^+$-cells in BALf on day 30 (Figure 3B). After surfactant pretreatment, more $CD3^+CD4^+$-cells were measured in lung tissue on day 30 as compared to unoperated, sham-operated and untreated LIRI animals (Figure 4A). The number of $CD45RA^+$-cells in lung tissue on day 30 and 90 was significantly higher than in untreated LIRI controls (Figure 4C). On day 90 the number of $CD3^+CD4^+$-cells in the surfactant pretreated group had returned to baseline values (Figure 4A & 4B).

3.6 Lymphocytes in thoracic lymph nodes

No significant differences between untreated LIRI animals and controls were found in $CD3^+CD8^+$, $CD3^+CD4^+$ and $CD45RA^+$-cells (Figure 5A, 5B, & 5C).
However, the lymphocyte population in TLN of surfactant pretreated animals showed elevated numbers of $CD3^+CD4^+$ and $CD3^+CD8^+$-cells on day 30 as compared to unoperated controls with numbers returning to normal on day 90 (Figure 5A & 5B)

3.7 Macrophages in BALf and lung tissue

While sham-operation had no effect on the number of macrophages found in BALf of the left lung, LIRI resulted in an increased number of macrophages 90 days postoperatively as compared to unoperated and sham-operated controls (Figure 3D).
In the surfactant pretreated group significantly more macrophages were found in BALf (Figure 3D) and lung tissue (Figure 4D) on day 30. Although the number of macrophages in lung tissue was still elevated on day 90 , it had returned to normal in the BALf on day 90 (Figure 3D).

3.8 Histology

No histological abnormalities were seen on H&E slides of sham-operated animals on day 30 and 90 (Figure 6, Table 1, Table 2). LIRI resulted in atelectasis, fibrosis and moderate inflammation, consisting mainly of histiocytes. The overall classification of LIRI animals on day 30 and 90 was fibroproliferative in 5 out of 6 animals. Although surfactant pretreatment resulted in mild inflammation with predominantly histiocytes and lymphocytes and mild atelectasis, it totally prevented the development of fibrosis. The overall classification after surfactant pretreatment was scored as resolving.

4 | Discussion

LIRI is suggested to be a major risk factor for the development of PGD following lung transplantation, thereby contributing profoundly to early morbidity and mortality after lung transplantation [2, 3]. The clinical course of PGD symptomatically resembles the acute phase

of the acute respiratory distress syndrome (ARDS) and consists of symptoms like hypoxemia, decreased lung compliance, increased pulmonary artery pressure, and development of non-cardiogenic pulmonary edema [4, 5]. The acute phase of PGD can either resolve quickly or result in death. If patients survive the acute phase of PGD, a 'chronic' fibroproliferative state, characterized by hyperplasia of alveolar type II cells, infiltration of macrophages and activated fibroblasts, collagen deposition and pulmonary remodelling, may develop within 4-7 days after onset of the first clinical symptoms [4, 5]. In an earlier report, we have demonstrated that 120 minutes warm ischemia resulted in our model in symptoms that resembled the symptoms seen in the acute phase of PGD, suggesting that LIRI is a major risk factor for PGD in the absence of other influencing factors, such as alloimmunity[1]. The 11% early mortality found in the present study was due to the development of severe pulmonary edema shortly after reperfusion. The 89% of the untreated LIRI animals that survived the acute phase developed chronic abnormalities, as demonstrated by impaired lung compliance up to 90 days after reperfusion and fibroproliferative changes on HE slides. Although we did not confirm collagen deposition with histology, a fibroproliferative state with tissue remodelling can further be illustrated by the increased levels of macrophages and $CD3^+CD8^+$-lymphocytes in BALf of untreated LIRI animals, which both play an important role in lung fibrosis. In addition, the decreased number of $CD45RA^+$-lymphocytes found in lung tissue of untreated LIRI animals on day 90 also suggests the occurrence of tissue remodelling after LIRI. Although extensive injury was found in the left lung of untreated LIRI animals, only mild hypoxemia was demonstrated on day 30. This discrepancy may be explained by the fact that PaO_2 depended on both lungs, and that the loss of left lung function may be compensated by the right lung.

While LIRI is suggested to be a major risk factor for the development of PGD, it is also thought to be one of the factors contributing to BOS, which affects 50% of patients surviving beyond 3 months after transplantation [2, 3, 6-8]. The exact aetiology of BOS is not fully understood, but its pathogenesis appears to involve a "response to injury" type of pattern. BOS probably develops as a result of multiple periods of injury, like brain death, LIRI, rejection and infection [3, 6, 8]. Although BOS is restricted to the pathology after lung transplantation, and not used in relation to ischemic injury per se, we clearly show that severe LIRI caused a progressive deterioration of the lung. Because LIRI is one of the first injuries the lung sustains in clinical lung transplantation, tackling LIRI is an important strategy to improve outcome following lung transplantation.

One approach to ameliorate LIRI is to treat the lung with surfactant. In both experimental models and in clinical lung transplantation, it has been shown that surfactant therapy, administered after the development of severe LIRI, is capable of restoring lung function within hours after administration [1, 9-16]. However, treatment with surfactant before the onset of ischemia has been suggested to be more beneficial than treatment after reperfusion, because an enlarged surfactant phospholipid pool inside the alveolus is already present before the onset of injury [17]. Surfactant pretreatment also results in a more homogeneous distribution in the lung as compared to treatment after reperfusion, when alveolar damage has already occurred and surfactant will accumulate in the already open areas of the lung instead of the atelectatic areas, where it is most needed. Previous data from our group demonstrated that surfactant treatment before the induction of ischemia resulted in improved

lung function in the first week after LIRI [1]. In the present study we demonstrate that surfactant pretreatment normalized lung compliance up to 3 months after reperfusion, restored the number of $CD45RA^+$-lymphocytes in lung tissue to the level observed in unoperated animals, and decreased lung damage on HE slides. However, more macrophages were found, accompanied by a $CD3^+CD4^+$-lymphocyte infiltrate in both lung tissue and TLN on day 30, which had disappeared on day 90.

Since reduction of the inflammatory reaction in the context of ischemia-reperfusion has proven to be successful in amelioration of injury, infiltration of leukocytes after LIRI should be considered as unwanted [18-20]. From the literature, it is known that surfactant is capable of down regulating the activation of macrophages and dendritic cells in vitro, and to decrease the production of cytokines [21]. The finding that surfactant pretreatment resulted in an increased number of $CD3^+CD4^+$-lymphocytes could be explained by the fact that amelioration of LIRI by surfactant may have unmasked a different inflammatory reaction, or, that surfactant itself elicited an inflammatory reaction. Another possibility is that surfactant induced a regulatory T-cell response, which warrants further analysis of this subset of cells using CD25 or FOX-P3. In our previous study we also observed increased numbers of macrophages after surfactant pretreatment in the BALf up to day 7 after reperfusion [1]. The fact that they are still present up to day 30 in the BALf may be explained by the surfactant recycling capacity of macrophages [1].

Although our study clearly demonstrates a long-term beneficial effect of surfactant pretreatment on lung function and pulmonary architecture following severe LIRI, this study has some limitations. Shortcomings of our experimental model are that warm instead of cold ischemia is used, and that it is impossible to easily administer pulmoplegia. However, this experimental model is an accepted and useful model to study the effect of LIRI in small rodents with acceptable mortality. Furthermore, it has been demonstrated that there are no major differences between short periods of warm and longer periods of cold ischemia [22].

5 | Conclusions

Severe LIRI causes extensive pulmonary injury up to 90 days after reperfusion, which can be ameliorated by surfactant pretreatment one hour before LIRI. Surfactant therapy may thus be a promising strategy to prevent short-term lung injury after ischemia and reperfusion of the lung, and to prevent a chronic fibroproliferative state.

6 | References

1. van der Kaaij NP, Haitsma JJ, Kluin J, et al. Surfactant pretreatment ameliorates ischemia-reperfusion injury of the lung. Eur J Cardiothorac Surg 2005;27:774-782.
2. Hosenpud JD, Bennett LE, Keck BM, et al. The Registry of the International Society for Heart and Lung Transplantation: seventeenth official report-2000. J Heart Lung Transplant 2000;19:909-931.
3. de Perrot M, Liu M, Waddell TK, et al. Ischemia-reperfusion-induced lung injury. Am J Respir Crit Care Med 2003;167:490-511.
4. Ware LB, Matthay MA. The acute respiratory distress syndrome. N Engl J Med 2000;342:1334-1349.
5. Ingbar DH. Mechanisms of repair and remodelling following acute lung injury. Clin Chest Med 2000;21:589-616.
6. Boehler A, Kesten S, Weder W, et al. Bronchiolitis obliterans after lung transplantation: A review. Chest 1998;114:1411-1426.
7. Fiser SM, Tribble CG, Long SM, et al. Ischemia-reperfusion injury after lung transplantation increases risk of late bronchiolitis obliterans syndrome. Ann Thorac Surg 2002;73:1041-1047.
8. Tullius SG, Tilney NL. Both alloantigen-dependent and -independent factors influence chronic allograft rejection. Transplantation 1995;59:313-318.
9. Haitsma JJ, Papadakos PJ, Lachmann B. Surfactant therapy for acute lung injury/acute respiratory distress syndrome. Curr Opin Crit Care 2004;10:18-22.
10. Novick RJ, Veldhuizen RA, Possmayer F, et al. Exogenous surfactant therapy in thirty-eight hour lung graft preservation for transplantation. J Thorac Cardiovasc Surg 1994;108:259-268.
11. Novick RJ, Gilpin AA, Gehman KE, et al. Mitigation of injury in canine lung grafts by exogenous surfactant therapy. J Thorac Cardiovasc Surg 1997;113:342-353.
12. Erasmus ME, Petersen AH, Hofstede G, et al. Surfactant treatment before reperfusion improves the immediate function of lung transplants in rats. Am J Respir Crit Care Med 1996;153:665-670.
13. Erasmus ME, Hofstede GJ, Petersen AH, et al. Effects of early surfactant treatment persisting for one week after lung transplantation in rats. Am J Respir Crit Care Med 1997;156:567-572.
14. Friedrich I, Borgermann J, Splittgerber FH, et al. Bronchoscopic surfactant administration preserves gas exchange and pulmonary compliance after single lung transplantation in dogs. J Thorac Cardiovasc Surg 2004;127:335-343.
15. Gunther A, Balser M, Schmidt R, et al. Surfactant abnormalities after single lung transplantation in dogs: impact of bronchoscopic surfactant administration. J Thorac Cardiovasc Surg 2004;127:344-354.
16. Struber M, Fischer S, Niedermeyer J, et al. Effects of exogenous surfactant instillation in clinical lung transplantation: a prospective, randomized trial. J Thorac Cardiovasc Surg 2007;133:1620-1625.
17. Hausen B, Rohde R, Hewitt CW, et al. Exogenous surfactant treatment before and after sixteen hours of ischemia in experimental lung transplantation. J Thorac Cardiovasc Surg 1997;113:1050-1058.
18. Uthoff K, Zehr KJ, Lee PC, et al. Neutrophil modulation results in improved pulmonary function after 12 and 24 hours of preservation. Ann Thorac Surg 1995;59:7-12.
19. de Perrot M, Young K, Imai Y, et al. Recipient T cells mediate reperfusion injury after lung transplantation in the rat. J Immunol 2003;171:4995-5002.
20. Krishnadasan B, Naidu B, Rosengart M, et al. Decreased lung ischemia-reperfusion injury in rats after preoperative administration of cyclosporine and tacrolimus. J Thorac Cardiovasc Surg 2002;123:756-767.
21. Thomassen MJ, Meeker DP, Antal JM, et al. Synthetic surfactant (Exosurf) inhibits endotoxin-stimulated cytokine secretion by human alveolar macrophages. Am J Respir Cell Mol Biol 1992;7:257-260.
22. Warnecke G, Sommer SP, Gohrbandt B, et al. Warm or cold ischemia in animal models of lung ischemia-reperfusion injury: is there a difference? Thorac Cardiovasc Surg 2004;52:174-179.

8

Surfactant treatment before ischemia is superior to treatment after reperfusion for ischemia-reperfusion injury of the lung

Based on
NP van der Kaaij, J Kluin, MA den Bakker, BN Lambrecht, B Lachmann, RWF de Bruin, AJJC Bogers
Submitted

Summary

Background: Lung ischemia-reperfusion injury (LIRI) contributes to primary graft dysfunction after lung transplantation. Treatment with surfactant ameliorates LIRI, but the optimal timing and lowest possible surfactant dose is unknown. We determined the lowest dose of surfactant that reduces LIRI and compared the efficacy of surfactant treatment before ischemia with treatment at and 24 hours after reperfusion. Methods: Surfactant dose study: Male Sprague-Dawley rats (n=24) received intratracheally administered porcine surfactant 1-hour before ischemia in a dose of 50 mg/kg, 100 mg/kg, 200 mg/kg or were not treated. Clamping the bronchus, pulmonary artery and veins of the left lung for 150 minutes induced LIRI. After 72 hours, arterial oxygenation and pulmonary pressure-volume-curves were recorded. Surfactant timing study: 61 rats received surfactant (200 mg/kg) either 1-hour before ischemia, at the start of reperfusion, or 24 hours after reperfusion. Sham-operated and untreated LIRI animals served as controls. After 72 hours, arterial oxygenation, pulmonary pressure-volume-curves, inflammatory cells in bronchoalveolar lavage fluid and histological pulmonary injury were assessed. Results: Surfactant dose study: In our model, pretreatment with 200 mg/kg surfactant improved pulmonary compliance and lung function, while 50 and 100 mg/kg had no effect. Surfactant timing study: LIRI resulted in 29% mortality. In contrast to surfactant treatment at or after reperfusion, surfactant pretreatment resulted in a mortality rate of 9%. Also, surfactant pretreatment improved lung function, averted fibroproliferation, and resulted in a lower histological pulmonary severity score of the ischemic lung. Surfactant treatment after reperfusion had no effect on pulmonary function. In general, surfactant therapy had a pro-inflammatory effect on the number of granulocytes and $MHCII^+$-antigen presenting cells in the right non-ischemic lung, while less T-lymphocytes were measured in the ischemic left lung after pretreatment. Conclusions: In our model, 200 mg/kg surfactant is the lowest dose that reduces severe LIRI. Surfactant pretreatment is superior to treatment at reperfusion or 24 hours after reperfusion.

Table 1. Experimental groups: Mortality and time of treatment

Group	**Operated animals** (N)	**Died** (N)	**Mortality** (%)
Sham	10	0	0
LIRI	14	4	29
Pretreatment	11	1	9
Reperfusion	14	4	29
24-hours	12	2	17

All animals that died, expired within one hour after reperfusion due to excessive pulmonary edema resulting in worsening arterial oxygenation and finally death despite mechanical ventilation. Note that the animals that died in the 24-hours group, died before the administration of surfactant. The number of operated animals per group was completed to have 10 surviving animals for analysis. LIRI: Lung Ischemia-Reperfusion Injury; N: Number of animals; Pretreatment: Surfactant treatment 1 hour before ischemia, 200 mg/kg; Reperfusion: Surfactant treatment at reperfusion, 200 mg/kg; 24-hours: Surfactant treatment 24 hours after reperfusion, 200 mg/kg.

1 | Goals of the study

1) To determine the lowest surfactant dose that reduces LIRI in our model.
2) To compare surfactant pretreatment with surfactant treatment at the start of reperfusion or 24 hours after reperfusion.

2 | Materials and methods in brief

For a full description of the materials and methods, see the materials and methods section at the end of this thesis.

2.1 Experimental design pilot

A total of 24 Male Sprague-Dawley rats (Harlan, the Netherlands, 334 ± 48 gram) were used to search for the minimal surfactant pretreatment dose that reduces LIRI, characterized by pulmonary compliance and blood gas values 72 hours after reperfusion. The lowest dose investigated was 50 mg/kg. If no significant effect was measured after 6 animals, the surfactant dose was doubled in another 6 rats until a significant effect was found. Untreated LIRI animals served as controls (n=6). Natural porcine surfactant (HL-10, Halas-Pharma, Germany) was dissolved in saline and administered intratracheally in 2 doses over 1 hour. Ischemia (150 minutes) was induced as described in the materials and methods section.

2.2 Experimental design timing study

Male Sprague-Dawley rats (n=61, Harlan, the Netherlands, 322 ± 15 gram), were randomized into 5 groups (Table 1): Sham-operated, untreated LIRI, surfactant treatment 1 hour before ischemia (pretreatment, 200 mg/kg), surfactant treatment at reperfusion (reperfusion, 200 mg/kg) and surfactant treatment 24 hours after reperfusion (24-hours, 200 mg/kg). LIRI

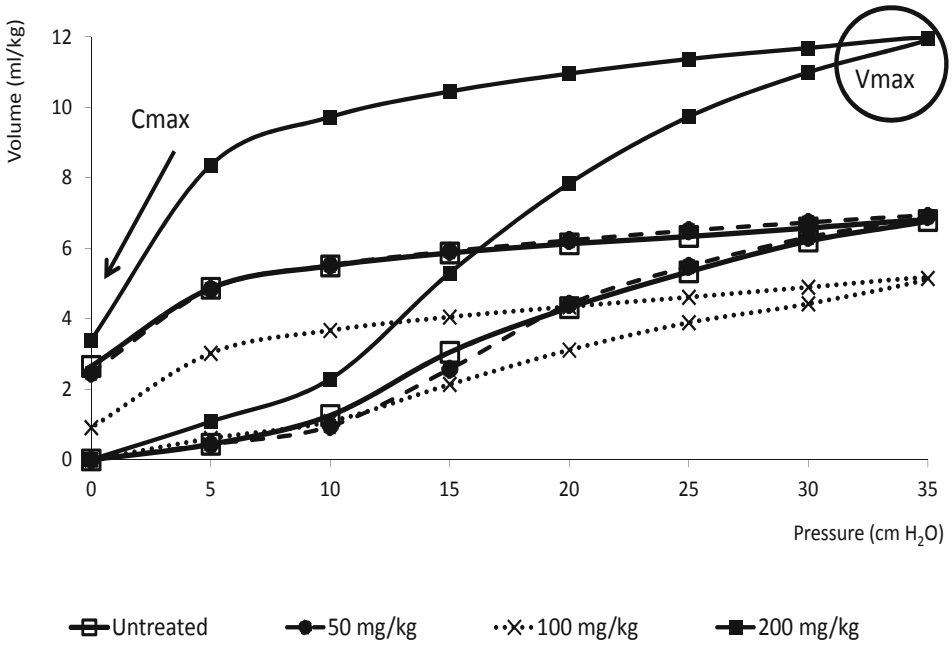

Figure 1. Pulmonary pressure-volume curves (PVC) of animals pretreated with 50 mg/kg, 100 mg/kg, 200 mg/kg surfactant or no surfactant pretreatment. Pretreatment with 50 or 100 mg/kg had no effect on pulmonary compliance. Pretreatment with 200 mg/kg significantly improved Vmax and Cmax as compared to untreated LIRI controls. Cmax: Maximal compliance of the expiration curve, corrected for body weight; LIRI: Lung Ischemia-Reperfusion Injury; Vmax: Maximal lung volume corrected for body weight at a pressure of 35 cm H_2O.

Table 2. Mean (SD) arterial blood gas and mean (SD) pressure-volume curves of untreated LIRI and 50, 100 or 200 mg/kg surfactant pretreated animals.

Lung Function	LIRI	50 mg/kg	100 mg/kg	200 mg/kg
Vmax L (ml/kg)	6,79 (1,6)	6,91 (1,6)	5,15 (2,1)	11,95 (1,1) *
Cmax L ((ml/kg)/cm H_20)	0,44 (0,11)	0,48(0,13)	0,42 (0,23)	0,99 (0,22) *
PaO_2/FiO_2 (mm Hg)	284 (136)	298 (88)	337 (71)	555 (51) *
$PaCO_2$ (mm Hg)	42 (7)	50 (14)	48 (12)	44 (4)
PH	7,37 (0,04)	7,36 (0,07)	7,34 (0,06)	7,41 (0,03)

PaO_2/FiO2, $PaCO_2$, PH, Vmax and Cmax of untreated and 50 mg/kg, 100 mg/kg or 200 mg/kg surfactant pretreated animals. Pretreatment with 50 or 100 mg/kg had no effect on pulmonary compliance. Pretreatment with 200 mg/kg significantly improved Vmax, Cmax and PaO_2/FiO_2 ratio as compared to untreated LIRI controls. Cmax: Maximal compliance of the expiration curve, corrected for body weight; LIRI: Lung Ischemia-Reperfusion Injury; SD: Standard Deviation; Vmax: Maximal lung volume corrected for body weight at a pressure of 35 cm H_2O.
*P<0.05 versus all other groups

animals received 150 minutes of warm ischemia. Sham-operated controls underwent surgery without applying ischemia. Between 30 minutes and 1 hour after reperfusion animals were weaned from the ventilator. Three days postoperatively measurements were performed. Each group was completed to have 10 surviving animals for analysis (Table 1). Of these 10 animals, 3 animals per group were used for histological analysis and 7 for flowcytometry.

2.3 Surfactant

Natural porcine surfactant (HL-10, Halas-Pharma, Germany) was dissolved in saline (50 mg/ml). In the pretreatment group, surfactant was administered intratracheally in 2 doses over 1 hour after the animals were anesthetized (65% nitrous oxide/33% oxygen/2% isoflurane) and intubated. After each dose, animals were detubated, recovered from anaesthesia and breathed spontaneously to allow the surfactant to be absorbed. One hour after the first and 30 minutes after the second dose, animals were again anesthetized, intubated and operated. The surfactant at reperfusion group received surfactant intratracheally in two doses after the start of reperfusion. In detail, directly after reperfusion, the lungs were recruited by a stepwise increase of peak inspiratory pressure (PIP) and positive end expiratory pressure (PEEP) (maximum of 50 and 18 cm H_2O, respectively) until the lung was visually expanded. Animals were disconnected from the ventilator and surfactant was instilled, where after animals were reconnected to the ventilator and lungs were again recruited. Thirty minutes after the first dose, the second was instilled by the same protocol. Animals in the 24-hours group were treated with surfactant 24 hours after reperfusion. These animals were anesthetized conform protocol, intubated and the surfactant was instilled intratracheally. Hereafter animals were detubated. Thirty minutes after the first dose, the animals received the second by the same protocol.

2.4 Measurements

Measurements include survival, blood gas values, static pulmonary compliance, flowcytometric analysis of infiltrating cells in BALf and histological analysis (in 3 additional animals per groups). The static pulmonary compliance is presented as Vmax and Cmax. Vmax is the maximal lung volume at a pressure of 35 cm H_2O, while Cmax is the maximal compliance of the expiration curve.

2.5 Statistical analysis

The results in text, tables and figures are presented as mean ± standard deviation (SD). Data were analysed using SPSS (version 11.1, SPSS, Illinois, USA). If an overall difference between groups was found by the Kruskal-Wallis test, Mann-Whitney-U tests were performed for intergroup comparison. Difference in mortality between groups was tested with a logistic regression model, with time of surfactant treatment as the independent factor.

3 | Results

3.1 Surfactant dose

Pretreatment with 50 mg/kg or 100 mg/kg had no effect on pulmonary compliance or PaO_2/FiO_2 ratio 72 hours after reperfusion (Figure 1, Table 2). Pretreatment with 200 mg/kg

Table 3. Lung function at 72 hours: Arterial blood gas and compliance

Group	**Vmax L** (ml/kg)	**Cmax L** ((ml/kg)/cm H_20)	**Vmax L&R** (ml/kg)	**Cmax L&R** ((ml/kg)/cm H_20)	**PaO_2/FiO_2** (mm Hg)	**$PaCO_2$** (mm Hg)	**PH**
Sham-operated	14,1 (2,1)	1,1 (0,2)	41,8 (2,9)	4,4 (0,4)	595 (26)	37 (6,5)	7,49 (0,03)
LIRI	7,7 **S** (1,6)	0,5 **S** (0,2)	34,9 **S** (2,5)	3,5 (0,4)	417 **S** (139)	53 **S** (20,1)	7,36 **S** (0,10)
Pretreatment	10,8 **SLR24** (1,5)	0,8 **SL** (0,2)	37,3 **S** (4,0)	3,8 (0,7)	527 **R24** (84)	47 **S** (4,8)	7,40 **SR24** (0,04)
Reperfusion	8,2 **S** (2,1)	0,6 **S** (0,2)	37,6 **S** (3,8)	3,8 (0,6)	399 **S** (140)	55 **S** (12,6)	7,34 **S** (0,09)
24-hours	8,7 **S** (2,2)	0,6 **S** (0,2)	37,0 **S** (3,9)	3,5 (0,6)	436 **S** (96)	61 **S** (28,9)	7,32 **S** (0,11)

Mean (SD) of PaO_2/FiO_2 ratio, $PaCO_2$, PH, Vmax of the single left lung and left and right lung together and Cmax of the single left lung and left and right lung together. Cmax: Maximal compliance of the expiration curve, corrected for body weight; LIRI: Lung Ischemia-Reperfusion Injury; Pretreatment: Surfactant treatment 1 hour before ischemia, 200 mg/kg; Reperfusion: Surfactant treatment at reperfusion, 200 mg/kg; 24-hours: Surfactant treatment 24 hours after reperfusion, 200 mg/kg; SD: Standard deviation; Vmax: Maximal lung volume corrected for body weight at a pressure of 35 cm H_2O.
S: $P<0.05$ versus sham-operated controls
L: $P<0.05$ versus LIRI controls
R: $P<0.05$ versus treatment at reperfusion
24: $P<0.05$ versus treatment 24 hours after reperfusion

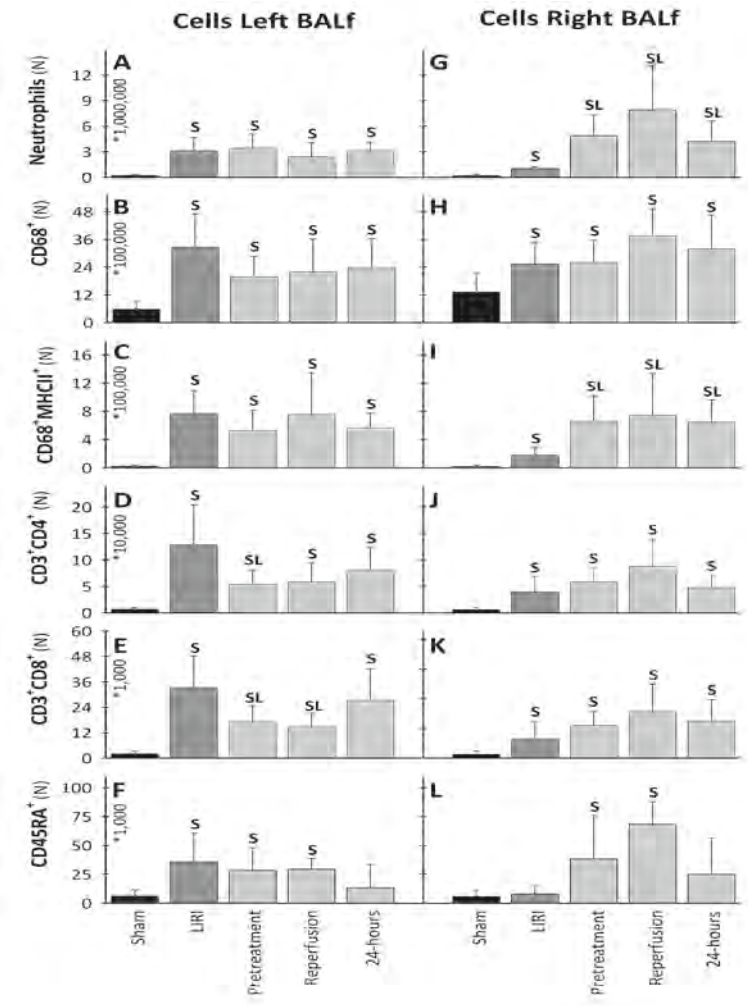

Figure 2. Mean number of inflammatory cells ± SD measured in the left and right BALf of sham-operated, untreated LIRI and surfactant treated groups. Presented are: A and G) neutrophils (HIS48$^+$), B and H) antigen-presenting cells (APC, CD68$^+$), C and I) MHCII$^+$-APC (MHCII$^+$CD68$^+$), D and J) helper T-lymphocytes (CD3$^+$CD4$^+$), E and K) cytotoxic T-lymphocytes (CD3$^+$CD8$^+$) and F and L) B-lymphocytes (CD45RA$^+$) measured in the left (A-F) and right (G-L) BALf. BALf: BronchoAlveolar Lavage Fluid; LIRI: Lung Ischemia-Reperfusion Injury; N: Number of cells; Pretreatment: Surfactant treatment 1 hour before ischemia, 200 mg/kg; Reperfusion: Surfactant treatment at reperfusion, 200 mg/kg; 24-hours: Surfactant treatment 24 hours after reperfusion, 200 mg/kg.
S: P<0.05 versus sham-operated animals
L: P<0.05 versus LIRI animals

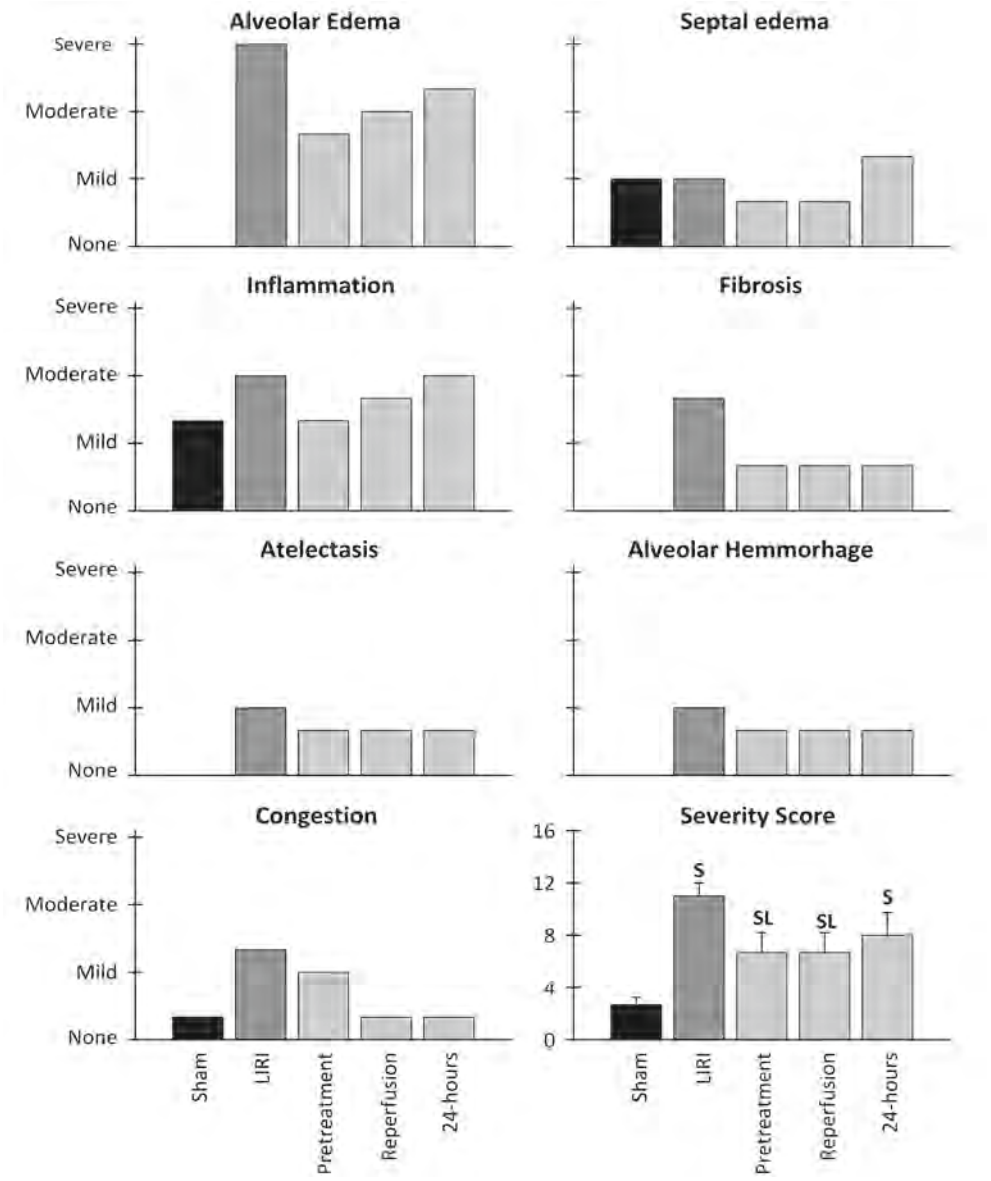

Figure 3. Mean histologic score of intra-alveolar edema, septal edema, severity of inflammation, fibrosis, atelectasis, intra-alveolar hemorrhage, congestion, and pulmonary severity, scored on HE sections of 3 animals per group. Each parameter was scored as: (0) absent, (1) mild, (2) moderate, or (3) severe. The pulmonary severity score is the sum of the individual scores of the eight categories, resulting in a possible score ranging from 0 for normal lungs to 24 for the most injured lungs. LIRI: Lung Ischemia-Reperfusion Injury; Pretreatment: Surfactant treatment 1 hour before ischemia, 200 mg/kg; Reperfusion: Surfactant treatment at reperfusion, 200 mg/kg; 24-hours: Surfactant treatment 24 hours after reperfusion, 200 mg/kg.
S: $P<0.05$ versus sham-operated animals
L: $P<0.05$ versus LIRI animals

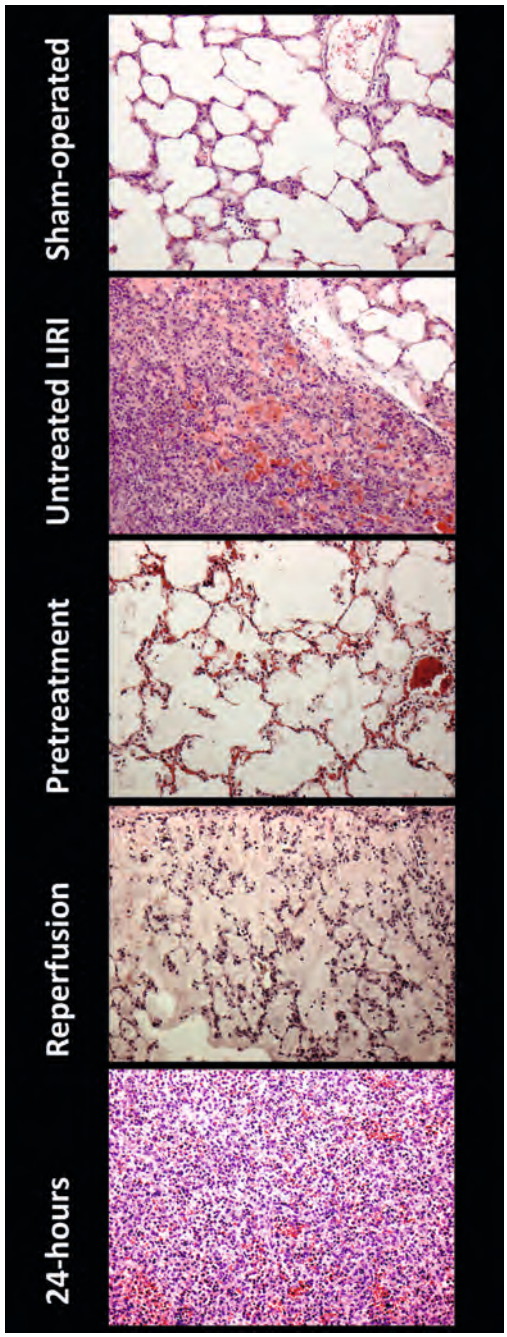

Figure 4. Representative histological samples (HE 100x) of sham-operated, untreated LIRI animals and surfactant treated groups. HE: Hematoxylin and Eosin staining; LIRI: Lung Ischemia-Reperfusion Injury; Pretreatment: Surfactant treatment 1 hour before ischemia, 200 mg/kg; Reperfusion: Surfactant treatment at reperfusion, 200 mg/kg; 24-hours: Surfactant treatment 24 hours after reperfusion, 200 mg/kg.

surfactant reduced LIRI as characterized by an improved Vmax, Cmax and PaO_2/FiO_2 ratio. Since 200 mg/kg was the lowest surfactant dose that reduced LIRI, a dose of 200 mg/kg was used in the timing study.

3.2 Timing study

3.2.1 Survival

All animals that died, expired within one hour after reperfusion due to excessive pulmonary edema resulting in worsening arterial oxygenation and death (Table 1). Thus, animals in the 24-hour group had not received surfactant at the time of death. Untreated LIRI resulted in 29% mortality while all sham-operated animals survived. Pretreatment led to a mortality rate of 9% with an odds ratio of 0.250 (0.024-2.648) for death compared to untreated LIRI. Due to the low numbers of animals this did not reach statistical significance (P=0.250). Surfactant treatment at and 24 hours after reperfusion also had no significant effect on mortality.

3.2.2 Arterial blood gas

Untreated LIRI resulted in hypoxemia and hypercapnia (Table 3). The PaO_2/FiO_2 ratio of surfactant pretreated animals was significantly higher than the ratio found in animals treated at or 24-hours after reperfusion. Hypercapnia was present in all treated and untreated LIRI animals.

3.2.3 Pulmonary pressure-volume-curves

Both in surfactant treated and untreated LIRI animals, lower Vmax and Cmax were found compared to sham-operated controls (Table 3). Of all surfactant groups, only surfactant pretreatment significantly improved the Vmax and Cmax of the left, ischemic lung relative to untreated controls.

3.2.4 Inflammation

LIRI resulted in infiltration with granulocytes ($HIS48^+$), antigen-presenting cells ($CD68^+$), $MHCII^+$-APC ($CD68^+MHCII^+$), helper T-lymphocytes ($CD3^+CD4^+$), cytotoxic T-lymphocytes ($CD3^+CD8^+$) and B-lymphocytes ($CD45RA^+$) in both the ischemic left and non-ischemic right lung (Figure 2). Less helper ($CD3^+CD4^+$) and cytotoxic ($CD3^+CD8^+$) T-cells were found in the BALf of the left, ischemic lung of surfactant pretreatment animals, while surfactant had no major effect on the number of granulocytes ($HIS48^+$), APC ($CD68^+$) and $MHCII^+$-APC ($CD68^+MHCII^+$) (Figure 2A-F). In the right lung, surfactant treatment, irrespective of the time of treatment, induced a pro-inflammatory effect, characterized by an elevated number of granulocytes ($HIS48^+$) and $MHCII^+$-APC ($CD68^+MHCII^+$) as compared to the number found in untreated animals (Figure 2G-L).

3.2.5 Histology

No significant changes were found in the right lung of all animals. In the left lung, mild septal edema and mild, mainly neutrophilic, inflammation was seen after sham-operation,

resulting in a pulmonary severity score of 2.7 ± 0.6 (Figure 3, Figure 4). LIRI resulted in diffuse alveolar damage consisting of severe intra-alveolar edema, mild septal edema, mild atelectasis, mild intra-alveolar hemorrhage, moderate lymphocytic and histiocytic inflammation, moderate fibrosis and mild congestion (Figure 3, Figure 4). The overall classification of LIRI lungs was proliferative and the pulmonary severity score added up to 11.0 ± 1.0.

Surfactant treatment decreased the level of fibrosis in all groups to less than mild and intra-alveolar edema to moderate. No major histological differences between untreated and surfactant treated animals were found in septal edema, severity of inflammation, atelectasis, intra-alveolar hemorrhage and congestion. The type of inflammation of all surfactant treated groups was scored as mainly histiocytic (and not lymphocytic as in untreated animals). Finally, after surfactant pretreatment, the pulmonary severity score was 6.7 ± 1.5 ($P<0.05$ versus untreated animals), the extent of injury ranged from patchy to focal, and the overall classification was exudative, in contrast to proliferative in untreated and other surfactant treated LIRI groups. The pulmonary severity score (6.7 ± 1.5) of the animals treated at the time of reperfusion was significantly lower than the pulmonary severity score of untreated animals ($P<0.05$), while surfactant treatment 24 hours after reperfusion had no significant effect (8.0 ± 1.7, $P=0.07$).

4 | Discussion

PGD clinically resembles ARDS. Both are associated with surfactant abnormalities, such as phospholipid and fatty-acid changes and reduced surfactant-associated proteins, which leads to surfactant dysfunction [1-9]. A normal surfactant function is important, since it minimizes the alveolar surface tension facilitating normal breathing and it suppresses the activity of inflammatory cells [10-12]. Therefore, surfactant replacement therapy is a rational approach in the prevention and treatment of PGD [13-17].

In our study, surfactant pretreatment resulted in a mortality rate of 9% and improved pulmonary function while surfactant treatment at or after reperfusion had no major effect on mortality, pulmonary compliance or the PaO_2/FiO_2 ratio. Thus, surfactant pretreatment in our study is superior to surfactant treatment at or after reperfusion, which can be explained by several factors. First of all, surfactant pretreatment results in a more homogeneous distribution than treatment later in the disease process. Randomized clinical trials investigating surfactant therapy in ARDS patients failed to show an effect on survival, although a trend in improved oxygenation was found [18-20]. In these trials, surfactant was delivered after development of ARDS resulting in surfactant accumulation in the open areas of the lung and not in the atelectatic parts, where it is most needed. The situation of lung transplantation is unique, since surfactant can be administered to a unoperated lung resulting in a more homogeneous distribution. A second explanation is that most of the 10-15 mg/kg endogenous surfactant in the lung is broken down during ischemia by phospholipases. A twenty-fold increase in concentration before ischemia prevents inactivation of the total surfactant pool [13, 16]. Finally, surfactant treatment at reperfusion causes fluid overload that is added to extensive pulmonary edema that develops immediately after reperfusion. Although pretreatment with 200 mg/kg surfactant improved pulmonary function compared to untreated animals,

pulmonary compliance was still impaired as compared to sham-operated controls. We previously demonstrated that 400 mg/kg pretreatment completely normalized lung compliance 3 days after reperfusion [3]. In this previous study a shorter ischemic interval of 120 minutes was used. The relationship between ischemic time and mortality is of cubic form, so that after a certain threshold pulmonary function decreases rapidly, resulting in severely impaired short-term survival [21]. Therefore, the animals in the present study may have had more severe LIRI, so that the results of the present and previous study are not fully comparable. To prove that the timing of surfactant administration is essential, the lowest surfactant dose that reduces LIRI was used.

Surfactant treatment increased the number of $MHCII^+$-APC and granulocytes in the right, non-ischemic lung regardless of the time of treatment, while pretreatment reduced the number of helper and cytotoxic T-lymphocytes in the ischemic lung. The discrepancy between the anti and pro-inflammatory effects of surfactant may reflect different entities of inflammation in this study. The proinflammatory effect of surfactant is consistent with other experimental studies [3, 4, 22]. An elevated number of $MHCII^+$-APC and granulocytes can be explained by a response to surfactant itself, since alveolar surfactant recycling mainly depends on the number of alveolar macrophages, but also on alveolar type II cells and granulocytes. This pro-inflammatory effect of surfactant administration is supported by the finding of less granulocytes, APC and $MHCII^+$-APC in the BALf of rats treated with 50 mg/kg surfactant before ischemia (data not shown). On the other hand, surfactant has been shown to inhibit cytokine production in vivo and in vitro which contributes to the anti-inflammatory effect of surfactant [10]. Whether this reduction is due to a targeted response of surfactant components, such as surfactant-associated proteins, or preserved lung architecture, resulting in less presentation of self-antigens, remains unanswered. However, the reduction in infiltration of lymphocytes after surfactant pretreatment likely contributes to the improved function observed in this study.

Severe LIRI histologically results in diffuse alveolar damage, characterized in the acute phase by pulmonary edema, inflammation, atelectasis, intra-alveolar hemorrhage and fibrosis [1-4]. Those surviving the acute phase may either recover from injury or enter a fibroproliferative state, which develops within 3-7 days after the onset of symptoms and is comparable to the process of ARDS [1-4]. Amelioration of the acute phase of LIRI is important since this prevents fibroproliferation up to 90 days after reperfusion [3, 4]. In the present study, surfactant treatment lowered the pulmonary severity score by decreasing alveolar edema and fibrosis and preventing a fibroproliferative state. This corresponds with the study by Dreyer et al which showed that the beneficial effect of surfactant is mainly due to the intra-alveolar activity of surfactant, since surfactant pretreatment reduced intra-alveolar edema and atelectasis while no changes were found in the ultrastructure of alveolar type 2 cells and lamellar body content [23].

Although 200 mg/kg surfactant pretreatment ameliorates LIRI, pretreatment with 50 and 100 mg/kg had no effect on pulmonary function. In other LIRI studies, low dose surfactant has been proven to be beneficial as well [14, 16]. Administration of 100 mg/kg surfactant to donor lungs before retrieval in a clinical randomized trial had a beneficial effect on early clinical outcome [14]. 20 mg/kg surfactant pretreatment improved oxygenation and pulmonary compliance in an experimental study by Hausen and colleagues [16]. The

discrepancy between these studies and our study may be explained by several factors. A natural porcine or bovine surfactant containing surfactant associated proteins is superior to surfactant without associated proteins [24]. Also, differences in the method of surfactant application results in variable intra-alveolar surfactant concentrations. Finally, the length of the ischemic interval has an important effect on surfactant and thus pulmonary function resulting in exponentially increasing mortality with ischemia beyond a certain threshold. Therefore, differences in the length of ischemia between studies may require different surfactant doses needed to reduce LIRI.

A limitation of our study is that we did not actually transplant the lung. We were thus unable to study the effect of cold ischemia. However, this model is an accepted and useful model to study LIRI and there seem no major differences between short periods of warm and longer periods of cold ischemia [25]. Finally, the optimal surfactant dose may be different in the rat than in humans.

5 | Conclusions

200 mg/kg surfactant is the lowest dose that reduces severe LIRI in our model. Surfactant pretreatment is superior to treatment at reperfusion or 24 hours after reperfusion.

6 | References

1. de Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia-reperfusion-induced lung injury. Am J Respir Crit Care Med 2003;167:490-511.
2. Lee JC, Christie JD. Primary graft dysfunction. Proc Am Thorac Soc 2009;6:39-46.
3. van der Kaaij NP, Haitsma JJ, Kluin J, et al. Surfactant pretreatment ameliorates ischemia-reperfusion injury of the lung. Eur J Cardiothorac Surg 2005;27:774-82.
4. van der Kaaij NP, Kluin J, Haitsma JJ, et al. Surfactant pretreatment decreases long-term damage after ischemia-reperfusion injury of the lung. Eur J Cardiothorac Surg 2009;35:304-312; discussion 312.
5. Erasmus ME, Petersen AH, Oetomo SB, Prop J. The function of surfactant is impaired during the reimplantation response in rat lung transplants. J Heart Lung Transplant 1994;13:791-802.
6. Gunther A, Balser M, Schmidt R, et al. Surfactant abnormalities after single lung transplantation in dogs: impact of bronchoscopic surfactant administration. J Thorac Cardiovasc Surg 2004; 127:344-354.
7. Lewis JF, Brackenbury A. Role of exogenous surfactant in acute lung injury. Crit Care Med 2003;31:S324-28.
7. Veldhuizen RA, Ito Y, Marcou J, Yao LJ, McCaig L, Lewis JF. Effects of lung injury on pulmonary surfactant aggregate conversion in vivo and in vitro. Am J Physiol 1997;272:L872-878.
9. Hohlfeld JM, Tiryaki E, Hamm H, et al. Pulmonary surfactant activity is impaired in lung transplant recipients. Am J Respir Crit Care Med 1998;158:706-712.
10. Thomassen MJ, Antal JM, Connors MJ, Meeker DP, Wiedemann HP. Characterization of exosurf (surfactant)-mediated suppression of stimulated human alveolar macrophage cytokine responses. Am J Respir Cell Mol Biol 1994;10:399-404.
11. Ahuja A, Oh N, Chao W, Spragg RG, Smith RM. Inhibition of the human neutrophil respiratory burst by native and synthetic surfactant. Am J Respir Cell Mol Biol 1996;14:496-503.
12. Ikegami M, Scoville EA, Grant S, et al. Surfactant protein-D and surfactant inhibit endotoxin-induced pulmonary inflammation. Chest 2007;132:1447-1454.
13. Novick RJ, MacDonald J, Veldhuizen RA, et al. Evaluation of surfactant treatment strategies after prolonged graft storage in lung transplantation. Am J Respir Crit Care Med 1996;154:98-104.
14. Strüber M, Fischer S, Niedermeyer J, et al. Effects of exogenous surfactant instillation in clinical lung transplantation: a prospective, randomized trial. J Thorac Cardiovasc Surg 2007;133:1620-1625.
15. Friedrich I, Börgermann J, Splittgerber FH, et al. Bronchoscopic surfactant administration preserves gas exchange and pulmonary compliance after single lung transplantation in dogs. J Thorac Cardiovasc Surg 2004;127:335-343.
16. Hausen B, Rohde R, Hewitt CW, et al. Exogenous surfactant treatment before and after sixteen hours of ischemia in experimental lung transplantation. J Thorac Cardiovasc Surg 1997;113:1050-1058.
17. Mühlfeld C, Schaefer IM, Becker L, et al. Preischemic exogenous surfactant reduces pulmonary injury in rat ischemia/reperfusion. Eur Respir J 2009;33:625-633.
18. Spragg RG, Lewis JF, Walmrath HD, et al. Effect of recombinant surfactant protein C-based surfactant on the acute respiratory distress syndrome. N Engl J Med 2004;351:884-892.
19. Anzueto A, Baughman RP, Guntupalli KK, et al. Aerosolized surfactant in adults with sepsis-induced acute respiratory distress syndrome. Exosurf Acute Respiratory Distress Syndrome Sepsis Study Group. N Engl J Med 1996;334:1417-1421.
20. Kesecioglu J, Beale R, Stewart TE, et al. Exogenous natural surfactant for treatment of acute lung injury and the acute respiratory distress syndrome. Am J Respir Crit Care Med 2009;180:989-994.
21. Thabut G, Mal H, Cerrina J, et al. Graft ischemic time and outcome of lung transplantation: a multicenter analysis. Am J Respir Crit Care Med 2005;171:786-791.
22. Kramer BW, Jobe AH, Bachurski CJ, Ikegami M. Surfactant protein A recruits neutrophils into the lungs of ventilated preterm lambs. Am J Respir Crit Care Med 2001;163:158-165.
23. Dreyer N, Muhlfeld C, Fehrenbach A, et al. Exogenous surfactant application in a rat lung ischemia-reperfusion injury model: effects on edema formation and alveolar type II cells. Respir Res 2008;9:5.
24. Wright JR, Borron P, Brinker KG, Folz RJ. Surfactant Protein A: regulation of innate and adaptive immune responses in lung inflammation. Am J Respir Cell Mol Biol 2001;24:513-517.
25. Warnecke G, Sommer SP, Gohrbandt B, et al. Warm or cold ischemia in animal models of lung ischemia-reperfusion injury: is there a difference? J Thorac Cardiovasc Surg 2004;52:174-179.

9

Exogenous surfactant attenuation of ischemia–reperfusion injury in the lung through alteration of inflammatory and apoptotic factors

Based on
BP van Putte, PM Cobelens, NP van der Kaaij, B Lachmann, A Kavelaars, CJ. Heijnen, J Kesecioglu
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Summary

Background. Lung ischemia–reperfusion injury (LIRI) is associated with impaired gas exchange from increased edema formation and surfactant inactivation. Surfactant replacement therapy is believed to improve gas exchange and lung function, but its effect on inflammation is less well understood. We therefore examined the effect of exogenous surfactant on inflammatory and apoptotic factors in the lung in a rat model of LIRI. Methods. The left lung in rats was subjected to ischemia for 120 minutes and reperfusion for as long as 240 minutes. Sham-treated animals underwent sham surgery and mechanical ventilation for equivalent times. Rats received porcine surfactant or saline solution intratracheally either before or just after ischemia. Lungs were analysed histopathologically and for expressions of inducible nitric oxide synthase, cytokines, and caspase-3. Results. LIRI resulted in worse lung histopathologic characteristics than in sham-operated animals. At 2 hours of reperfusion, LIRI animals showed increased pulmonary caspase-3 expression. Moreover, LIRI resulted in inducible nitric oxide synthase expression at all time points. Exogenous surfactant resulted in less inflammatory cell infiltration and edema in the lungs relative to saline-treated animals. Surfactant decreased activated caspase-3 expression and increased inducible nitric oxide synthase expression relative to saline-treated animals. At 4 hours of reperfusion, surfactant increased interleukin 6 and 10 expressions in the lung. Conclusions. This study showed a significant improvement in lung histological characteristics after surfactant therapy, accompanied by reduced apoptosis and increased anti-inflammatory cytokine levels. Interestingly, surfactant therapy also increased pulmonary inducible nitric oxide synthase expression.

1 | Goal of the study

To investigate the effect of surfactant on inflammatory mediators and apoptosis in a rat model of LIRI.

2 | Materials and methods in brief

For a full description of the materials and methods, see the materials and methods section at the end of this thesis.

2.1 Experimental design

Male Sprague-Dawley rats (N=84), obtained from Harlan (Harlan Netherlands BV, Horst, the Netherlands), were used. Except for the sham-operated group, which underwent all operative interventions but without induction of ischemia, all rats underwent 120 minutes of warm ischemia of the left lung, followed by 30, 120, or 240 minutes of reperfusion (all groups n=7). Rats were treated by intratracheal instillation of surfactant (natural porcine surfactant, HL-10, 200 mg/kg; LEO Pharma A/S, Ballerup, Denmark) either before

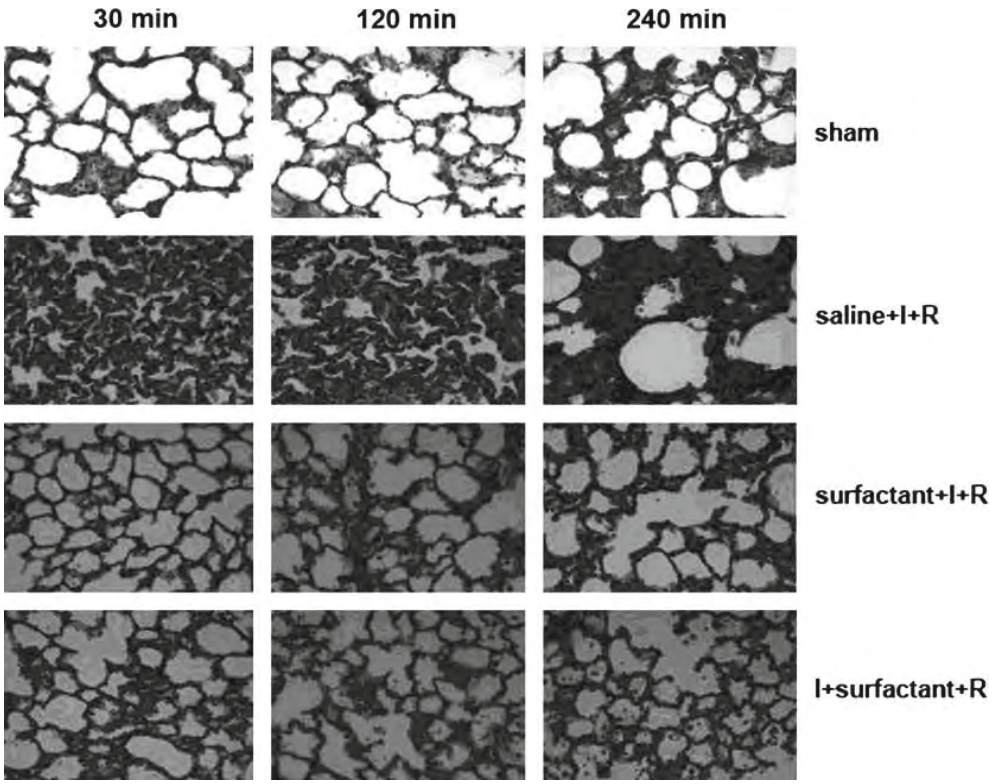

Figure 1. Representative paraffin sections of rat lung tissue stained with hematoxylin and eosin as function of time for sham-operated group (sham), control group (saline+I+R), and groups with surfactant administered before (surfactant+I+R) and after (I+surfactant+R) ischemia. Original magnification X200. I: Ischemia; R: Reperfusion.

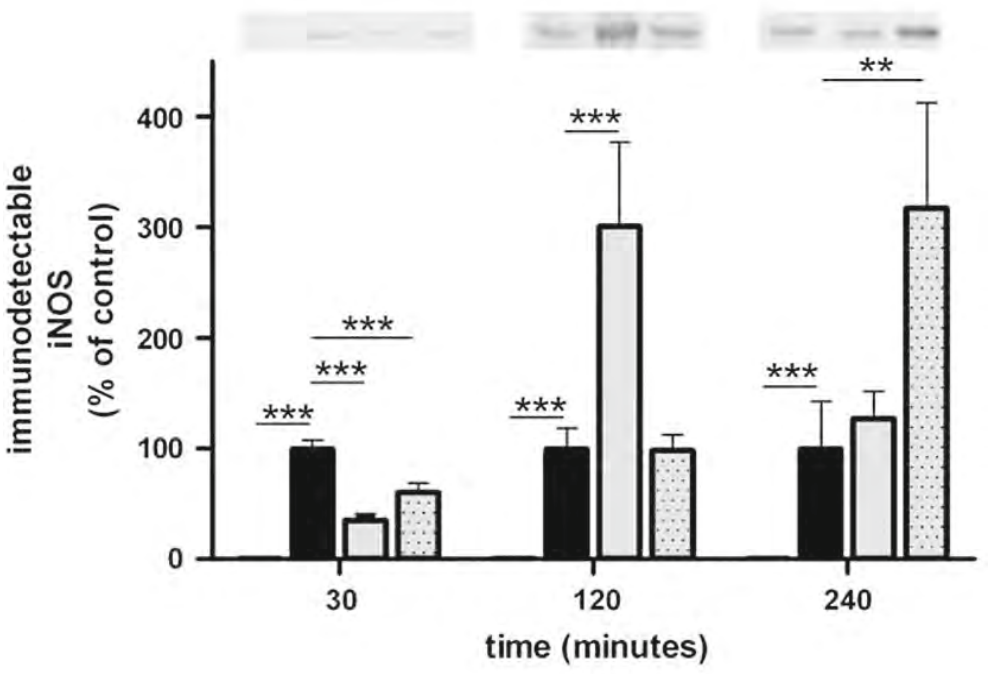

Figure 2. Inducible nitric oxide (iNOS) concentrations as function of time. Inducible nitric oxide expression levels were assessed in total cell lysates resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and analyzed by Western blot. Data (mean ± SEM) were normalized to b-actin expression and depicted as percentage of control values (black bars). Insets are representative Western blots depicting immunodetectable inducible nitric oxide. Sham (white bars); Saline+I+R (black bars); Surfactant+I+R (grey bars); I+surfactant+R (mottled bars). I: Ischemia; R: Reperfusion; SEM: Standard Error of the Mean.
**: P<0.01; ***: P<0.001; N=7 per group.

(surfactant, then ischemia, then reperfusion (SIR)) or just after (ischemia, then surfactant, then reperfusion (ISR)) ischemia. Control rats received saline solution intratracheally before start of ischemia.

2.2 Surfactant

Surfactant was dissolved in saline solution (50 mg/mL) and administered intratracheally at a dose of 200 mg/kg, resulting in surfactant exposure of both lungs. All SIR rats received surfactant intratracheally after they were briefly anesthetized and intubated. Two doses (each instillation included half of the total volume per rat) were administered to each rat during 1 hour before the start of ischemia. After each surfactant instillation, the animals recovered from anaesthesia while breathing spontaneously, resulting in homogeneous distribution in both lungs. The control rats received saline solution in the same volume and according to the same protocol as the SIR rats. The ISR animals had surfactant instillation after 2 hours of ischemia. Immediately after release of the clamp at the start of reperfusion, the whole dose of 200 mg/kg was administered intratracheally in a single instillation. After recruitment,

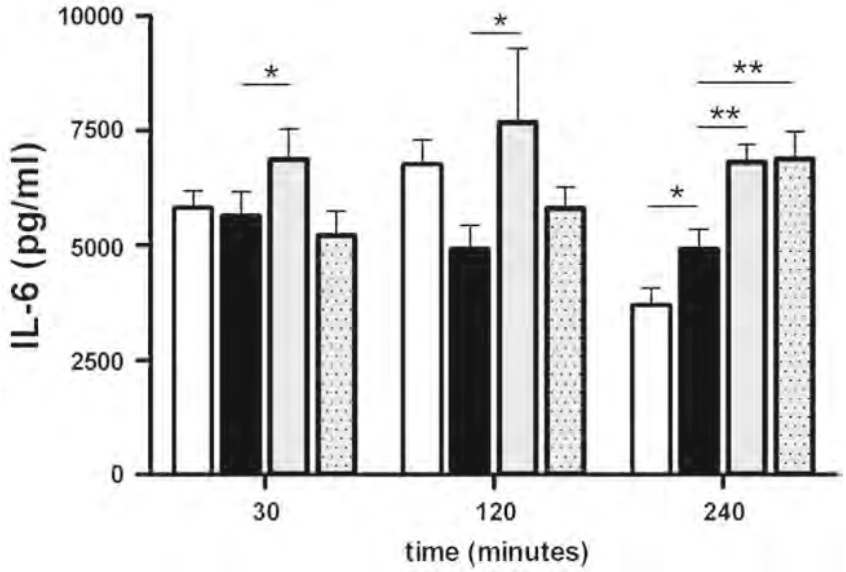

Figure 3. Interleukin 6 (IL-6) concentrations as function of time. Interleukin 6 levels were determined by standard enzyme-linked immunosorbent assay. Sham (white bars); Saline+I+R (black bars); Surfactant+I+R (grey bars); I+surfactant+R (mottled bars). I: Ischemia; R: Reperfusion.
*P<0.05; **P<0.01; N=7 per group.

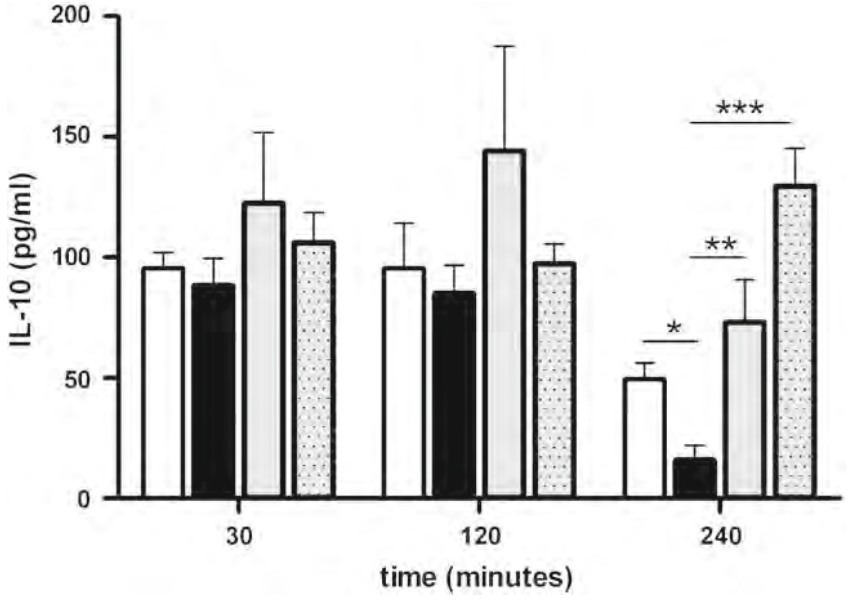

Figure 4. Interleukin 10 (IL-10) concentrations as function of time. Interleukin 10 levels were determined by standard enzyme-linked immunosorbent assay. Sham (white bars); Saline+I+R (black bars); Surfactant+I+R (grey bars); I+surfactant+R (mottled bars). I: Ischemia; R: Reperfusion.
*: P<0.05; **: P<0.01; ***: P<0.001; N=7 per group.

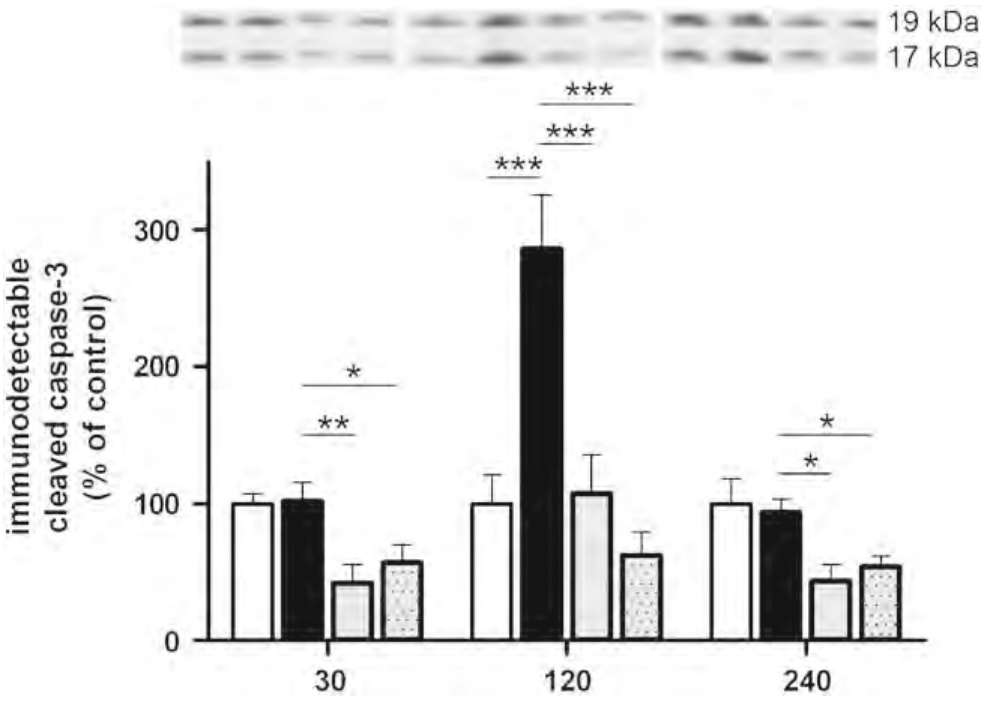

Figure 5. Effect of lung ischemia–reperfusion injury on cleaved caspase-3 expression as function of time. Cleaved caspase-3 expression levels were assessed in total cell lysates resolved by 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis and analyzed by Western blot. Data (mean ± SEM) were normalized to b-actin expression and depicted as percentage of values in sham-operated group. Insets are representative Western blots depicting immunodetectable cleaved caspase-3. Sham (white bars); Saline+I+R (black bars); Surfactant+I+R (grey bars); I+surfactant+R (mottled bars). I: Ischemia; R: Reperfusion. SEM: Standard Error of the Mean.
*: P<0.05; **: P<0.01; ***: P<0.001; N=7 per group.

pressure-controlled ventilation was temporarily augmented to achieve homogeneous distribution of surfactant.

2.3 Measurements

Measurements include histological analysis, inflammatory mediators, and apoptosis.

2.4 Statistical Analysis

All data are presented as mean ± SEM. All parameters were analysed by 1-way analysis of variance, followed by Bonferroni post hoc test.

3 | Results

3.1 Histological Study

Lung specimens of the control animals were characterized by severe pathologic changes at all reperfusion time points. At 30 minutes of reperfusion, atelectasis and thickened alveolar septa that had been infiltrated by inflammatory cells were observed (Figure 1). Alveolar edema and bleeding appeared to be more prominent at 240 minutes of reperfusion. Interestingly, both surfactant groups (SIR and ISR) only showed slightly thickened alveolar septa, slight alveolar edema, and minimal infiltration of inflammatory cells at all reperfusion time points relative to saline-treated animals (Figure 1).

3.2 Inflammatory Mediators

Western blot analysis revealed inducible nitric oxide synthase (iNOS) expression in the control group at all reperfusion time points, whereas iNOS was undetectable in the sham-operated group ($P<0.001$; Figure 2). Surfactant treatment decreased iNOS expression at 30 minutes of reperfusion in both the SIR and ISR groups ($P<0.001$). Interestingly, iNOS expression was significantly increased at 120 minutes of reperfusion in the SIR group relative to the control group ($P<0.001$), and a delayed response was observed in the ISR group, resulting in higher levels of iNOS at 240 minutes of reperfusion ($P<0.01$; Figure 2). IL-6 expression in the control group remained stable at all reperfusion time points, being significantly higher at 240 minutes of reperfusion relative to the sham-operated group ($P < 0.05$; Figure 3). Surfactant treatment increased IL-6 expression relative to control at all time points only in the control group ($P<0.05$). In the ISR group, IL-6 was only elevated relative to control at 240 minutes of reperfusion ($P<0.01$; Figure 3). IL-10 expression in the control group dropped significantly as a function of time, being significantly lower at 240 minutes of reperfusion relative to the sham-operated group ($P<0.05$; Figure 4). After surfactant treatment, significantly higher IL-10 expressions were observed at 240 minutes of reperfusion in both the SIR and ISR groups ($P<0.01$ SIR, $P<0.001$ ISR; Figure 4).

3.3 Apoptosis

Control animals showed increased levels of the proapoptotic marker cleaved caspase-3 relative to the sham-operated group only at 120 minutes of reperfusion ($P<0.001$). At 30 and 240 minutes of reperfusion, caspase-3 levels were similar to those in the sham-operated group (Figure 5). Surfactant reduced cleaved caspase-3 expression at 120 minutes of reperfusion relative to control ($P<0.001$ SIR and ISR). Also at 30 and 240 minutes of reperfusion, caspase-3 levels were diminished in both surfactant groups (SIR and ISR) relative to both the control and sham-operated groups ($P<0.05$; Figure 5).

4 | Discussion

In this study, we determined the effects of exogenous surfactant on inflammation and apoptosis in an animal model of LIRI. Although many studies have described the protective effect of exogenous surfactant on lung function and outcome in experimental models of

acute lung injury, little is known about the immunomodulatory effects of surfactant in vivo [1-3]. The effects of surfactant seem to depend on the experimental model, dose, and time of administration [4-6]. To address the time of administration, we treated rats either before ischemia or just after ischemia. We show here that surfactant increases IL-6 and IL-10 in the lung at 240 minutes after ischemia. IL-6 is a pleiotropic cytokine that can either induce proinflammatory mediator expression or exert cytoprotective effects, depending on the in vivo environmental circumstances [7]. IL-6 is transcriptionally regulated by neutral factor kB, but at high levels IL-6 protein in turn may limit neutral factor kB activity in a negative feedback loop, thereby dampening the inflammatory response. We therefore suggest that the increased IL-6 response seen after surfactant treatment may be anti-inflammatory. This hypothesis is supported by data of Farivar and colleagues, who showed that recombinant IL-6 improved LIRI [8]. Moreover, in a rodent model of hemorrhagic shock, exogenous IL-6 decreased lung and liver tissue injury [9].

In addition to increased levels of IL-6, we also observed augmented IL-10 expression after surfactant treatment. IL-10 has a strong anti-inflammatory effect by limiting the secretion of proinflammatory cytokines by macrophages and T-cells. Several studies have shown that exogenous IL-10 attenuates experimental reperfusion injury of the intestine, liver, lung, and hind limb [10-13]. In this study, IL-10 decreased dramatically with time in the control animals, whereas surfactant treatment normalized or even increased IL-10 expression, suggesting that surfactant induces a strong anti-inflammatory environment. We also looked at the expression of iNOS. Baron and associates demonstrated that expression of iNOS in lung epithelial cells is critical for the development of lung injury and furthermore mediates surfactant dysfunction [14]. Some investigators have suggested that increased lung apoptosis could be one of the mechanisms through which iNOS causes lung injury [15]. We report that iNOS was specifically upregulated in the control group, whereas it was not upregulated in the sham-operated group, which supports the notion that iNOS is an important mediator during lung injury. In line with this finding, we observed increased cleaved caspase-3 expression in the control group. Interestingly, 2 and 4 hours after ischemia iNOS was strongly upregulated by surfactant either before (SIR) or after (ISR) ischemia. The enhanced expression of iNOS after surfactant treatment was not associated with enhanced apoptosis, because at all time points both surfactant treatment schedules significantly decreased apoptosis in the lung. Interestingly, there are some indications that iNOS expression may be involved in antiapoptotic pathways, depending on cell type, stimulus, and duration (as reviewed by Mehta [15]). In our study, the early expression of iNOS, 30 minutes after ischemia, was significantly downregulated by surfactant treatment. We therefore speculate that early expression of iNOS triggers the proinflammatory response, whereas longer periods of iNOS expression trigger antiapoptotic pathways. This speculation is supported by the data of Mikawa and co-workers, who showed that selective inhibition of iNOS with ONO-1714 improved lung oxygenation, pulmonary edema, and leukocyte sequestration when administered before or within 2 hours after induction of acute lung injury but not if administered 3 or 4 hours after induction [16]. Interestingly, comparison of the effects of surfactant administered before and after ischemia on the parameters measured showed a time-dependent relationship. The delayed response in the ISR group is explained by the time sequence of the experimental setting. It is known that the reperfusion period plays a

significantly more important role in causing injury than does the ischemic period [17]. This might explain the equivalent responses, but with different time kinetics, that we observed after both surfactant treatment schedules. From a clinical point of view, it may be interesting for future studies to analyse the effect on ischemia–reperfusion injury of surfactant administered after the onset of reperfusion. Interestingly, however, Strüber and colleagues showed that exogenous surfactant instilled in donor lungs before retrieval had protective effects on posttransplant surfactant function and on clinical outcome [18].

5 | Conclusions

We have shown surfactant to have pleiotropic effects during LIRI. First, surfactant therapy efficiently improves lung architecture. Second, surfactant results in anti-inflammation, accompanied by decreased apoptosis. Finally, surfactant decreases the short-term iNOS expression but increases the long-term iNOS expression. Surfactant replacement therapy thus may provide a good approach to treat LIRI in patients, although more studies are warranted to further delineate the long-term effects of surfactant and the mechanisms involved.

6 | References

1. Chen CM, Fang CL, Chang CH. Surfactant and corticosteroid effects on lung function in a rat model of acute lung injury. Crit Care Med 2001;29:2169-2175.
2. Erasmus ME, Petersen AH, Hofstede G, et al. Surfactant treatment before reperfusion improves the immediate function of lung transplants in rats. Am J Respir Crit Care Med 1996;153:665-670.
3. Verbrugge SJ, Vazquez de Anda G, Gommers D, et al. Exogenous surfactant preserves lung function and reduces alveolar Evans blue dye influx in a rat model of ventilation-induced lung injury. Anesthesiology 1998;89:467-474.
4. Hausen B, Rohde R, Hewitt CW, et al. Exogenous surfactant treatment before and after sixteen hours of ischemia in experimental lung transplantation. J Thorac Cardiovasc Surg 1997;113:1050-1058.
5. Novick RJ, MacDonald J, Veldhuizen RA, et al. Evaluation of surfactant treatment strategies after prolonged graft storage in lung transplantation. Am J Respir Crit Care Med 1996;154:98-104.
6. van Helden HP, Kuijpers WC, Langerwerf PE, et al. Efficacy of Curosurf in a rat model of acute respiratory distress syndrome. Eur Respir J 1998;12:533-539.
7. Heinrich PC, Behrmann I, Haan S, et al. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J 2003;374:1-20.
8. Farivar AS, Merry HE, Fica-Delgado MJ, et al. Interleukin-6 regulation of direct lung ischemia reperfusion injury. Ann Thorac Surg 2006;82:472-478.
9. Meng ZH, Dyer K, Billiar TR, et al. Distinct effects of systemic infusion of G-CSF vs. IL-6 on lung and liver inflammation and injury in hemorrhagic shock. Shock 2000;14:41-48.
10. Dinant S, Vetelainen RL, Florquin S, et al. IL-10 attenuates hepatic I/R injury and promotes hepatocyte proliferation. J Surg Res 2007;141:176-182.
11. Engles RE, Huber TS, Zander DS, et al. Exogenous human recombinant interleukin-10 attenuates hindlimb ischemia-reperfusion injury. J Surg Res 1997;69:425-428.
12. Hess PJ, Seeger JM, Huber TS, et al. Exogenously administered interleukin-10 decreases pulmonary neutrophil infiltration in a tumor necrosis factor-dependent murine model of acute visceral ischemia. J Vasc Surg 1997;26:113-118.
13. Eppinger MJ, Ward PA, Bolling SF, et al. Regulatory effects of interleukin-10 on lung ischemia-reperfusion injury. J Thorac Cardiovasc Surg 1996;112:1301-1305; discussion 1305-1306.
14. Baron RM, Carvajal IM, Fredenburgh LE, et al. Nitric oxide synthase-2 down-regulates surfactant protein-B expression and enhances endotoxin-induced lung injury in mice. Faseb J 2004;18:1276-1278.
15. Mehta S. The effects of nitric oxide in acute lung injury. Vascul Pharmacol 2005;43:390-403.
16. Mikawa K, Nishina K, Takao Y, et al. ONO-1714, a nitric oxide synthase inhibitor, attenuates endotoxin-induced acute lung injury in rabbits. Anesth Analg 2003;97:1751-1755.
17. Ng CS, Wan S, Arifi AA, et al. Inflammatory response to pulmonary ischemia-reperfusion injury. Surg Today 2006;36:205-214.
18. Struber M, Fischer S, Niedermeyer J, et al. Effects of exogenous surfactant instillation in clinical lung transplantation: a prospective, randomized trial. J Thorac Cardiovasc Surg 2007;133:1620-1625.

10

Alveolar preservation with high inflation pressure and intermediate oxygen concentration reduces ischemia-reperfusion injury of the lung

Based on
NP van der Kaaij, J Kluin, RA Lachmann, MA den Bakker, B Lachmann, RWF de Bruin, AJJC Bogers
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Summary

Background. The aim of this study was to investigate the optimal alveolar oxygen concentration and inflation pressure during ischemia that reduces lung ischemia reperfusion injury (LIRI). Methods. Male Sprague-Dawley rats (n=66) underwent 150 minutes of left lung ischemia by hilar clamping at an airway inflation pressure (P) of 5 or 30 cm H_2O and an oxygen (O) concentration of 0%, 30% or 100% (P_5O_0, P_5O_{30}, P_5O_{100}, $P_{30}O_0$, $P_{30}O_{30}$ and $P_{30}O_{100}$ groups). Lungs preserved with 0% oxygen were inflated with 100% N_2. Measurements, consisting of arterial blood gas values, pulmonary compliance, histology and flowcytometry of bronchoalveolar lavage , were performed on day 2 postoperatively. Results. Inflation with 30 cm H_2O resulted in improved PaO_2/FiO_2 ratio and lung compliance, less diffuse alveolar damage and less infiltration of $CD4^+$-and $CD8^+$-lymphocytes and major histocompatibility complex positive antigen presenting cells ($MHCII^+$-APC) in the left lung on day 2 as compared to clamping at a pressure of 5 cm H_2O. Furthermore, the 100% oxygen groups demonstrated a lower PaO_2 and a decreased pulmonary compliance than 30% oxygen groups. More $CD8^+$-lymphocytes and $MHCII^+$-APC were found in the P_5O_{100} group than in the P_5O_0 and P_5O_{30} groups. Conclusions. Alveolar inflation with a pressure of 30 cm H_2O and an oxygen concentration of 30% decreases the severity of LIRI. The protective effect is mainly due to hyperinflation and to a lesser extent through oxygen concentration.

1 | Goal of the study

To identify the optimal inflation pressure and oxygen content that minimizes LIRI 48 hours after reperfusion.

2 | Materials and methods in brief

For a full description of the materials and methods, see the materials and methods section at the end of this thesis.

2.1 Experimental design

The experimental protocol was approved by the Animal Experiments Committee under the national Experiments on Animals Act and complied with the 1986 directive 86/609/EC of the Council of Europe. Male Sprague-Dawley rats (n=66, Harlan, Horst, The Netherlands), weighing 312±17 gram, were randomized into 6 groups (11 animals per group). Lungs were clamped at a pressure of 5 cm H_2O with 0% oxygen (100% nitrogen) (P_5O_0), 30% oxygen (P_5O_{30}) or 100% oxygen (P_5O_{100}) or at a pressure of 30 cm H_2O with 0% oxygen (100% nitrogen) ($P_{30}O_0$), 30% oxygen ($P_{30}O_{30}$) or 100% oxygen ($P_{30}O_{100}$). All animals received 150 minutes of warm ischemia. Measurements were performed 48 hours after reperfusion.

2.2 Measurements

Measurements include survival, blood gas values, static pulmonary compliance, flowcytometric analysis of infiltrating cells in BALf and histological analysis (in 4 additional animals per group). The static pulmonary compliance is presented as Vmax and Cmax. Vmax is the maximal lung volume at a pressure of 35 cm H_2O, while Cmax is the maximal compliance of the expiration curve.

2.3 Statistical analysis

The results in text, tables and figures are presented as mean ± standard error of the mean (SEM). Data were analysed using SPSS version 11.1 statistical software (SPSS Inc., Chicago, Illinois, USA). If an overall difference between groups was found by the Kruskal-Wallis test, Mann-Whitney-U tests were performed for intergroup comparison. P values <0.05 were considered to be significant.

3 | Results

3.1 Survival

Mortality of animals in which lungs were preserved with P_5O_{100} was 21% compared to 0% in all other groups.

3.2 Arterial blood gas, pulmonary compliance

PaO_2, alveolar-arterial oxygen difference (AAOD) and pulmonary compliance (Vmax

and Cmax) significantly improved if lungs were inflated with 30 cm H_2O as compared to inflation with 5 cm H_2O, independent of the oxygen level (Table 1). Within both the low and high pressure groups, lungs inflated with 100% oxygen showed a lower pulmonary compliance (Vmax and Cmax) and PaO_2 and a higher AAOD than lungs inflated with 30% oxygen (Table 1).

3.3 Inflammatory cells in left BALf

No major difference in the number of neutrophils was found (Figure 1A). However, more $MHCII^+$-APC (Figure 1C), $CD3^+CD4^+$-lymphocytes (Figure 1D), and $CD3^+CD8^+$-lymphocytes (Figure 1E) were present in the P_5O_{30} and P_5O_{100} groups as compared to inflation with $P_{30}O_{30}$ and $P_{30}O_{100}$ groups. Within the P_5 groups, inflation with 100% O_2 resulted in infiltration of more $CD3^+CD8^+$-lymphocytes than inflation with 0% and 30% O_2 (Figure 1E) and more APC (Figure 1B) and $MHCII^+$-APC (Figure 1C) than inflation with 0% O_2. Also, the number of APC (Figure 1B), $CD3^+CD4^+$-cells (Figure 1D) and $CD3^+CD8^+$-lymphocytes (Figure 1E) was significantly higher in the $P_{30}O_0$ group than in the other high alveolar inflation pressure groups, although the number of $MHCII^+$-APC (Figure 1C) failed to reach a statistically significant level. The number of $CD45RA^+$-cells (B-lymphocytes) did not differ between the groups (data not shown).

3.4 Inflammatory cells in right BALf

The number of $CD3^+CD4^+$-lymphocytes (Figure 1I), $CD3^+CD8^+$-lymphocytes (Figure 1J), and $MHCII^+$-APC (Figure 1H) was significantly higher in lungs that were preserved with P_5O_{100} as compared to $P_{30}O_{100}$. Inflation with P_5O_{100} also resulted in more alveolar accumulation of neutrophils (Figure 1F), $CD3^+CD8^+$-lymphocytes (Figure 1J) and $MHCII^+$-APC (Figure 1H) than inflation with lower oxygen concentration. The number of $CD45RA^+$-cells (B-lymphocytes) did not differ between the groups (data not shown).

3.5 Histology

Pulmonary inflation with 5 cm H_2O resulted in diffuse alveolar damage characterized by moderate to severe intra-alveolar edema, septal edema and inflammation, mild to moderate intra-alveolar hemorrhages, and mild atelectasis and congestion (Figure 2, Figure 3). The inflammatory pattern of low pressure inflated groups largely confirmed the data found with flowcytometry demonstrating mixed inflammation, consisting of neutrophils, lymphocytes and histiocytes in all groups. Inflation with 30 cm H_2O caused less intra-alveolar edema, septal edema, and inflammation, although mild atelectasis and intra-alveolar hemorrhages were still present (Figure 2, Figure 3). As opposed to 0% and 100%, inflation with 30% oxygen prevented development of intra-alveolar hemorrhage and congestion. Furthermore, the inflammatory pattern of lungs preserved with 30 cm H_2O mainly consisted of histiocytes but not lymphocytes. The histological severity score was significantly higher in low pressure inflated than in hyperinflated groups, although no major differences were found between the different oxygen concentrations (Table 2).

Table 1. Lung function: Mean (SEM) arterial blood gas and pulmonary compliance

Lung function	P_5O_0	P_5O_{30}	P_5O_{100}	$P_{30}O_0$	$P_{30}O_{30}$	$P_{30}O_{100}$
PaO_2 (mm Hg)	352 (29)	439 (31) †	263 (46)	548 (28) *	605 (22) *†	508 (34) *
$PaCO_2$ (mm Hg)	49 (4)	52 (2)	49 (4)	41 (2) †	40 (3) *†	51 (3)
PH	7.37 (0.03)	7.37 (0.01)	7.37 (0.02)	7.42 (0.02) †	7.44 (0.02) *†	7.33 (0.02)
AAOD	300 (27)	230 (36) †	389 (45)	159 (37) *	58 (22) *†#	141 (32) *
V_{max} L&R (ml/kg)	33.1 (1.6)	37.8 (1.2) #	34.5 (2.1)	39.9 (1.5) *	41.4 (1.1) †	37.3 (1.0)
V_{max} L (ml/kg)	6.7 (0.7)	8.8 (0.6) †	4.8 (1.1)	11.9 (0.5)*	12.6 (0.4) *†	10.1 (0.7) *
C_{max} L&R ((ml/kg)/cm H_20)	3.3 (0.2)	4.1 (0.2) #	3.8 (0.4)	4.3 (0.3) *	4.5 (0.2) †	3.9 (0.3)
C_{max} L ((ml/kg)/cm H_20)	0.5 (0.08) †	0.7 (0.05) †	0.2 (0.09)	1.1 (0.09) *†	1.0 (0.07) *†	0.6 (0.13)

PaO_2, $PaCO_2$, PH, AAOD, and Vmax and Cmax of the left and total lung of P_5O_0, P_5O_{30}, P_5O_{100}, $P_{30}O_0$, $P_{30}O_{30}$ and $P_{30}O_{100}$ groups. AAOD: Alveolar-arterial oxygen difference; Cmax: Maximal compliance of the expiration curve, corrected for body weight; L: Left lung; L&R: Left and Right lung; O: Oxygen concentration; P: Alveolar inflation Pressure; SEM: Standard Error of the Mean; Vmax: Maximal lung volume corrected for body weight at a pressure of 35 cm H_2O.
*: $P<0.05$ versus P_5 with similar oxygen concentration
†: $P<0.05$ versus O_{100} with similar inflation pressure
#: $P<0.05$ versus O_0 with similar inflation pressure

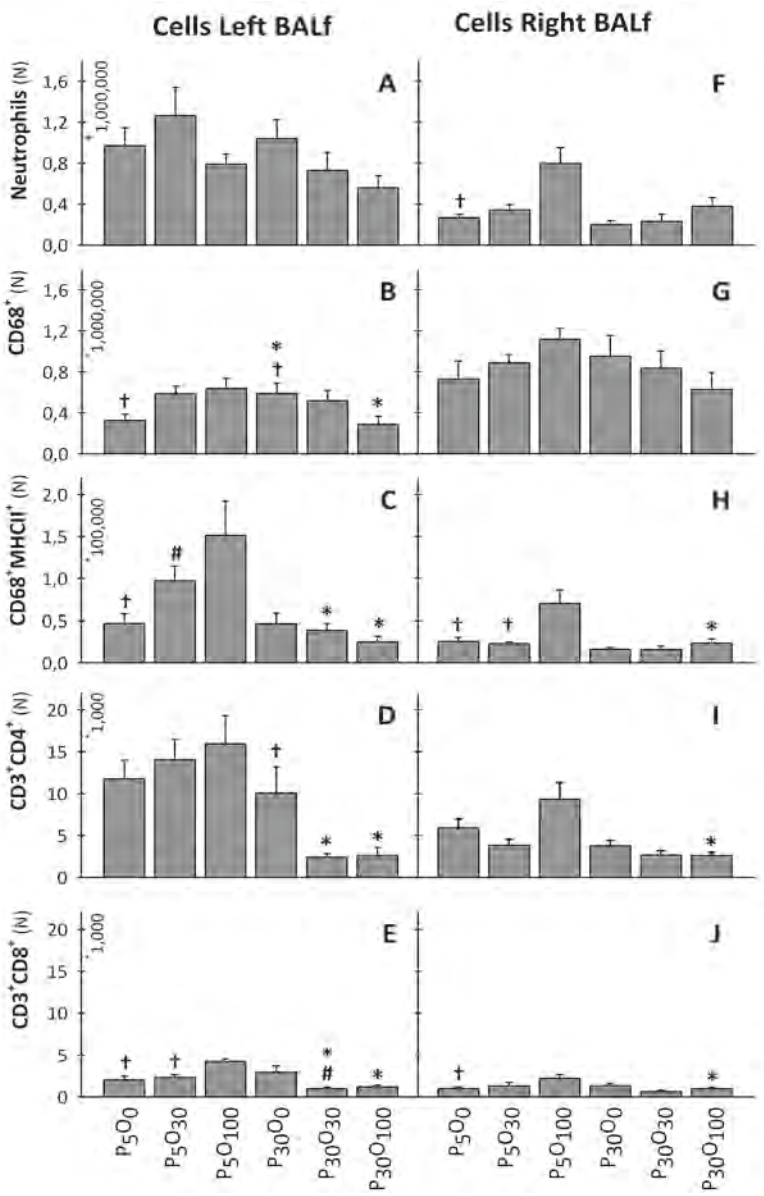

Figure 1. Mean number of inflammatory cells ± SEM in BALf of the left (A-E) and right lung (F-J) of P_5O_0, P_5O_{30}, P_5O_{100}, $P_{30}O_0$, $P_{30}O_{30}$ and $P_{30}O_{100}$ groups. Presented are: (A and F) neutrophils, (B and G) $CD68^+$-cells (antigen presenting cells), (C and H) $MHCII^+CD68^+$-cells (antigen presenting cells expressing major histocompatibility complex II), (D and I) $CD3^+CD4^+$-lymphocytes (helper T-cells), (E and J) $CD3^+CD8^+$-lymphocytes (cytotoxic T-cells). Presented on the Y-axis is the number of cells, multiplied by 1,000,000 (A, B, F, G), 100,000 (C, H), or 1,000 (D, E, I, J). BALf: BronchoAlveolar Lavage Fluid; N: Number of cells; O: Oxygen concentration; P: Alveolar inflation pressure; SD: Standard Deviation.

*: P<0.05 versus P5 with similar oxygen concentration

†: P<0.05 versus O100 with similar inflation pressure

#: P<0.05 versus O0 with similar inflation pressure

Table 2. Mean (SEM) histologic everity score of the left lung

Group	Severity Score
P_5O_0	9.3 (1.5)
P_5O_{30}	8.3 (0.5)
P_5O_{100}	9.6 (0.8)
$P_{30}O_0$	4.2 (2.2) +
$P_{30}O_{30}$	2.9 (1.3) *
$P_{30}O_{100}$	5.9 (1.7)

Histologic severity score of left lungs of P_5O_0, P_5O_{30}, P_5O_{100}, $P_{30}O_0$, $P_{30}O_{30}$ or $P_{30}O_{100}$ groups. HE sections were scored for intra-alveolar and septal edema, hyaline membrane formation, inflammation, fibrosis, atelectasis, intra-alveolar hemorrhage and congestion. Each parameter was scored as: (0) absent, (1) mild, (2) moderate/scattered, or (3) severe/frequent. The pulmonary severity score is the sum of the individual scores of these eight categories, resulting in a possible score ranging from 0 for normal lungs to 24 for the most injured lungs**.** HE: Hematoxylin and Eosin staining; O: Oxygen concentration; P: Alveolar inflation pressure; SEM: Standard Error of the Mean; SS: Severity Score.
*: $P<0.05$ versus P_5 with similar oxygen concentration;
+: $P=0.05$ versus P_5 with similar oxygen concentration

4 | Discussion

This study demonstrates that alveolar inflation with 30 cm H_2O during ischemia resulted in improved survival, better pulmonary compliance and arterial oxygenation, less diffuse alveolar damage and a reduced number of $CD3^+CD4^+$-lymphocytes and $CD3^+CD8^+$-cells and $MHCII^+$-APC as compared to inflation with 5 cm H_2O. Furthermore, the P_5O_{100} and $P_{30}O_{100}$ groups showed a lower PaO_2 and pulmonary compliance as compared to the 30% oxygen preserved groups. Also, a more pronounced inflammation was observed in the lungs of the P_5O_{100} than in the P_5O_0 and P_5O_{30} groups, as characterized by an elevated level of $CD3^+CD8^+$-lymphocytes and $MHCII^+$-APC.

The protective effect of hyperinflation during ischemia may be due to an increased intra-alveolar surfactant concentration and less atelectasis, resulting in a preserved alveolo-capillary barrier, increased pulmonary vascular resistance, and more efficient vascular preservation. The concentration of intra-alveolar surfactant is directly related to inflation pressure during preservation [1]. Inflation to total lung capacity before ischemia stimulates surfactant release into the alveolus [1]. Since surfactant is important in minimizing the surface tension in the alveolus and protects against the formation of intra-alveolar and septal edema, it is important to optimize the concentration of surfactant before or during the ischemic period [2]. In this regard, administration of exogenous surfactant before the induction of ischemia reduces LIRI [2]. Secondly, atelectatic areas of the lung are particularly vulnerable to LIRI, since not only less surfactant is secreted, but surfactant is also degraded at a higher rate [3]. Together, this will result in a lower concentration of surfactant in the alveolus at the start of reperfusion, which causes increased surface tension at the alveolo-capillary barrier leading to alveolar collapse, accumulation of anaerobic metabolites, hypoxic vasoconstriction and hypoperfusion. Furthermore, higher shear forces are developed due to surfactant injury

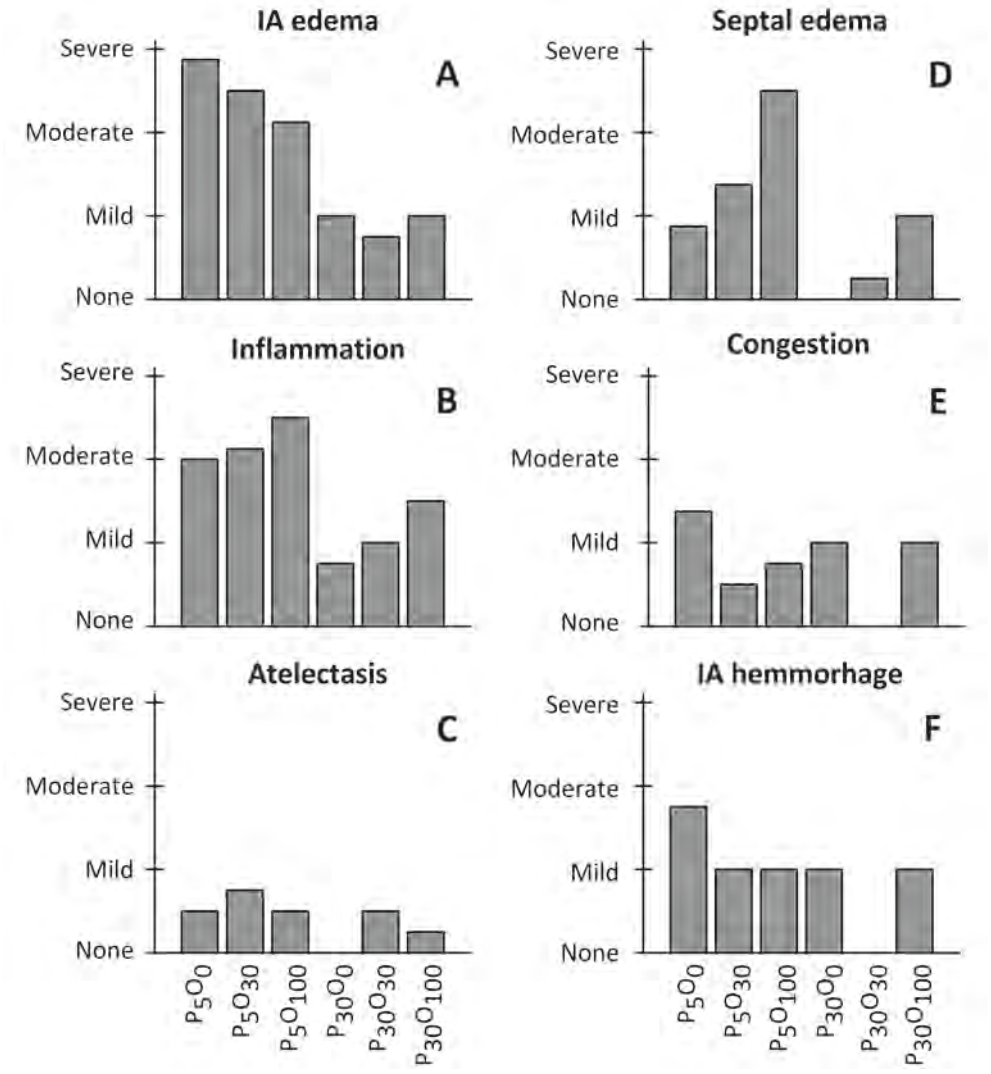

Figure 2. Mean score of (A) intra-alveolar edema, (B) severity of inflammation, (C) atelectasis, (D) septal edema, (E) congestion, and (F) intra-alveolar hemorrhage, scored on HE sections of 4 animals per group. Each parameter was scored as: Absent, mild, moderate/scattered, or severe/frequent. O: Oxygen concentration; P: Alveolar inflation pressure.

leading to stress failure and injury of the alveolar epithelium and capillary endothelium during reperfusion, subsequent increased capillary permeability and protein rich edema formation [4, 5]. Since surfactant is rate limiting for the transfer of proteins across the alveolo-capillary membrane, a further influx of proteins is facilitated, which dose-dependently further inhibits surfactant resulting in a cascade of surfactant inactivation [6]. In the third place, hyperinflation causes a temporary increase in pulmonary vascular resistance during ischemia and in the early stages of reperfusion, thereby allowing the damaged endothelium

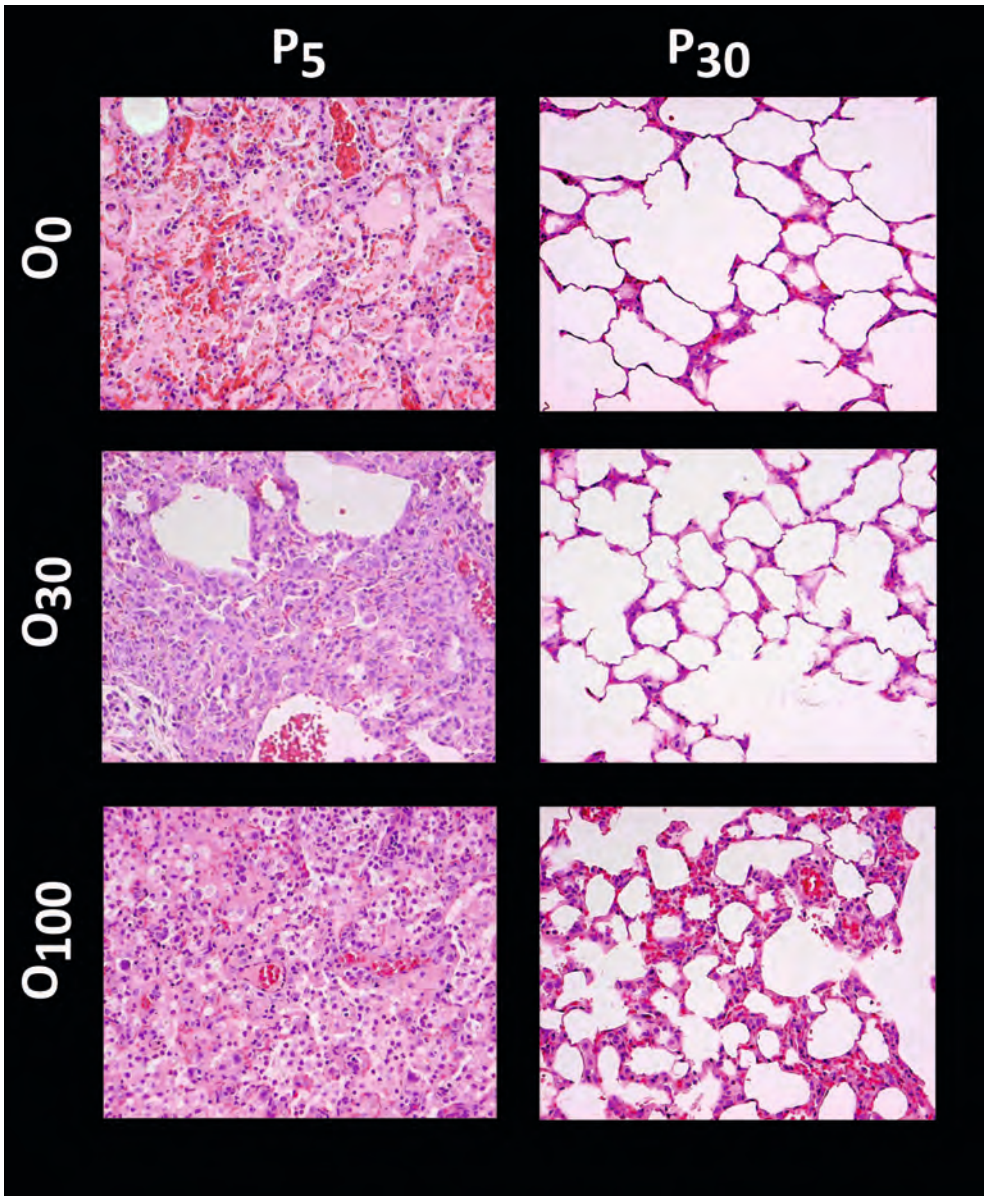

Figure 3. Histologic examples (HE staining, 200*) of P_5O_0, P_5O_{30}, P_5O_{100}, $P_{30}O_0$, $P_{30}O_{30}$ and $P_{30}O_{100}$ groups. HE: Hematoxylin and Eosin staining; O: Oxygen; P: Alveolar inflation pressure.

to (partly) recover in the early phase of reperfusion [7]. Finally prevention of atelectasis before the induction of ischemia causes less hypoxic vasoconstriction and endothelial cell swelling and may therefore result in a more efficient pulmonary vascular perfusion [8].

Inflation with 30% O_2 minimizes LIRI and the effect is most pronounced in lungs inflated with 30 cm H_2O. Alveolar inflation with 100% O_2 results in more severe LIRI than inflation with 30% O_2, which may be due to formation of reactive oxygen species (ROS)

and resorption atelectasis. Although the oxygen consumption of the lung tissue is lower than that of the myocardium or muscle, the lung still requires oxidative metabolism to carry out a variety of functions such as protein and phospholipid synthesis [9]. During cold storage only a small quantity of O_2 is required to maintain metabolism [10]. In this regard, alveolar inflation under hypoxic conditions (5% O_2) maintains intrapulmonary metabolism and attenuates LIRI [11]. Fisher et al demonstrated that metabolism in the cold preserved lung did not change until the alveolar O_2 concentration decreased to less than 0,7 mm Hg (1% O_2) [10]. However, anaerobic metabolism alone is not capable of maintaining the pulmonary metabolism and it is associated with more severe LIRI [12]. Generation of ROS in lungs preserved with high oxygen concentration is the most important contributor to the severity of LIRI, consisting of damage to particular the cellular membrane, alveolar epithelial and capillary endothelial cells and mitochondria, which are especially susceptible to oxidative stress [4, 13]. Furthermore, resorption atelectasis is induced in airways with higher O_2 concentrations, since O_2 diffuses easily into the plasma, while N_2 is poorly soluble. This will result in collapse of the alveolus, which is undesirable during the ischemic period, as discussed above. This was macroscopically evident in our study, since lungs inflated with 100% oxygen were completely atelectatic at the end of the ischemic period, independent of the inflation pressure. Alveolar inflation with N_2 in our experiment prevents resorption atelectasis and reduces LIRI. However, if lungs were inflated with 100% N_2 with 5 cm H_2O, LIRI was still severe, indicating that the most important component in minimizing LIRI is the alveolar inflation pressure. This is supported by the finding that no major differences exist between the hyperinflated 100% N_2 and 30% O_2 groups, which is confirmed by other studies [4, 7, 12, 14]. Thus, a combination of high inflation pressure with a low oxygen concentration that minimizes resorption atelectasis and ROS production, is the optimal alveolar inflation protocol.

Hyperinflation did not influence the number of neutrophils and APC in the left lung. However, the number of $MHCII^+$-APC, $CD3^+CD4^+$-cells and $CD3^+CD8^+$-lymphocytes were significantly reduced. Inflammation with T-cells in our autologous experimental model may be explained by released self-antigens after LIRI, like myosin, heat shock proteins and type V collagen, which is further supported by MHCII expression on APC, as confirmed by others [15-17]. Since involvement of T-cells and $MHCII^+$-APC may be the link between LIRI and BOS, a reduction in the number of these cells in the hyperinflated groups may be relevant for the long-term outcome after transplantation.

Although the majority of experimental studies supports the inflation of lungs during ischemia, the outcome of clinical lung transplantation in centers where grafts were preserved with low alveolar pressures did not differ from the outcome in centers where hyperinflation is common practice [18]. Also, Steen et al demonstrated excellent pulmonary function after cold atelectatic storage for 12 hours in an experimental pig study [19]. Differences between these and our study could be explained by differences between warm and cold ischemia, since there is a positive correlation between metabolic rate and temperature during ischemia [20]. However, no major differences are found between short periods of warm ischemia and long periods of cold ischemia, so that the use of warm ischemia models is accepted as an accelerated model of clinically relevant cold IRI in models of lung, liver and kidney IRI [21]. Differences in outcome between our and other studies may therefore be explained by the

relative short period of cold ischemia. In the study by Haverich et al cold ischemic intervals under 4 hours were usually achieved [18]. Moreover, the 12 hour cold ischemic period in the study by Steen et al may even be too short too induce symptoms of PGD in that model [19]. In our model, the goal was to induce a severe ischemic hit, which resulted in symptoms comparable to PGD. The animals in the P_5O_5 group did not tolerate warm ischemia beyond 150 minutes in our model.

Next to the use of warm instead of cold ischemia, other limitations of our study are that surfactant subtype analysis could not be performed, and ROS and ATP levels were not recorded. Furthermore, Decampos et al showed optimal outcome if lungs were stored at 50% of total lung capacity, which corresponds to 15-20 cm H_2O in our model [22]. However, intermediate alveolar inflation pressures were not included in our study. Although the values found in the $P_{30}O_{30}$ group were close to values found in normal animals, it could be that lower inflation pressures also prevent the development of PGD grade 3. To shed more light on the protective mechanisms involved, these are subject of future studies.

5 | Conclusions

Alveolar inflation with a pressure of 30 cm H_2O and an oxygen concentration of 30% decreases the severity of LIRI. The major effect is due to hyperinflation and to a lesser extent oxygen concentration. Therefore, a combination of an inflation pressure that stimulates surfactant release with an oxygen concentration that minimizes resorption atelectasis and ROS production is the optimal alveolar inflation protocol. The optimal alveolar inflation protocol reduces the number of T-lymphocytes and MHCII expression on APC after reperfusion. This observation may be of particular relevance since these cells are thought to be a link between ischemia-reperfusion injury and the subsequent development of BOS.

6 | References

1. Hildebran JN, Goerke J, Clements JA. Surfactant release in excised rat lung is stimulated by air inflation. J Appl Physiol 1981;51:905-910.
2. van der Kaaij NP, Haitsma JJ, Kluin J, et al. Surfactant pretreatment ameliorates ischemia-reperfusion injury of the lung. Eur J Cardiothorac Surg 2005;27:774-782.
3. Levine BE, Johnson RP. Effects of atelectasis on pulmonary surfactant and quasi-static lung mechanics. J Appl Physiol 1965;20:859-864.
4. Sakuma T, Takahashi K, Ohya N, et al. Ischemia-reperfusion lung injury in rabbits: Mechanisms of injury and protection. Am J Physiol 1999;276:L137-145.
5. Novick RJ, Gehman KE, Ali IS, Lee J. Lung preservation: The importance of endothelial and alveolar type ii cell integrity. Ann Thorac Surg 1996;62:302-314.
6. van der Kaaij NP, Lachmann RA, Bogers AJJC, Lachmann B. Surfactant alterations and treatment of lung transplant ischemia–reperfusion injury. Journal of organ dysfunction 2006;2:221-229.
7. Van Raemdonck DE, Jannis NC, De Leyn PR, Flameng WJ, Lerut TE. Alveolar expansion itself but not continuous oxygen supply enhances postmortem preservation of pulmonary grafts. Eur J Cardiothorac Surg 1998;13:431-440; discussion 40-1.
8. Puskas JD, Hirai T, Christie N, Mayer E, Slutsky AS, Patterson GA. Reliable thirty-hour lung preservation by donor lung hyperinflation. J Thorac Cardiovasc Surg 1992;104:1075-1083.
9. D'Armini AM, Tom EJ, Roberts CS, Henke DC, Lemasters JJ, Egan TM. When does the lung die? Time course of high energy phosphate depletion and relationship to lung viability after "death". J Surg Res 1995;59:468-474.
10. Fisher AB, Dodia C. Lung as a model for evaluation of critical intracellular po2 and pco. Am J Physiol 1981;241:E47-50.
11. Fukuse T, Hirata T, Ishikawa S, et al. Optimal alveolar oxygen concentration for cold storage of the lung. Transplantation 2001;72:300-304.
12. Fukuse T, Hirata T, Nakamura T, Kawashima M, Hitomi S, Wada H. Influence of deflated and anaerobic conditions during cold storage on rat lungs. Am J Respir Crit Care Med 1999;160:621-627.
13. Fisher AB, Dodia C, Tan ZT, Ayene I, Eckenhoff RG. Oxygen-dependent lipid peroxidation during lung ischemia. J Clin Invest 1991;88:674-679.
14. Watanabe A, Kawaharada N, Kusajima K, Komatsu S, Abe T, Takahashi H. Influence of oxygen in inflation gas during lung ischemia on ischemia-reperfusion injury. J Thorac Cardiovasc Surg 1997;114:332-338.
15. van der Kaaij NP, Kluin J, Haitsma JJ, et al. Ischemia of the lung causes extensive long-term pulmonary injury: An experimental study. Respir Res 2008;9:28.
16. de Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia-reperfusion-induced lung injury. Am J Respir Crit Care Med 2003;167:490-511.
17. Waddell TK, Gorczynski RM, DeCampos KN, Patterson GA, Slutsky AS. Major histocompatibility complex expression and lung ischemia-reperfusion in rats. Ann Thorac Surg 1996;62:866-872.
18. Haverich A, Scott WC, Jamieson SW. Twenty years of lung preservation--a review. J Heart Transplant 1985;4:234-240.
19. Steen S, Sjöberg T, Ingemansson R, Lindberg L. Efficacy of topical cooling in lung preservation: Is a reapprisal due? Ann Thorac Surg 1994;58:1657-1663.
20. Date H, Matsumura A, Manchester JK, Cooper JM, Lowry OH, Cooper JD. Changes in alveolar oxygen and carbon dioxide concentration and oxygen consumption during lung preservation. The maintenance of aerobic metabolism during lung preservation. J Thorac Cardiovasc Surg 1993;105:492-501.
21. Warnecke G, Sommer SP, Gohrbandt B, et al. Warm or cold ischemia in animal models of lung ischemia-reperfusion injury: is there a difference? J Thorac Cardiovasc Surg 2004;52:174-179.
22.Decampos KN, Keshavjee S, Liu M, Slutsky AS. Optimal inflation volume for hypothermic preservation of rat lungs. J Heart Lung Transplant 1998;17:599-607.

11

Open lung ventilation reduces ischemia-reperfusion injury of the lung

Based on
NP van der Kaaij, RA Lachmann, MA den Bakker, B Lachmann, RWF de Bruin, J Kluin, AJJC Bogers
Submitted

Summary

Background. Lung ischemia-reperfusion injury (LIRI) contributes to development of primary graft dysfunction (PGD) after lung transplantation. This study compares the effect of open lung concept ventilation (OLV) with ventilation according to the ARDS network (conventional ventilation (CV)) and direct extubation (DE) in a rat LIRI model. Methods. Male Sprague-Dawley rats (n=72) underwent 150 minutes warm ischemia of the left lung. At reperfusion, lungs were recruited and animals received CV (tidal volume 6 ml/kg, 5 cm H_2O positive end-expiratory pressure (PEEP)), OLV (collapsed alveoli were recruited, PEEP was set to keep the lung open, and tidal volume was kept as low as possible) for 3 hours or were directly extubated (DE). Measurements, consisting of arterial blood gas values, pulmonary compliance, histology and inflammatory cells in bronchoalveolar lavage and lung tissue, were performed 3, 24 and 72 hours after reperfusion. Results. No difference in PaO_2/FiO_2 ratio and lung compliance was found between CV and DE 24 hours after reperfusion. CV resulted in more severe inflammation with neutrophils and antigen presenting cells, but also more MHCII expression as compared to DE. After OLV, a normal PaO_2/FiO_2 ratio, less diffuse alveolar damage on HE slides, and an increased pulmonary compliance were found. Also, OLV resulted in less MHCII expression and less infiltration of neutrophils, antigen presenting cells, and lymphocytes in the BALf of the left lung as compared to CV and DE. Conclusions. CV results in additional lung injury. OLV, started directly at the start of reperfusion, decreases symptoms of PGD in our model. It may hereby prevent the development of PGD and decrease the risk on long-term organ dysfunction.

1 | Goals of the study

1) Although our rat LIRI model has been used extensively in earlier reports, we have consequently extubated our animals as soon as possible after the start of reperfusion to prevent possible interference of ventilator-induced-lung injury. Therefore, the first goal of this study was to further validate our experimental model regarding the use of postoperative ventilation in combination with severe LIRI at a single time point.
2) To test the hypothesis that ventilation according to the ARDS network, viewed as conventional ventilation (CV), results in ventilator-induced-lung injury. Therefore we compared CV with direct extubation (DE) after reperfusion.
3) To test the hypothesis that open lung concept ventilation (OLV), started directly after the start of reperfusion, is superior to CV in reduction of ventilator-induced-lung injury, but may also prevent PGD grade 3 development.

2 | Materials and methods in brief

For a full description of the materials and methods, see the materials and methods section at the end of this thesis.

2.1 Experimental design

Sprague-Dawley rats (Harlan, the Netherlands, weighing 307 ± 24 grams) were used in this study. Experiment 1 was conducted to further validate our LIRI model. Animals were either sham-operated (n=9) or subjected to 150 minutes of left lung warm ischemia (n=9), where after both groups were ventilated for 3 hours according to the CV protocol, as explained in the CV section. Measurements were performed 72 hours after surgery. Sham-operated animals underwent exactly the same operation protocol as LIRI animals, but without applying ischemia to the left lung.
The 2nd experiment was performed to assess whether CV results in ventilator-induced-lung injury on top of LIRI. LIRI was induced and animals were either directly extubated (DE, n=9) following reperfusion or ventilated according to the CV protocol for 3 hours (n=9). Measurements were performed 24 hours after reperfusion.
Finally, in the 3rd experiment, we assessed whether OLV reduces ventilator-induced-lung injury following LIRI as compared to CV. Animals were randomized into the LIRI OLV group (n=27) or the LIRI CV group (n=27) and were ventilated for 3 hours after reperfusion. Three time points were studied: 3, 24 and 72 hours (n=9 per time point) following reperfusion.

2.2 Open lung concept positive pressure ventilation

The ventilation pressures required for OLV in our model were assessed in a pilot study by using the following protocol: The animals were pressure-control ventilated and LIRI was induced. At the start of reperfusion, the FiO_2 was set to 1.00 and a recruitment manoeuvre was performed by a stepwise increase in PIP (2 cm H_2O per step) and PEEP until PaO_2 reached 450 mm Hg, assessed by an arterial blood gas sample taken from the carotid artery. The PIP at this point was defined as the opening pressure. Then, PIP and PEEP were stepwise decreased until the PaO_2 fell again below 450 mm Hg, indicating alveolar collapse. The

PEEP at this level was defined as the closing pressure. After a second recruitment manoeuvre, PEEP was set 2 cm H_2O above the closing pressure. Finally the pressure amplitude was minimized to prevent alveolar over-distension, as confirmed by plethysmography. The ventilation frequency was adjusted to keep $PaCO_2$ within normal range. After 1, 2 and 2.5 hours, optimal ventilator settings were again determined by the same protocol. The ventilation pressures needed to keep the lung open decreased over time, so that ventilator settings were adjusted accordingly. Table 1 summarizes the ventilation pressures required to recruit the alveoli and to keep the lung open during the 3-hour ventilation period. To control the pressures found in the pilot study, PaO_2 was also measured in the 3-hour reperfusion OLV and CV groups (at 15, 60, 120 and 180 minutes after reperfusion). Animals with air leak were excluded.

2.3 Conventional positive pressure ventilation (CV)

The CV protocol is a pressure controlled BiPAP ventilation protocol. After removal of the clamp, the lung was recruited exactly in the same manner as in the OLV group. Hereafter animals were ventilated with a PEEP of 5 cm H_2O and a tidal volume of 6 ml/kg, which resulted in a PIP of 12 cm H_2O, as assessed in a pilot study by plethysmography. The ventilation frequency was adjusted to keep $PaCO_2$ within normal range. Table 1 summarizes the ventilation pressures in the CV group. Animals with air leak were excluded.

2.4 Plethysmography

Since an EVITA XL ventilator is not capable of measuring tidal volumes in small animals, tidal volumes were assessed in a pilot study by plethysmography (n=5 per protocol). Briefly, after induction of LIRI, the lung was recruited and the rats were ventilated in a bodybox with both ventilator protocols to determine the tidal volume (Table 2).

2.5 Measurements

Measurements include survival, blood gas values, static pulmonary compliance, flowcytometric analysis of infiltrating cells (BALf and lung), histological analysis (in 4 additional animals per group) and plethysmography. The static pulmonary compliance is presented as Vmax and Cmax. Vmax is the maximal lung volume at a pressure of 35 cm H_2O, while Cmax is the maximal compliance of the expiration curve.

2.6 Statistical analysis

The results in text, tables and figures are presented as mean ± standard error of the mean (SEM), unless otherwise specified. Data were analysed using SPSS version 11.1 statistical software (SPSS Inc., Chicago, Illinois, USA). If an overall difference between groups was found by the Kruskal-Wallis test, Mann-Whitney U tests were performed for intergroup comparison. P values <0.05 were considered to be significant.

3 | Results

3.1 Validation of the model

3.1.1 Ventilation settings

Both the sham-operated group and the LIRI group were ventilated for 3 hours according to the CV protocol with a tidal volume of approximately 6 ml/kg, as assessed by plethysmography (Table 1, Table 2). This resulted in a PIP of 12 and a PEEP of 5 cm H_2O in both groups. Ventilation frequency was adjusted to keep the $PaCO_2$ between 35 and 50 mm Hg, resulting in ventilation with 40 breaths/minute in sham-operated controls and 80 breaths/minute in LIRI animals with a FiO_2 of 1.00 in both groups.

3.1.2 Lung physiology

LIRI resulted in a decrease in PaO_2/FiO_2 ratio, and a lower Vmax and Cmax 72 hours after reperfusion, as compared to sham-operated controls (Figure 1A-E).

3.1.3 Inflammation

Three days after reperfusion, more neutrophils, helper T-lymphocytes ($CD3^+CD4^+$), cytotoxic T-lymphocytes ($CD3^+CD8^+$), macrophages ($CD68^+$) and $MHCII^+$-antigen presenting cells (APC) were found in left and right BALf and left lung tissue of LIRI animals as compared to sham-operated controls. (Figure 1F-O). Also, the MIP-2 concentration was elevated in left BALf after LIRI, while no differences were found in IL-6 and TNF-alpha (Figure 1P-R).

Table 1. Ventilator settings of the CV and OLV protocols.

Group	**PIP** (cm H_2O)	**PEEP** (cm H_2O)	**Freq** (bpm)	**FiO_2**	**Vt** (ml/kg)
CV Sham	12	5	40	1.00	6.0
CV LIRI	12	5	80	1.00	6.0
OLV 0-59 min	35	18	90	1.00	4.2
OLV 60-149 min	30	15	90	1.00	4.2
OLV 150-180 min	17	9	100	1.00	4.5

Ventilator settings of the CV and OLV protocols. Ventilation of the CV group did not change during the 3-hour ventilation interval. The pressures needed in the OLV group to keep the lung open changed during the ventilation interval, as assessed by plethysmography in a pilot study. The ventilation frequency was adjusted to keep $PaCO_2$ within normal range. CV: Conventional Ventilation; FiO_2: Fraction of inspired Oxygen; LIRI: Lung Ischemia Reperfusion Injury; Min: Minutes; OLV: Open Lung concept Ventilation; PIP: Peak Inspiratory Pressure; PEEP: Positive End Expiratory Pressure; Vt: Tidal volume (ml/kg)

3.1.4. Histology

While sham-operation did not cause any histological abnormalities, LIRI resulted in diffuse alveolar damage, consisting of intra-alveolar- and septal edema, intra-alveolar hemorrhage, inflammation, and fibrosis (Table 3, Figure 2). Inflammation was classified as histiocytic in

Table 2. Bodybox measurements of lung ischemia-reperfusion injury animals.

Group	**PIP** (cm H_2O)	**PEEP** (cm H_2O)	**Tidal volume** (ml/kg)
CV	12	5	6.09 (0.24)
CV	14	5	8.03 (0.75)
OLV	35	18	4.15 (0.94)
OLV	30	15	4.20 (0.83)
OLV	17	9	4.51 (0.69)

Mean (SEM) tidal volumes were assessed in a pilot study by plethysmography (n=5 per protocol). Briefly, LIRI was induced, whereafter the lung was recruited and the rats were ventilated in a bodybox with both ventilator protocols to determine the tidal volume. CV: Conventional ventilation; OLV: Open lung concept ventilation; PIP: Peak inspiratory pressure; PEEP: Positive End Expiratory Pressure; Vt:Tidal volume (ml/kg).

all 3 LIRI animals. The pulmonary severity score of respectively the LIRI and sham-operated group was 7,0 ± 0.8 versus 0.0 ± 0.

3.2 Direct extubation versus conventional ventilation

3.2.1 Ventilation settings

Animals were either directly extubated after reperfusion or ventilated for 3 hours according to the CV protocol, implicating a tidal volume of 6 ml/kg (Table 1, Table 2). Therefore, PIP was set to a level of 12 cm H_2O with a PEEP set at 5 cm H_2O. A ventilation frequency of 80 breaths/minute was required to keep $PaCO_2$ within normal range.

3.2.2 Lung physiology

DE animals had a higher PaO_2/FiO_2 ratio than animals in the CV group. Pulmonary compliance of both groups was not significantly different (Figure 3A-E).

3.2.3 Inflammation

Ventilation for 3 hours after the start of reperfusion resulted in additional infiltration of neutrophils and increased numbers of $CD68^+$ cells in both left and right BALf (Figure 3H & 3I) To investigate the possible negative effects of OLV on right lung inflammation, we also measured cellular differentiation in BALf of the right lung. One day after reperfusion, and left lung tissue (Figure 3M & 3N). Also, more $MHCII^+$-APC was found in left BALf and left lung tissue after CV as compared to the DE group (Figure 3J & 3O). Although no differences between the DE and the CV group were found in $CD3^+CD4^+$ cells (Figure 3F & 3K), more $CD3^+CD8^+$ cells were measured in left BALf of the CV group 24 hours after reperfusion (Figure 3G). Ventilation according to the CV protocol resulted in increased concentration of IL-6 and TNF-alpha in the BALf of the left lung, although MIP-2 was not significantly elevated (Figure 3P-R)

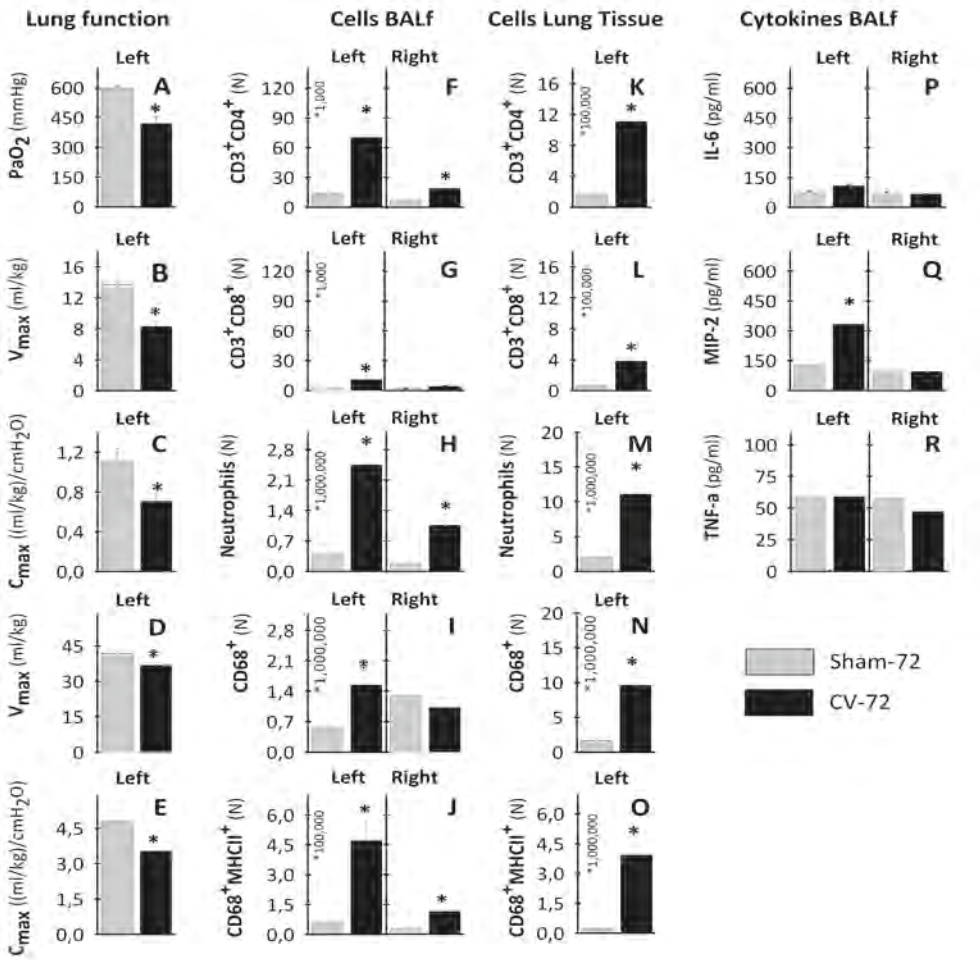

Figure 1. Validation of the model. Animals were either sham-operated or subjected to 150 minutes left lung warm ischemia, whereafter both groups were ventilated for 3 hours according to the conventional ventilation (CV) protocol. Ischemia followed by conventional ventilation resulted in a decrease in PaO_2/FiO_2 ratio (A), a lower Vmax and Cmax of left lung only (B and C) and both lungs together (D and E), more helper T-lymphocytes ($CD3^+CD4^+$) in BALf (F) and lung tissue (K), more cytotoxic T-lymphocytes ($CD3^+CD8^{+)}$ in BALf (G) and lung tissue (L), more neutrophils ($HIS48^+$) in BALf (H) and lung tissue (M), more antigen presenting cells ($CD68^{+)}$ in BALf (I) and lung tissue (N), and more $MHCII^+$-antigen presenting cells ($CD68^+$ $MHCII^+$) in BALf (J) and lung tissue (O). Also, the MIP-2 concentration was elevated in BALf of the left lung (Q), while no difference was found in IL-6 and TNF-alpha (P and R). All data is presented as mean ± standard error of the mean. BALf: BronchoAlveolar Lavage Fluid; Cmax: Maximal compliance of the expiration curve, corrected for body weight; N: Number of cells; Vmax: Maximal lung volume corrected for body weight at a pressure of 35 cm H_2O.
*: $P<0.05$ versus sham operated controls

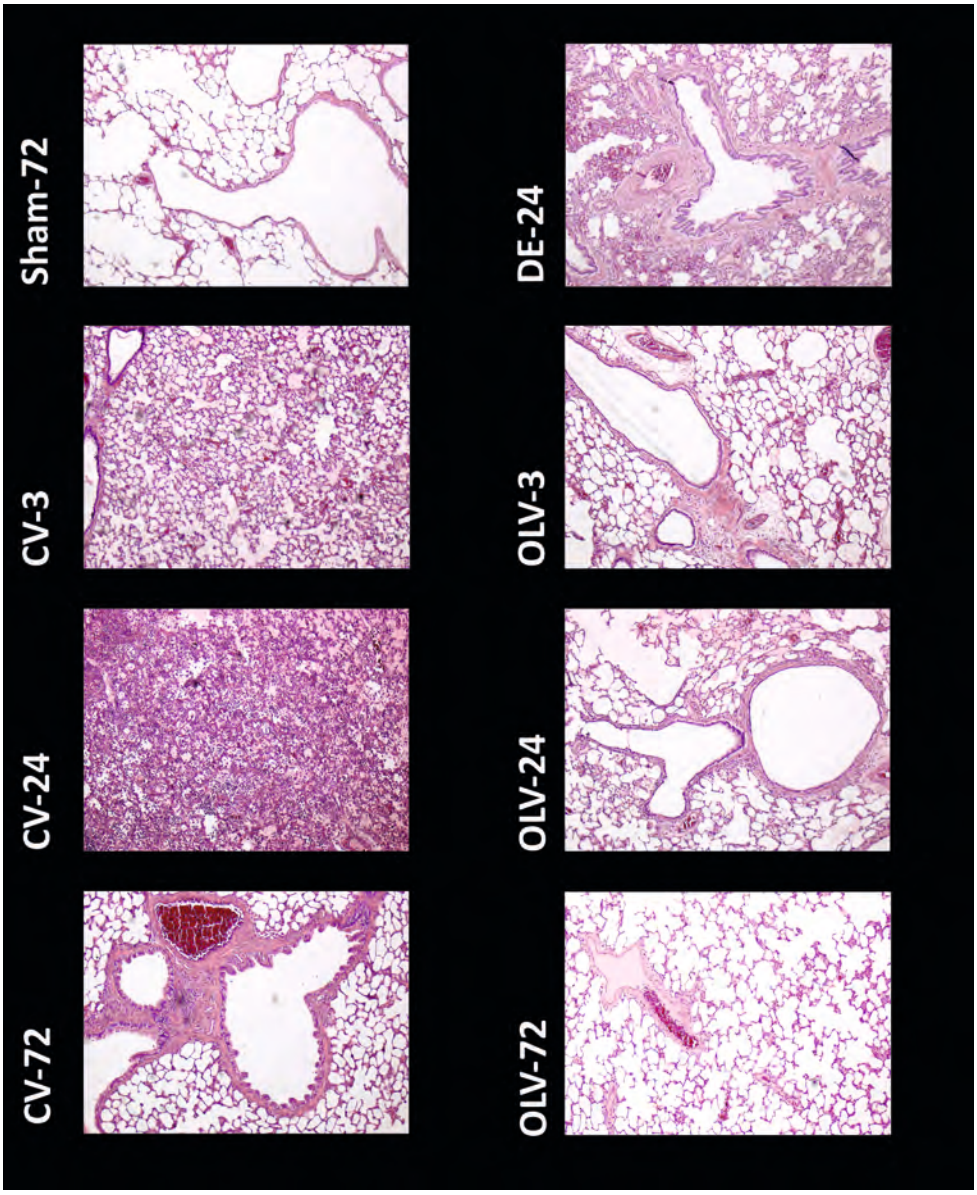

Figure 2. Representative histological samples (HE 50x) of sham-operated controls (72 hours after reperfusion), directly extubated (DE) controls (24 hours after reperfusion), conventional ventilated (CV) controls (3, 24 and 72 hours after reperfusion) and open lung concept ventilated (OLV) animals (3, 24 and 72 hours after reperfusion). HE: Hematoxylin and Eosin staining.

Table 3A. Histologic score of the left lung.

Group	IAE				SE				IAH				FIB				ATL				CON			
	0	**1**	**2**	**3**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**
Sham	3				3				3				3				3							
CV 3		1	2		2	1			1	1	1		3				3				1	1	1	
OLV 3		3			3				2	1			3				3				1	2		
DE 24			1	2	1	2			2		1		3				3				1		2	
CV 24			1	2		3				2	1		3				3						3	
OLV24			2	1	3					3			3				3					2	1	
CV 72	1	2				3			1	2				1	2		3					3		
OLV 72	3					3			3					1	2			3				3		

0 None
1 Mild
2 Moderate
3 Severe

Hematoxylin-eosin sections of 3 animals per group were scored for intra-alveolar edema (IAE), septal edema (SE), intra-alveolar hemorrhage (IAH), fibrosis (FIB), atelectasis (ATL), and congestion (CON). Presented is the number of animals within each group with a particular parameter score. Each parameter was scored as: (0) absent, (1) mild, (2) moderate, or (3) severe. CV: Conventional ventilation; DE: Direct extubation; OLV: Open lung concept ventilation; SD: Standard deviation.

Table 3B. Histologic score of the left lung.

Group	INF				INF CLS			PSS (SD)
	0	1	2	3	L	H	N	
Sham								0 (0)
CV 3		3			2	2	2	5,0 (2,2)
OLV 3	2	1			1	1		2,0 (0,5)
DE 24		3			3	3	1	6,3 (2,1)
CV 24			1	2		1	3	9,7 (1,3)
OLV 24		2	1		2	3	2	6,0 (1,3)
CV 72			3		1	3		7,0 (0,8)
OLV 72			3		3	3		6,0 (0,2)

Scoring		Inflammation	
0	None	L	Lymphocytic
1	Mild/scattered	H	Histiocytic
2	Moderate/occasional	N	Neutrophilic
3	Severe/frequent		

Hematoxylin-eosin sections of 3 animals per group were scored for inflammation (INF), and classification of inflammation (INF CLS). Presented is the number of animals within each group with a particular parameter score. Each parameter was scored as: (0) absent, (1) mild, (2) moderate/scattered, or (3) severe/frequent. Some groups may contain more than 3 scores, since some animals had mixed inflammatory patterns. The pulmonary severity score (PSS) is the sum of the individual scores of the eight categories, resulting in a possible score ranging from 0 for normal lungs to 24 for the most injured lungs. CV: Conventional Ventilation; DE: Direct Extubation; OLV: Open Lung concept Ventilation; SD: Standard Deviation.

and left lung tissue (Figure 3M & 3N). Also, more $MHCII^+$-APC was found in left BALf and left lung tissue after CV as compared to the DE group (Figure 3J & 3O). Although no differences between the DE and the CV group were found in $CD3^+CD4^+$ cells (Figure 3F & 3K), more $CD3^+CD8^+$ cells were measured in the left BALf of the CV group 24 hours after reperfusion (Figure 3G). Ventilation according to the CV protocol resulted in increased concentration of IL-6 and TNF-alpha in the BALf of the left lung, although MIP-2 was not significantly elevated (Figure 3P-R)

3.2.4 Histology

Twenty-four hours after reperfusion and CV, histological analysis revealed the following scores: Severe intra-alveolar edema, mild septal edema, mild intra-alveolar hemorrhages, no fibrosis, no atelectasis and severe, mainly neutrophilic, inflammation (Figure 2). Animals in the DE group showed less intra-alveolar hemorrhage and only mild, mainly lymphocytic and histiocytic, inflammation. The mean pulmonary severity score of the DE and CV group were respectively 6,33 ± 2,1 and 9.7 ± 1,3 (Table 3).

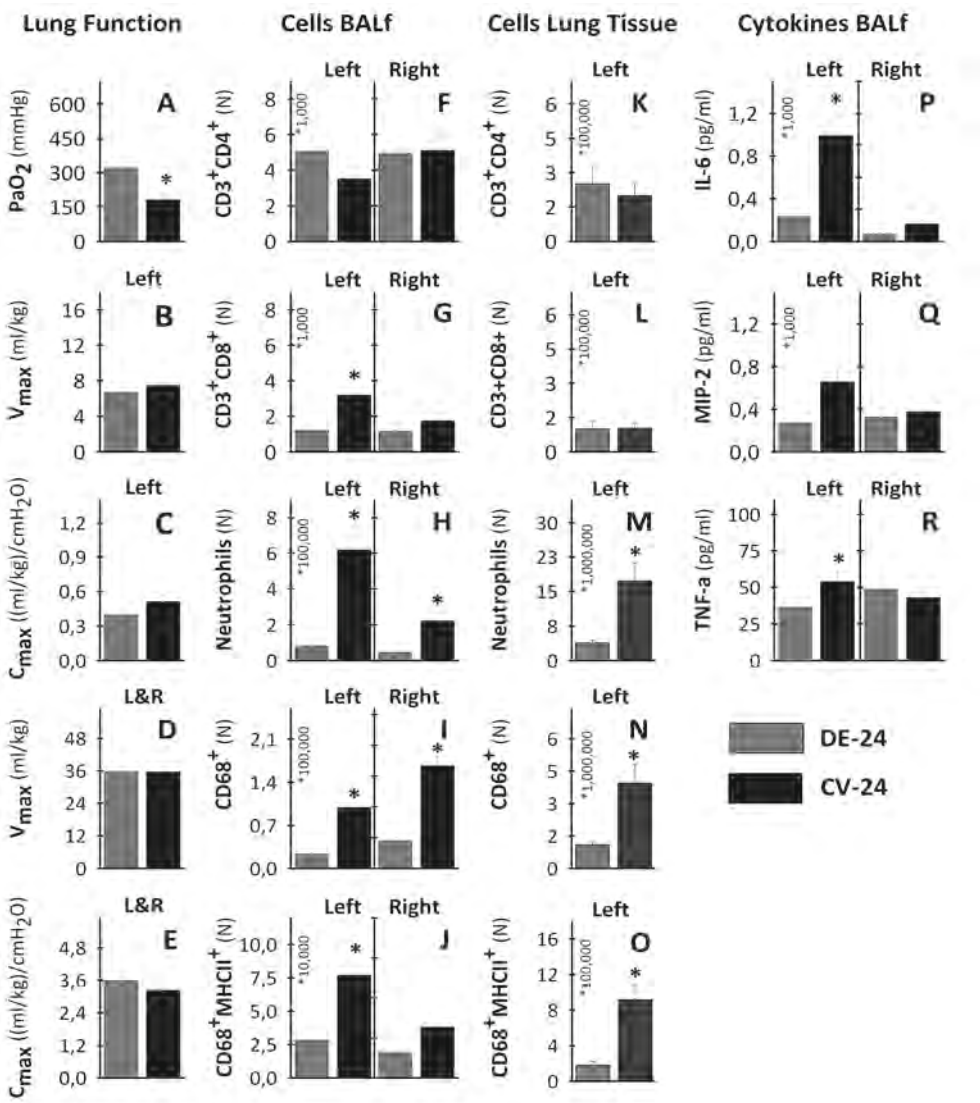

Figure 3. Conventional ventilation (CV) versus direct extubation (DE). Ischemia was induced and animals were either directly extubated following reperfusion or ventilated according to the conventional ventilation protocol for 3 hours. Ischemia followed by CV resulted in a decrease in PaO_2/FiO_2 ratio (A), but had no effect on Vmax and Cmax of left lung only (B and C) and both lungs together (D and E). CV caused additional infiltration of neutrophils ($HIS48^+$) in BALf (H) and left lung tissue (M), cytotoxic T-lymphocytes ($CD3^+CD8^+$) in BALf (G) but not in lung tissue (L), antigen presenting cells ($CD68^+$) in BALf (I) and lung tissue (N) and $MHCII^+$-antigen presenting cells ($CD68^+MHCII^+$) in BALf (J) and lung tissue (O). Also, the IL-6 (P) and TNF-alpha (R) concentration were elevated in BALf of the left lung. No differences were found in the number of helper T-lymphocytes ($CD3^+CD4^+$) in BALf (F) and lung tissue (K) and the concentration of MIP-2 (Q). All data is presented as mean ± standard error of the mean. BALf: BronchoAlveolar Lavage Fluid; Cmax: Maximal compliance of the expiration curve, corrected for body weight; N: Number of cells; Vmax: Maximal lung volume corrected for body weight at a pressure of 35 cm H_2O.
*: $P<0.05$ versus DE controls

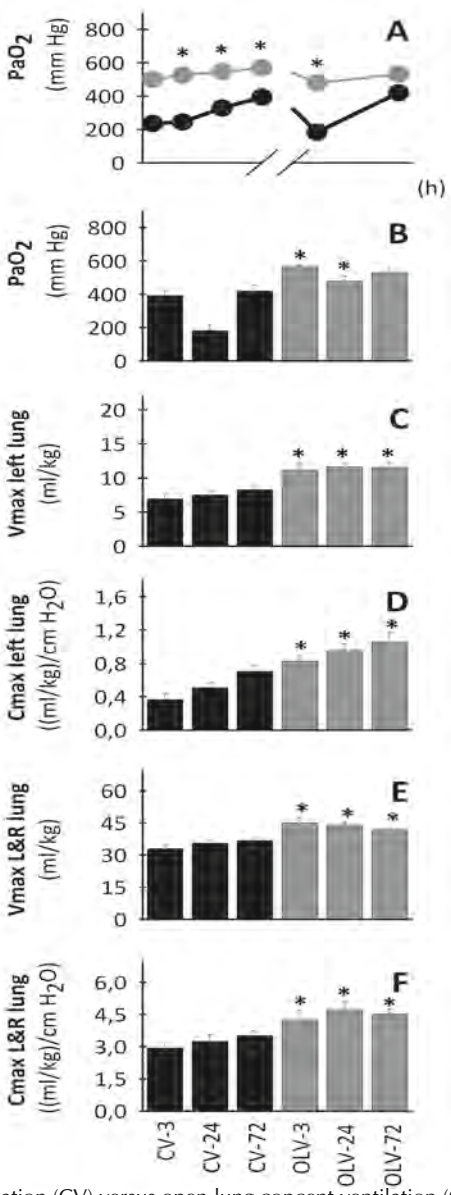

Figure 4. Conventional ventilation (CV) versus open lung concept ventilation (OLV). Following 150 minutes of ischemia, animals were ventilated for 3 hours according to the conventional ventilation (CV) protocol or open lung concept ventilation (OLV) protocol. PaO_2/FiO_2 ratio, which is a parameter for shunting and therefore a parameter for alveolar recruitment, was higher than 450 mm Hg in the OLV group during the ventilation period and significantly higher than in the CV group (A). One day after reperfusion, the PaO_2/FiO_2 ratio was still superior in the OLV group (A and B). Vmax and Cmax of the left lung only (C and D) and left and right lung together (E and F) were superior 3, 24, and 72 hours after reperfusion in the OLV group as compared to the CV group. All data is presented as mean ± standard error of the mean. Cmax: Maximal compliance of the expiration curve, corrected for body weight; Vmax: Maximal lung volume corrected for body weight at a pressure of 35 cm H_2O.
*: $P<0.05$ versus CV controls at corresponding time point

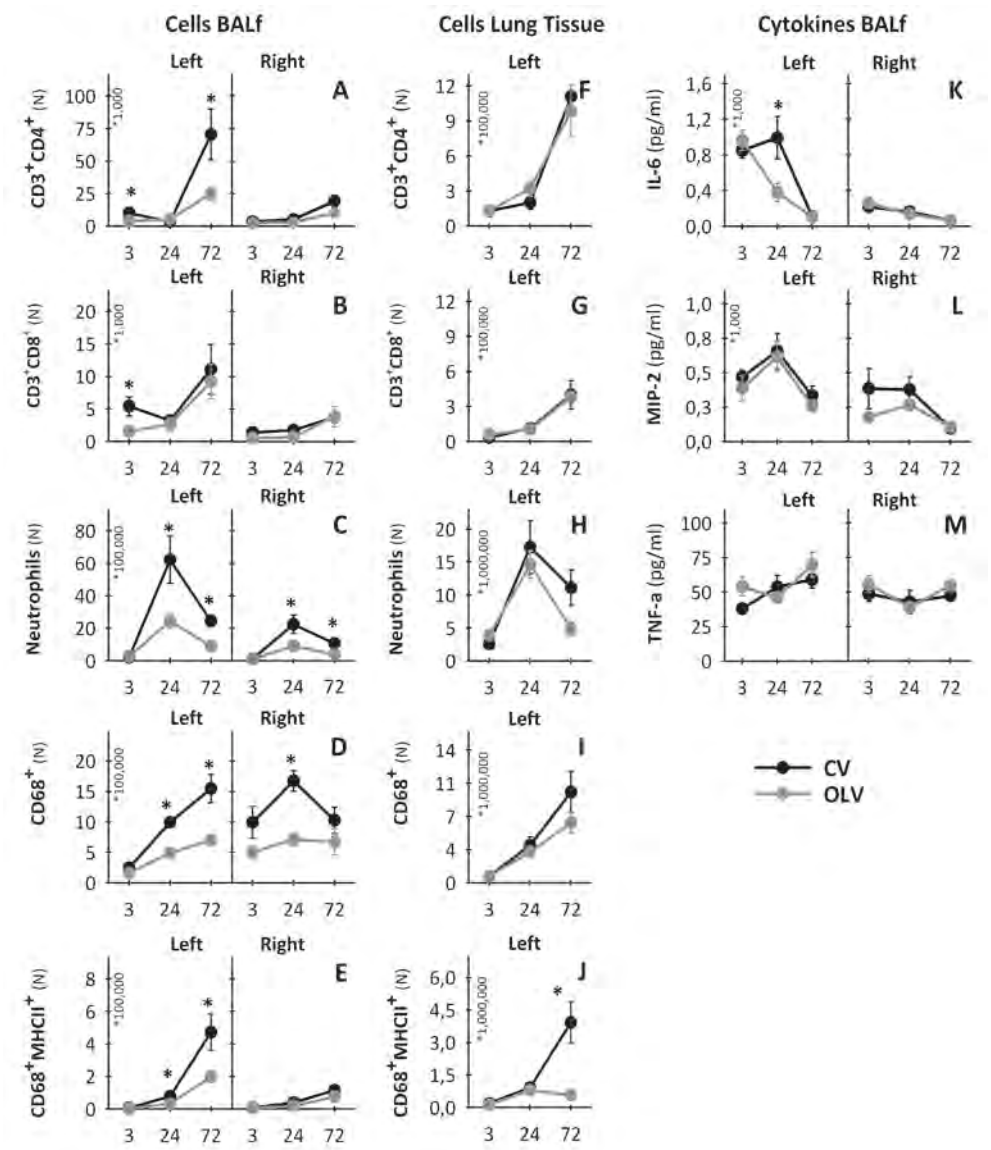

Figure 5. Conventional ventilation (CV) versus open lung concept ventilation (OLV). Following 150 minutes of ischemia, animals were ventilated for 3 hours according to the conventional ventilation (CV) protocol or open lung concept ventilation (OLV) protocol. OLV resulted in less influx of neutrophils (HIS48$^+$) in BALf (C), less helper T-lymphocytes ($CD3^+CD4^+$) in BALf 3 and 72 hours after reperfusion (A), less cytotoxic T-lymphocytes ($CD3^+CD8^+$) in BALf 3 hours after reperfusion (B), less antigen presenting cells ($CD68^+$) in BALf (D) and less $MHCII^+$-antigen presenting cells ($CD68^+MHCII^+$) in BALf (E) and lung tissue (J). In left lung parenchyma no differences in helper T-lymphocytes (F, $CD3^+CD4^+$), cytotoxic T-lymphocytes (G, $CD3^+CD8^+$), neutrophils (H, HIS48$^+$) and antigen presenting cells (I, $CD68^+$) were found. There are no differences in IL-6 (K), MIP-2 (L) and TNF-alpha (M), except for IL-6 in left BALf of the CV group 24 hours after reperfusion (K). All data is presented as mean ± standard error of the mean. BALf: Bronchoalveolar lavage fluid; N: Number of cells.

*: $P<0.05$ versus CV controls at corresponding time point

3.2.4 Histology

Twenty-four hours after reperfusion and CV, histological analysis revealed the following scores: Severe intra-alveolar edema, mild septal edema, mild intra-alveolar hemorrhages, no fibrosis, no atelectasis and severe, mainly neutrophilic, inflammation (Figure 2). Animals in the DE group showed less intra-alveolar hemorrhage and only mild, mainly lymphocytic and histiocytic, inflammation. The mean pulmonary severity score of the DE and CV group were respectively 6,33 ± 2,1 and 9.7 ± 1,3 (Table 3).

3.3 Open lung concept ventilation versus conventional ventilation

3.3.1 Ventilation settings

CV animals were ventilated for 3 hours with a PEEP of 5 cm H_20, a PIP of 12 cm H_2O and a frequency of 80 breaths/minute (Table 1, Table 2). The OLV group was ventilated in the first hour after reperfusion with a PIP of 35 cm H_2O, a PEEP of 18 cm H_2O and a frequency of 90 breaths/minute, which corresponds with a tidal volume of 4,15 ml/kg. Hereafter, pressures were reduced to 30 over 15 cm H_2O (4,2 ml/kg), followed by a further reduction to 17 over 9 cm H_2O (4,5 ml/kg) for the last 30 minutes of the 3-hour ventilation period.

3.3.2 Weight and lung physiology

No differences in weight change were found between both groups. PaO_2/FiO_2 ratio, which is a parameter for shunting and therefore a parameter for alveolar recruitment, was higher than 450 mm Hg in the OLV group during the ventilation period and significantly higher than in the CV group (Figure 4A). One day after reperfusion, PaO_2/FiO_2 ratio was still higher in the OLV group (Figure 4B). At 72 hours, PaO_2/FiO_2 ratio had increased so that no significant differences were found. Vmax and Cmax of the left lung only and left and right lung together were superior 3, 24, and 72 hours after reperfusion in the OLV group as compared to the CV group (Figure 4C-F).

3.3.3 Inflammation

In left BALf 24 and 72 hours after reperfusion OLV resulted in less influx of neutrophils, APC, and $MHCII^+$-APC (Figure 5C-E). Also, 3 hours after reperfusion, less $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes were measured in left BALf (Figure 5A & 5B). While no differences between groups were found in $CD3^+CD8^+$ lymphocytes on day 3, $CD3^+CD4^+$ cells were again elevated in the CV group (Figure 5A & 5B). In left lung parenchyma no differences in number of inflammatory cells were found between the CV and OLV group, except for $MHCII^+$-APC on day 3 (Figure 5F-J). To investigate the possible negative effects of OLV on right lung inflammation, we also measured cellular differentiation in BALf of the right lung. One day after reperfusion, more neutrophils, $CD68^+$-cells and MHC^+-APC were measured in the right alveolar compartment of the CV group as compared to the OLV group (Figure 5C-E). Differences hereafter disappeared. No differences in IL-6, MIP-2 and TNF-alpha were found between the groups, except for IL-6 in the left BALf of the CV group 24 hours after reperfusion (Figure 5K-M).

3.3.4 Histology

OLV prevented development of septal edema and reduced the severity of intra-alveolar edema, intra-alveolar hemorrhage and inflammation as compared to the CV group. Inflammation was mainly classified in the OLV group as mixed (lymphocytic, histiocytic and neutrophilic), which is comparable to the CV group. No differences between the groups were found in fibrosis, atelectasis and congestion. The pulmonary severity scores of the OLV group were lower 3 and 24 hours after reperfusion, while no difference was found 72 hours after reperfusion.

4 | Discussion

Development of PGD stage 3 occurs in 15-30% of lung transplant recipients and is the main cause for early morbidity and mortality after lung transplantation, resulting in a one-year survival rate of approximately 85% [1, 2]. LIRI is a major risk factor for PGD, although other factors like donor brain death, mechanical ventilation, pneumonia, hypotension, aspiration, donor trauma and allo-immunity have been found to interplay with LIRI [1-3]. Symptoms of PGD usually develop within 72 hours after reperfusion and consist of hypoxemia, which cannot be corrected by supplemental oxygen, non-cardiogenic pulmonary edema, increased pulmonary artery pressure, decreased lung compliance and diffuse alveolar damage on histological slides [1-3]. We have previously demonstrated that 150 minutes of warm ischemia in our model results in symptoms comparable to PGD symptoms [3]. Ventilator-induced-lung injury is a complication of mechanical ventilation and can be characterized by damage to the alveolar lining cells and both local and systemic inflammation [3-6]. It also results in a loss of active surfactant components and increased conversion of surface-active large surfactant aggregates into non-surface active small surfactant aggregates [5]. As a consequence transmural filtration pressure is increased and the permeability of the alveolo-capillary barrier is increased resulting in pulmonary edema [3,5]. The use of small tidal volume ventilation (4-6 ml/kg) is propagated to decrease the risk of ventilator-induced-lung injury [7]. In the present model, CV with a tidal volume of 6 ml/kg did not improve lung function or histological score as compared to direct extubation. In contrast, CV resulted in an increased pulmonary severity score and it induced more severe inflammation with neutrophils and APC but also more MHCII expression on APC. Especially the increased expression of MHCII on APC is important. Since we use an autologous experimental model, upregulation of MHCII may be explained by the release of self-antigens, like myosin, heat shock proteins and type V collagen [8, 9]. Since involvement of T-cells and $MHCII^+$-APC may be the link between LIRI and BOS, an increase in MHCII expression must be seen as unwanted for the long-term outcome after transplantation. Thus, CV does result in ventilator-induced-lung injury, so that an alternative approach is needed.

OLV is such an alternative approach. In the present study, OLV was started directly after reperfusion and recruitment leading to a PaO_2/FiO_2 ratio that was continuously higher than 450 mm Hg, proving that minimal atelectasis and shunting was present. OLV resulted in improved oxygenation, better pulmonary compliance and a dampened inflammatory response 3, 24 and 72 hours after reperfusion. Importantly, both the number of helper T-lymphocytes and MHCII upregulation were reduced. Still, although OLV reduced lung

injury, pulmonary compliance and pulmonary severity score had not normalized to values found in sham-operated controls.
The goal of OLV is to open up the lung and to keep it open with as low as possible ventilation pressures [10, 11]. In the clinical setting development of PGD often occurs shortly after the start of reperfusion. During the ischemic period several parts of the lungs become atelectatic and surfactant is degraded. After removal of the clamp pulmonary edema may develop fast, resulting in alveolar flooding, hypoxemia and problems to adequately ventilate the lungs. Therefore, it is desirable to perform a recruitment manoeuvre to open up the lung as soon as possible after the start of reperfusion, followed by adjustment of ventilation pressures so that the alveolus is stabilized. Permissive atelectasis should be prevented, even if this results in the use of high ventilation pressures. In this regard, the use of high PEEP levels in ARDS patients is controversial [12-14]. Although high PEEP improves survival in ARDS patients, it is detrimental in patients with acute lung injury [12]. By definition, acute lung injury patients have a less severe form of lung injury than ARDS patients, so that high PEEP may result in alveolar overdistension and thus ventilator-induced-lung injury. Since OLV is an individual approach, high ventilation pressures will only be needed in the most affected patients and low pressures can be used in those who are less affected. Since inflammatory parameters after OLV were reduced as compared to CV, overdistension of the alveolus is not likely, although much higher pressures were used in the OLV group.
In the case of single lung transplantation, OLV may cause overdistension of the native lung. Therefore, we also performed measurements in the right, non-ischemic, lung. OLV did not result in increased inflammatory cells in the right BALf, the pulmonary severity score of the right lung was close to zero and no inflammation was seen on HE slides. Therefore, overdistension of the right lung is not likely in our experiment.
As with all experimental models, our model has some disadvantages. First of all, it is a warm ischemia model without the use of vascular (cold) preservation. Also, we used a FiO_2 of 1.00 in both ventilator protocols. The ARDS network recommends ventilation with 100% oxygen only in combination with high PEEP levels [7]. Since the percentage of oxygen influences the severity of LIRI, we decided to ventilate both experimental groups with the same FiO_2. Nevertheless, only 3-hours of ventilation according to the open lung concept is capable of reducing LIRI. It can be expected that a longer interval of OLV can further reduce injury. The pulmonary severity score in the 3-hour OLV was very low, while it had increased 24 hours after reperfusion. After extubation, some atelectasis may have occurred leading to alveolar shunting and shear stress. A longer interval of OLV preserves alveolar stability and could thus minimize pulmonary injury.

5 | Conclusions

Conventional ventilation results in ventilator induced lung injury. Open lung ventilation, started directly at the start of reperfusion, decreases symptoms of LIRI in our experimental model. OLV may be an important concept to prevent the development or dampen the severity of PGD and decrease the risk on long-term pulmonary dysfunction after lung transplantation.

6 | References

1. de Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia-reperfusion-induced lung injury. Am J Respir Crit Care Med 2003;167:490-511.
2. Lee JC, Christie JD. Primary graft dysfunction. Proc Am Thorac Soc 2009;6:39-46.
3. van der Kaaij NP, Kluin J, Haitsma JJ, et al. Ischemia of the lung causes extensive long-term pulmonary injury: an experimental study. Respir Res 2008;9:28.
4. Fiser SM, Tribble CG, Long SM, et al. Ischemia-reperfusion injury after lung transplantation increases risk of late bronchiolitis obliterans syndrome. Ann Thorac Surg 2002;73:1041-1047; discussion 1047-1048.
5. de Perrot M, Imai Y, Volgyesi GA, et al. Effect of ventilator-induced lung injury on the development of reperfusion injury in a rat lung transplant model. J Thorac Cardiovasc Surg 2002;124:1137-1144.
6. Halter JM, Steinberg JM, Gatto LA, et al. Effect of positive end-expiratory pressure and tidal volume on lung injury induced by alveolar instability. Crit Care 2007;11:R20.
7. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network. N Engl J Med 2000;342:1301-1308.
8. Lachmann B. Open up the lung and keep the lung open. Intensive Care Med 1992;18:319-321.
9. Spieth PM, Güldner A, Carvalho AR, et al. Open lung approach vs acute respiratory distress syndrome network ventilation in experimental acute lung injury. Br J Anaesth 2011;107:388-397.
10. Spieth PM, Carvalho AR, Pelosi P, et al. Variable tidal volumes improve lung protective ventilation strategies in experimental lung injury. Am J Respir Crit Care Med 2009;179:684-693.
11. Verbrugge SJ, Lachmann B, Kesecioglu J. Lung protective ventilatory strategies in acute lung injury and acute respiratory distress syndrome: From experimental findings to clinical application. Clin Physiol Funct Imaging 2007;27:67-90.
12. Dreyfuss D, Saumon G. Ventilator-induced lung injury: Lessons from experimental studies. Am J Respir Crit Care Med 1998;157:294-323.
13. Hudson LD. Progress in understanding ventilator-induced lung injury. Jama 1999;282:77-78.
14. Waddell TK, Gorczynski RM, DeCampos KN, Patterson GA, Slutsky AS. Major histocompatibility complex expression and lung ischemia-reperfusion in rats. Ann Thorac Surg 1996;62:866-872.
15. Hartog A, Vazquez de Anda GF, Gommers D, Kaisers U, Lachmann B. At surfactant deficiency, application of “the open lung concept” prevents protein leakage and attenuates changes in lung mechanics. Crit Care Med 2000;28:1450-1454.
16. Briel M, Meade M, Mercat A, et al. Higher vs lower positive end-expiratory pressure in patients with acute lung injury and acute respiratory distress syndrome: systematic review and meta-analysis. JAMA 2010;303:865-873.
17. Mercat A, Richard JCM, Vielle B, et al. Positive end-expiratory pressure setting in adults with acute lung injury and acute respiratory distress syndrome: A randomized controlled trial. JAMA 2008;299:646-655.
18. Brower RG, Lanken PN, MacIntyre N, et al. Higher versus lower positive end-expiratory pressures in patients with the acute respiratory distress syndrome. N Engl J Med 2004;351:327-336.

12

General discussion

Primary graft dysfunction

At the start of this thesis, the term primary graft dysfunction (PGD) did not exist in its current form. Several groups had described a syndrome after lung transplantation with comparable symptoms, consisting of severe pulmonary edema, hypoxemia and low pulmonary compliance, which started shortly after reperfusion [1-4]. Different terms were used to describe this syndrome, like the pulmonary reimplantation response, donor lung dysfunction, primary acute graft failure, ischemia-reperfusion injury, and non-immune acute graft injury [1-5]. Although several attempts were made to identify risk factors for PGD, this was complicated by different definitions and a small number of patients included in these studies [1-4]. In 2005 a PGD working group of the International Society of Heart and Lung Transplantation (ISHLT) published consensus documents on the definition of PGD, donor related risk factors, recipient related risk factors and treatment options [6-11]. Since then, much work has been done to further characterize PGD.

The first and most important contemplation in lung transplantation and PGD is that you do not recognize PGD if you are unaware of its existence and its symptoms. Since the mortality and morbidity of PGD is high and since PGD increases the risk of bronchiolitis obliterans syndrome (BOS), PGD should be prevented as far as possible or treated as soon as possible after the first symptoms. In the present thesis we focused on minimizing the effect of lung ischemia-reperfusion injury and thereby PGD by treatment before the induction of ischemia and after onset of reperfusion. In the clinical setting, minimizing the risk of PGD already starts at the moment the lung is allocated. Medical staff responsible for allocation of the lung, harvesting, implantation and postoperative care should be aware of the risk factors and treatment options of PGD. In this general discussion we will review the factors that may contribute to pulmonary PGD. Furthermore, we give some recommendations that can help to reduce the incidence of PGD, extrapolated from our experimental findings and/or based on the available literature.

1 | Recipient risk factors for primary graft dysfunction

1.1 Underlying disease

COPD patients have the lowest risk of PGD with an incidence of PGD between 3 and 11% [12-14]. Both a recipient diagnosis of primary pulmonary hypertension (PPH) and sarcoidosis is a clear risk factor for PGD [12-20]. However the effect of secondary pulmonary hypertension due to end stage lung disease or cardiac failure is more controversial. Christie et al noted a less strong association between pulmonary artery pressure and PGD after statistical adjustment for the diagnosis of PPH, suggesting that it is the disease state rather than the elevated pulmonary artery pressure [13]. On the opposite, for every 10 mm Hg increase in systolic pulmonary pressure, an increase in odds ratio between 1.3 and 1.6 for PGD development has been found [14]. Others confirmed that secondary pulmonary hypertension with a systolic pressure above 50-60 mm Hg is a risk factor for PGD [15, 17, 21, 22]. This is because of the presence of recipient ventricular hypertrophy causing increased shear stress on the pulmonary endothelium after the transplantation when the afterload of the right ventricle is reduced. Also hepatic failure, enhanced coagulation and impaired fibrinolysis, which are more often present in patients with pulmonary hypertension, have been suggested to be part of the mechanism by which pulmonary hypertension leads to PGD [23, 24]. Recently idiopathic pulmonary fibrosis has been identified as an intermediate risk for PGD [12], although this was not found earlier [13, 15-18, 22].

1.2 Type of transplantation

While some studies did find a relation between single lung transplantation (SLT) and PGD [14, 15, 25], there are numerous studies not describing an effect. The recent meta-analysis by Liu, that included 4554 bilateral lung transplantation (BLT) patients and 5190 SLT patients in eleven studies, failed to show an association between PGD and type of transplantation [12]. Thus, there is insufficient evidence that the type of transplantation has an effect on PGD, although it will continue to be subject of debate. Importantly, patients with BLT have a significantly superior long-term survival than SLT patients [26].

1.3 Age, race and gender

There is no significant age difference between recipients with PGD and those without PGD [12-16, 18, 21, 22, 27]. Afro-American race may be a risk factor for PGD with a reported incidence of PGD in 19% of Afro-American recipients and an odds ratio of 1.82 [12], although this was not reported by others [15 14]. Recipient gender was not identified as a clear risk factor for PGD in 2005 [7]. In the following years, only one study found an increased risk of PGD in female recipients [15]. A recent meta-analysis included 12 studies reporting on the influence of recipient gender on PGD development confirmed that female gender is indeed a risk factor for PGD with an odds ratio of 1.38 [12].

1.4 Body mass index

In the consensus document of 2005, body mass index was not identified as a risk factor for PGD [7]. Recent reports showed increasing risk of PGD with increasing BMI starting from

25 [14, 15]. Patients with the highest risk are patients with a BMI above 30 kg/m^2 [12, 14, 15]. The risk of PGD in these patients is probably increased by the higher probability of technical difficulties during implantation and thus more surgical manipulation of the lung. Also, elevated adipokines levels, such as leptin, have been found in obese patients undergoing surgery [28-30]. Adipokines have a pro-inflammatory effect and can thereby contribute to PGD [28-30].

1.5 Pre-transplant mechanical ventilation

Historically, patients awaiting lung transplantation who became ventilator dependent were removed from the waiting list. At present, pre-operative ventilation is not an absolute contraindication anymore. In CF patients, prolonged pre-operative mechanical ventilation is a relative contra-indication for lung transplantation since 1-year mortality rates between 30 and 42% after lung transplantation have been described, mainly due to sepsis [31]. Also, a 56% incidence of graft failure has been reported in a paediatric group with CF as the indication for transplantation [32]. A registry study in 2005 confirmed that preoperative mechanical ventilation decreases 1-year survival of lung transplant patients, irrespective of the recipient pulmonary disease, but no influence on PGD development per se was described [33]. In the consensus report of the ISHLT in 2005, preoperative mechanical ventilation was not regarded as risk factor for PGD. Importantly, this conclusion was based on studies up to 2001; a time in which no agreement on the definition of PGD existed [7, 34-36]. A more recent report confirmed that preoperative mechanical ventilation had no influence on the chance of PGD after adult lung transplantation, although these patients required longer post-operative mechanical ventilation [37]. It must be noted that the percentage of CF patients in this study was very small [37]. Thus, there is insufficient evidence that preoperative mechanical ventilation and duration of preoperative ventilation are risk factors for PGD. Still, since preoperative mechanical ventilation in CF patients could have unwanted effects and since 1-year mortality after adult lung transplantation is increased, pre-operative mechanical ventilation may in some cases still be regarded as a contra-indication for lung transplantation [33].

1.6 Pre-transplant extra corporeal life support

Pre transplant extra corporeal life support (ECLS) was seen as a contra-indication for lung transplantation because of the poor outcomes. The 1-year mortality rate of patients on ECLS who receive a lung transplant is between 33-50% as compared to 17-21% in patients who do not need ECLS preoperatively [38, 39]. The major impediment to survival is development of PGD in these patients. Toyoda confirmed that 54% of the preoperative ECLS patients required postoperative ECLS because of PGD (6% in the control group), with no significant difference between venovenous and venoarterial procedures [40]. ECLS contributes to PGD since it induces a systemic inflammatory status and since it stimulates coagulation disorders requiring more blood transfusions. It is clear that the shorter the preoperative ECLS interval, the better the outcome. Remarkably, Toyoda found no effect of pre-transplant ECLS on short and long-term mortality, even though half of the patients developed PGD grade 3 [40]. A shorter waiting time for transplantation in the LAS era and adequate ECLS treatment after the development of PGD may have contributed to this improved outcome. Other

studies confirmed that patients, who survived the first year, had similar long-term survival as compared to patients who did not need ECLS [39]. Thus, pre-operative ECLS is an important risk factor for PGD, although ECLS before transplantation should not be seen as an absolute contra-indication for transplantation. In these cases, PGD must be expected and treated accordingly.

1.7 Factors not related to development of PGD

Left ventricular dysfunction, renal impairment, previous thoracic surgery, and the presence of cytomegalovirus have not been identified as PGD risk factor.

2 | Donor risk factors and donor management

The consensus document on donor related risk factors of PGD, published in 2005, included donor age, race, smoking history, gender and underlying lung disease [9]. In recent years, other risk factors were identified that could also contribute to PGD, like the type of donation procedure and the increasing use of marginal donors.

2.1 Donor risk factors

2.1.1 Donor age and race

As was already suggested in 2005, several studies have confirmed that donor age beyond 45 years or an age under 21 years is an independent risk factor for PGD [9, 13, 15, 22, 41]. It is somewhat surprising that if lungs are taken from donors younger than 21 years old, this results in a higher PGD incidence. However, recently it has been discovered that lungs are still developing and growing by neoalveolarization up to adolescence, so that lungs from these donors are more vulnerable as compared to adult lungs [42]. Less surprising is that the use of lungs taken from donors older than 45 years contributes to a higher incidence and severity grade of PGD, since deterioration in pulmonary function goes along with increasing age [9, 13,15, 22, 41].

Christie et al identified Afro-American donor race as a risk factor of PGD with an odds ratio of 5.6, although numerous other studies did not find a relation [13]. Therefore donor race should be regarded as irrelevant when a donor lung is accepted or rejected.

2.1.2 Donor smoking history

The perception has always been that a history of smoking is undesirable and non-smoking is best in donor selection. Although a donor smoking history of less than 20 pack-years may prevent PGD, a smoking history beyond 40 pack-years is an independent risk factor for PGD [13, 14, 16, 41]. Besides, donor smoking can have an additive chance on developing a lung malignancy or emphysema after the transplantation. Recipients of donor lungs with a positive smoking history have a lower 3–year survival, are longer on the ICU and in hospital and have a lower FEV1 than recipients from non-smoking donors. Nevertheless, since waiting list mortality is still around 30%, patients awaiting lung transplantation are likely to survive longer if lungs from donors that smoked are used [43]. However, one should be cautious using lungs from a donor with a smoking history of more than 20 pack years.

2.1.3 Donor gender

Although female donor gender has been identified as risk factor in some studies, the high numbered studies failed to show an effect of gender on PGD outcome. Therefore, donor gender should not be taken into account when accepting or rejecting a donor lung.

2.1.4 Donation after brain death and donation after cardiac death

Heart-beating donation (donation after brain death (DBD)) was the mainstream option up to 1995 for clinical transplantation [44]. However, in clinical practice, only 15-30% of the lungs of brain dead donors are suitable for transplantation, resulting in a significant shortage of organ donors and therefore high waiting list mortality [44, 45]. As a consequence, interest in non-heart-beating donation procedures (NHBD/donation after cardiac death (DCD)) started again in 1995. Most DCD procedures worldwide are performed in category III patients according to the Maastricht classification, which means that cardiac arrest has not yet occurred. In this procedure supportive therapy is stopped after the patient is accepted as a potential donor. If irreversible interruption of circulatory and respiratory function develops within one hour after the end of supportive therapy, the lungs may be used for transplantation. A maximum of 1-hour warm ischemia, characterized as the time from cardiac arrest until cold pulmonary flush, is accepted. In DCD, warm ischemic time is longer than in the DBD procedure. Despite this, PGD incidence is comparable [46-49]. This may be explained by the fact that the potential significant effect of a longer ischemic interval in the DCD group is countered by the effect of brain death on lung injury in DBD procedures. After brain death, hemodynamic instability occurs due to a sympathetic storm and changes in vasomotor tone. In combination with the release of pro-inflammatory cytokines, this contributes to neurogenic pulmonary edema. Thus, both procedures may contribute to PGD, but by a different pathway. PGD has been described more often after Maastricht Classification Category II DCD [50]. In this case, patients with failed cardiopulmonary resuscitation are used as donors. A maximum of 120 minutes of warm ischemia, from the time of cardiac arrest until topical cooling, is tolerated for acceptance of the lungs. Hereafter, a maximum of 240 minutes from topical cooling until harvest is permitted to get next-of-kin permission. Thus, warm and cold ischemia time is much longer than in category III donors. Although graft ischemic time is a controversial risk factor for PGD in several studies, we found that a significant warm ischemic hit resulted in PGD grade 3 like symptoms, suggesting that ischemia is an independent risk factor for PGD development. Thus, we recommend not to use Maastricht category II DCD patients for donation. Both DBD and DCD category III procedures should be continued, but the warm ischemic time must be as short as possible in DCD category III procedures.

2.1.5 Marginal donors

The use of marginal donors has been advocated due to a shortage of organ donors and increasing waiting list mortality. A donor is regarded marginal if two or more of the following criteria are present: Age beyond 55 years, a PaO_2/FiO_2 ratio lower than 300-350 mm Hg, an infiltrate on chest X-ray, smoking history of more than 20 pack years, purulent secretions on bronchoscopy, presence of pulmonary contusion, positive sputum culture, and evidence of aspiration [51]. The effect of the use of marginal donors on PGD development, mortality,

postoperative mechanical ventilation and ICU and hospital stay is controversial, since numerous studies have found no significant effect [52-56], while others have found a higher mortality rate [57, 58], persistent inferior pulmonary function [59] and a higher incidence of PGD grade 3 [51, 57, 60]. The factors that have the highest impact on PGD are a donor PaO_2/FiO_2 ratio lower than 300-350 mm Hg and donor smoking history [51]. Thus, the use of a marginal donor may contribute to PGD development. Using marginal donors for a low PGD risk receiver may be safe, while allocating a donor with a P/F ratio lower than 300 mm Hg could be a significant burden for a high-risk receiver. In these cases ex-vivo lung perfusion should be considered in combination with other treatment options like OLV or surfactant therapy.

2.1.6 Size mismatch

Donor and recipient size match is based on a donor-to-recipient predicted total lung capacity ratio (pTLC). Implanting an oversized lung allograft, characterized by a ratio greater than 1.0, can be technically demanding, since there is a higher risk of the inability to close the thorax without severe compression of the lung and risk of hemodynamic instability leading to impaired outcome. However, few studies support this concern [61-63]. On the contrary, Eberlein et al found that donation procedures with a mean pTLC ratio of 0.89 resulted in a higher incidence of PGD (25%) as compared to oversized grafts (5%) [64]. Hyperinflation of the undersized grafts and increased lung perfusion resulting in more shear stress are suggested to contribute to PGD in these cases [64].

2.1.7 Factors not related to development of PGD

Some potential factors that could theoretically contribute to PGD development have not been identified in multivariate logistic regression models as independent risk factors, like donor body mass index, cytomegalovirus and cause of death.

2.2 Donor management

2.2.1 Donor and recipient matching

When a lung is allocated, several injuries related to PGD development are not taken into account. Donor allocation depends on the lung allocation score, blood type, size, and the number of lungs needed. After introduction of the lung allocation score (LAS), a higher incidence of primary graft dysfunction and a significant longer ICU stay have been suggested [65]. Especially patients with a LAS higher than 46 have a reduced 1-year survival expectancy between 65% and 80% [66-68]. Moreover, PGD has a negative impact on pulmonary function up to years after transplantation and contributes significantly to development of BOS. At the moment a potential donor lung is allocated, it is important to be aware of the PGD risk factors and to take them into account when a lung is accepted or rejected for a certain patient. Matching of donor and acceptor should be in such a way that the chance of PGD is minimized. Thus, if a recipient has a LAS higher than 50 or an intermediate LAS with important recipient related risk factors for PGD, it is not advisable to accept a donor with important risk factors for PGD. Furthermore, ischemic time in such a patient should be kept as short as possible and the use of surgical manipulation minimized without the help of CPB, whenever possible.

2.2.2 Surfactant treatment

Strüber reported in 1995 on a 26-year-old woman, who underwent right-sided lung transplantation and developed severe PGD 5 hours after transplantation [69]. She was treated with an intrapulmonary nebulized synthetic surfactant. Shortly hereafter, lung compliance, PaO_2, and tidal volume increased. 24 hours after therapy, the edematous infiltrate of the transplanted lung on chest X-ray film had resolved. The rationale behind surfactant replacement therapy is to ameliorate the inflammatory reaction which is a key feature of PGD, to protect the alveolo-capillary barrier, to preserve the levels of dipalmitoyl-phosphatidylcholine and surfactant associated proteins, and to decrease the inhibitory effects of serum proteins [70]. Surfactant therapy has also been investigated in clinical trials on ARDS treatment [71-73]. Surfactant therapy after the onset of the disease failed to show an effect on survival in these studies, although a trend in improved oxygenation was found [71-73]. In our studies, only surfactant treatment of the donor (thus before the onset of ischemia) resulted in less pulmonary injury, which is in line with these ARDS trials. There are, however, some reports that surfactant treatment is also favourable after reperfusion [74-76]. Nevertheless, this thesis confirms that surfactant pretreatment is more beneficial. A lung transplant procedure is unique in its possibility to administer surfactant before the possible onset of PGD/ARDS. If surfactant is administered to a unoperated lung, it will more likely result in homogenous distribution of surfactant, whereas surfactant administered to already injured lungs will accumulate in the open areas of the lung and not in the atelectatic parts, where it is most needed. Furthermore, if patients develop PGD grade 3, both extensive intra-alveolar and interstitial edema will be present, so that intratracheal administration of surfactant will cause more fluid overload with a negative impact on the outcome. We confirmed this in our studies. Also, the surfactant concentration will be lowered due to dilution, which is undesirable. Finally, pretreatment also has the advantage of preservation of the endogenous surfactant associated proteins, although some (natural) surfactants may contain surfactant associated proteins B&C itself [77, 78]. A randomized clinical trial is the definite way to investigate whether surfactant pretreatment is capable of reducing the incidence of PGD in a clinical setting. The optimal dose and concentration of surfactant in such a trial is still controversial. We have found that 200 mg/kg is the lowest possible dose capable of reducing PGD alike symptoms in our model. In a clinical randomized trial, administration of 100 mg/kg surfactant to donor lungs had a beneficial effect on early clinical outcome [76]. Furthermore, 20 mg/kg surfactant pretreatment improved oxygenation and pulmonary compliance in an experimental study by Hausen et al [79]. The discrepancy between these studies and our study may be explained by the type of surfactant used, the method of surfactant application resulting in variable intra-alveolar surfactant concentrations and, most importantly, the length of the ischemic interval. As mentioned before, mortality after 330 minutes ischemia increases exponentially [80]. A normal lung contains 10-15 mg/kg endogenous surfactant. During ischemia, the endogenous surfactant pool is broken down by phospholipases. The longer the ischemic time, the more surfactant is degraded. Therefore, differences in the length of ischemia may require different surfactant doses. The goal of surfactant pretreatment is to prevent inactivation of the total endogenous and exogenous surfactant pool so that atelectasis during ischemia is prevented.

The clinical use of pretreatment in human lung transplantation is still troubled by the cost

of the material. If surfactant pretreatment of all donors would be considered in the clinical setting, the cost of surfactant must be weighed against the potential improved outcome. Furthermore, there are lung transplantation procedures in which surfactant pretreatment may have marginal benefit. If optimal allocation is possible with a very low risk of PGD, surfactant pretreatment might be futile, while suboptimal allocation with a significant risk of PGD may require surfactant pretreatment to optimize the outcome. Also surfactant therapy may be very useful to increase the use rate of ex-vivo perfused lungs.

3 | Lung harvest and transport

3.1 Warm and cold ischemia

The influence of ischemic time on PGD development is controversial. The International Society of Heart and Lung Transplantation concluded in 2005 that: "Prolonged ischemia should probably be seen as an additional risk factor, but not as a direct cause of primary graft dysfunction" [9]. This conclusion was based on several studies that failed to demonstrate a relationship between cold ischemic time and the outcome after lung transplantation [81, 82]. However, an important paper by Thabut et al showed that the relationship between cold graft ischemic time and survival was of cubic form with a cut-off value of 330 minutes [80]. Thereafter short-term mortality increases rapidly mainly due to development of PGD. This is supported by our findings in this thesis. We have demonstrated that a significant warm ischemic hit results in symptoms comparable to PGD, even though PGD is always thought to be the end result of several injuries the lung may sustain during a transplantation procedure. In accordance with the study by Thabut, there seems to be a threshold for development of PGD grade III symptoms [80]. During the development of our experimental model, an interval shorter than 120 minutes of warm ischemia did not result in PGD stage 3, but milder forms of PGD. Although warm ischemia is not fully comparable to cold ischemia, warm ischemia can be seen as an accelerated form of cold ischemia [83]. Therefore, ischemic time should be seen as an important risk factor of PGD.

3.2 Surgical manipulation

The influence of surgical manipulation on PGD development is hard to determine in clinical studies, since surgical manipulation is inevitable at lung harvest and difficult to grade. It seems clear that the less pulmonary trauma, the lower the pulmonary damage. To optimize alveolar preservation, barotrauma and injury to the pulmonary parenchyma resulting in an air leak is undesirable.

3.3 Alveolar preservation

In the consensus statement of the ISHLT regarding PGD, it is advised to prevent hyperinflation of the lung, since this has been suggested to increase the pulmonary capillary filtration coefficient [84]. Therefore it was propagated to limit lung inflation during storage to 50% of the total lung capacity or to an airway pressure of 10 to 15 cm H_2O to avoid barotrauma. However, this advice was based on a single experimental study in 1996 [84]. In the literature controversy exists on the optimal alveolar preservation protocol, since both hyperinflation

and inflation with low airway pressures have been found to be beneficial or detrimental. Also, there is no consensus about the ideal oxygen percentage during storage. If one askes several cardiothoracic surgeons about their optimal alveolar preservation protocol, it becomes clear that there is no consent. In clinical practice, inflation of the lung with 100% oxygen is still advocated by some surgeons. We show in this thesis the detrimental effects of atelectasis and high oxygen content on postoperative pulmonary function, while we have found a clear protective effect of inflation of the lung with 30 cm H_20 with an oxygen concentration of 30%. Therefore we recommend inflating the lungs with an inflation pressure around 30 cm H_2O and with an oxygen concentration of 30%. The protective effect of hyperinflation during ischemia is probably due to increased intra-alveolar surfactant concentration, less atelectasis formation, and a higher pulmonary vascular resistance during ischemia and at the start of reperfusion. Alveolar inflation with 100% O_2 should be prevented since it increases the risk of reactive oxygen species (ROS) formation and resorption atelectasis.

3.4 Vascular preservation

Graft temperature, preservation volume, preservation pressure and type of preservation solution all have an important impact on LIRI [9]. The aim of vascular preservation is to uniformly reduce the temperature and to clear the vascular bed of all red and white blood cells. Hypothermia to 4 °C reduces the metabolic activity of the graft to 5% of its value at 37°C, although some groups advocate cooling the lungs less aggressively. Preservation volume should be between 60 and 150 ml/kg and preservation pressure as low as 10-15 mm Hg [9]. Euro-Collins, which originally was developed for kidney preservation, had been used mostly for flush perfusion of lung grafts until the development of low-potassium dextran solution, which is an extracellular type solution. Since superior effects of a particular type of low-potassium dextran glucose solution (Perfadex) had been found in experimental studies, most centers worldwide now routinely use Perfadex in lung transplantation. Another extracellular solution containing mannitol, albumin, heparin, Ringer's solution and donor blood, Papworth, has been used until 2006. Of these preservation solutions, Perfadex is negatively associated with PGD grade 2-3, whereas the incidence of PGD grade 3 increases with the use of Papworth and Euro-Collins [15, 18, 25, 85]. The superior effect of Perfadex is due to less vasoconstriction because of a low potassium concentration, less interstitial edema formation with dextran, decreased cytotoxicity to alveolar type II cells, reduction in lipid peroxidation and less thrombocyte aggregation leading to preserved microcirculation [86]. Thus, Perfadex seems to be better in addressing oxygen free radical-mediated reperfusion injury and preserving the alveolar surfactant pool, thereby contributing to either less severe PGD or a lower incidence of PGD in general. We recommend antegrade perfusion of the donor lung with Perfadex at 4 °C with a perfusion pressure of 10-15 mm Hg in a volume of 60-150 ml/kg to reduce the risk of PGD. Retrograde pulmonary perfusion through the pulmonary veins with cold perfusion solution after cold antegrade perfusion has no additional effect in the prevention of PGD, although residual pulmonary emboli can be flushed out of the pulmonary circulation [87, 88].

3.5 Prostaglandins

Prostaglandins have vasodilatation and immune modulation effects. It is used in the

harvesting procedure before the induction of ischemia to allow a better distribution of the preservation fluid [10, 89, 90]. Furthermore, in experimental studies, prostaglandins were able to reduce lung ischemia-reperfusion injury, characterized in these studies by decreased capillary permeability, less neutrophil adhesions, less platelet aggregation and preserved surfactant function [89-92]. No significant adverse effects of prostaglandins have been found. Therefore, we advocate the use of prostaglandins before the induction of ischemia.

3.6 Storage temperature

During the transplantation period, the lung is hypothermically stored to reduce the rate of metabolism, which results in a decreased degradation of important cellular components. However, hypothermia may also induce tissue edema and pulmonary vasoconstriction [93]. Pulmonary function after storage between 10 °C to 15 °C was found to be superior to storage at 4 °C in experimental setting, although this was not supported by a more recent report [82, 94-96]. In conclusion, the optimal storage temperature remains to be decided. Until then, we recommend to store the lungs at 4 °C.

3.7 Ex-vivo lung perfusion

Ex-vivo lung perfusion (EVLP) has been developed to increase the number of possible donor lungs by using organs that normally were not suitable for transplantation [97]. Normally, between 15 to 20% of the lungs available for transplantation are used [97]. A significant part of this rejection pool consists of lungs that are rejected because of an inadequate PaO_2/FiO_2 ratio (<300 mm Hg), pulmonary edema or an infiltrate on chest X-ray, so that the estimated risk of PGD is high. These lungs may be eligible for an EVLP procedure. The rationale behind EVLP is to keep the lung in its most physiologic state at a normal temperature [97]. Reconditioning during EVLP occurs by dehydration of the lung tissue, removal of waste products and prevention of atelectasis [97]. Next to the possibility to repair a damaged lung, EVLP prevents possible adverse effects of hypothermic storage and it creates a window for optimal assessment of the quality of the donor lung [97]. Criteria to accept a graft for transplantation after 4-6 hours of EVLP differ between hospitals, but generally include a stable PaO_2/FiO_2 ratio above 400 mm Hg of the effluent in the left atrium and an improving trend in pulmonary artery pressure, airway pressure and pulmonary compliance [97]. There are several studies that have evaluated EVLP, but most have included a low number of EVLP procedures [98-103]. Interestingly, EVLP has led to a 20-30% increase in the donor pool and reduced the rate of PGD from 30% to 15% in a randomized clinical trial including 20 lungs transplanted after EVLP [99]. The largest study up to now has included 50 lung transplantations after EVLP and reported promising results with a 2% incidence of PGD grade 3, although this was not statistically different as compared to the control group (8.5%) because of the relatively low number of procedures [104]. Thirty day and 1 year survival were similar and no differences in median time to extubation, intensive care unit stay, and hospital length of stay were found [97, 104]. Thus, 4-6 hours of EVLP seems a promising approach to reduce PGD. Still, EVLP is expensive, time-consuming and outcomes beyond 1 year have not been established. Also, in some reports, between 31 and 54% of the lungs that were treated with EVLP did not improve over time [100, 102]. Therefore, a combination of EVLP with other prevention strategies, like surfactant therapy and/or open lung ventilation as described in

this thesis, can further increase the donor pool and reduce the incidence of PGD.

4 | The implantation procedure

4.1 Hospital volume

Increasing surgical volume is associated with improved survival, shorter length of stay, lower costs and less readmissions in general surgical procedures [105-107]. With high-risk procedures like lung transplantations, this is true not only for the surgical part but for all supportive care for lung transplant patients. In cardiac transplantation, mortality significantly increases in centers performing less than 9-12 procedures annually [107, 108]. Centers that perform more than 20 lung transplantations annually have superior short- and long-term outcomes [109]. Also, in centers of expertise, donor utilization rate is higher and more complex recipients are accepted on the waiting list with good results. Therefore, it is advised to perform lung transplantations in dedicated and designated high volume hospitals.

4.2 Cardiopulmonary bypass

If a lung recipient patient does not tolerate mechanical ventilation of one lung during implantation or is hemodynamically unstable, surgeons must use cardiopulmonary bypass (CPB) during the transplant procedure. However, CPB may be started also because of the possibility of controlled reperfusion or to make it technically easier to implant the lung. One of the downsides of CPB is that the patient must be heparinized with the potential risk of postoperative difficulties in hemostasis and the increased risk of transfusion of blood products. Also CPB results in the systemic inflammatory response syndrome, which is characterized by systemic inflammation due to a cytokine storm, in which there is abnormal regulation of various cytokines. The association between CPB and PGD is controversial with studies propagating an increased PGD risk [14] and studies describing no adverse effect [16-18, 21, 22, 27, 110, 111]. However, in a large meta-analysis, PGD grade 3 was found in 32% in patients with CPB as compared to 25% in patients who were not supported by CPB [12]. These conflicting results may be due to bias in some of the earlier reports, since it is difficult to interpret why CPB was initiated (severity of the patient's illness, operative difficulties, or planned on forehand). Overall, CPB must be regarded as a risk factor for PGD, although it may have a pleiotropic effect, since in some patients it can be used to reduce the PGD risk (e.g. in patients with PPH).

Some groups have advocated the use of ECLS instead of CPB during the implantation procedure, since ECLS requires less heparinization, has a less severe pro-inflammatory effect (due to less blood turnover in a suction system) and can be prolonged after the operation if necessary. However, ECLS is not superior to CPB in terms of the incidence of PGD and survival [39, 112-114]. Still, if patients are on ECLS before transplantation as a bridge to transplantation, they have a high risk for postoperative ECLS because of PGD. If ECLS is extended in these patients after the transplantation, the outcome is improved. Also, in patients with pulmonary hypertension, extended ECLS may help to reduce the risk of PGD [113].

4.3 Surgical manipulation

Although the extent to which a pulmonary graft can be manipulated is unclear, surgical manipulation of the pulmonary graft must be kept to a minimum to reduce trauma to the graft. This is supported by the finding of an increased risk of PGD in patients with a body mass index of 30 or higher due to an increased risk of technical difficulties and consequently more surgical manipulation [14, 15]. Also, alveolar pressure decreases after the donor bronchus is opened at implantation, so that the lung becomes atelectatic. Atelectatic lungs are even more vulnerable to surgical manipulation and ischemia. Thus this atelectatic period should be as short as possible with minimal manipulation of the graft.

4.4 Controlled reperfusion

Reperfusion of the graft can be a damaging process. Therefore, strategies to reduce reperfusion injury have been developed over the last decades, including reperfusion with alternative solutions and lowering of the reperfusion pressure [115-120]. Schnickel et al performed 100 lung transplantations with an alternative reperfusion approach [117]. After insertion of a catheter in the pulmonary artery, lungs were reperfused with leukocyte depleted but NTG/aspartate/glutamate/dextrose enriched blood for 10 minutes at a pressure lower than 20 mm Hg [117]. This approach resulted in an incidence of only 2% PGD grade 3 and excellent early and 1-year survival of respectively 97 and 91% [117]. If central venoarterial ECLS or CPB is used during the implantation procedure, modified reperfusion with a gradual increase in reperfusion pressure can be partly achieved.

4.5 Surgical complications

Surgical complications that have a clear effect on the risk of PGD development include vascular and bronchial anastomotic obstruction, orientation of the graft, and an air leak. Difficulties in optimal ventilation of the graft can be due to stenosis of the bronchial anastomosis or a large air leak. Venous obstruction causes pulmonary hypertension and subsequent pulmonary edema and may result in infarction of the lung [121, 122]. Pulmonary artery stenosis causes hypoperfusion of the lung grafts and thus hypoxia; no relationship between pulmonary artery stenosis and PGD is described. Lobar torsion is associated with severe PGD and infarction [123, 124]. It is clear that these complications must be prevented at all costs.

5 | Peri –and postoperative care

5.1.1 Mechanical ventilation

The protocol of mechanical ventilation is important in the reduction of ventilator induced lung injury on top of ischemia-reperfusion injury. This already starts in the operation room. When the clamp on the pulmonary artery and veins is released, it is essential to choose a ventilation strategy that minimizes the risk of PGD. Ventilator-induced-lung injury (VILI) is a complication of mechanical ventilation and is a form of acute lung injury [125-129]. It is characterized by damage to the alveolar lining cells, a pro-inflammatory state, and injury to the endogenous surfactant system [125-128] . Since VILI is a subsequent hit the lung

sustains, it may contribute to PGD [130]. In this regard, VILI should be prevented. The use of small tidal volume ventilation (4-6 ml/kg) is propagated to decrease the risk of VILI in ARDS patients [131, 132]. In this thesis, mechanical ventilation according to this protocol had no effect on pulmonary function, but it resulted in an increased histological pulmonary severity score and aggravated pulmonary inflammation, suggestive of increased pulmonary injury. Open lung ventilation is an alternative mechanical ventilation approach that aims to open up the lung and to keep it open with as low as possible ventilation pressures [133-135]. We have demonstrated that open lung ventilation resulted in improved oxygenation, better pulmonary compliance and a dampened inflammatory response. In the clinical setting development of PGD often occurs shortly after the start of reperfusion. During the ischemic period several parts of the lungs become atelectatic and surfactant is degraded. After removal of the clamp pulmonary edema may develop fast, resulting in alveolar flooding, hypoxemia and problems to adequately ventilate the lungs. Therefore, it is best to perform a recruitment manoeuvre to open up the lung as soon as possible after the start of reperfusion, followed by adjustment of ventilation pressures so that the alveolus is stabilized. Permissive atelectasis should be prevented, even if this results in the use of high ventilation pressures. Unfortunately, open lung ventilation is not without risks. The use of high PEEP levels in ARDS patients is controversial [136-138]. Although high PEEP improves survival in ARDS patients, it is detrimental in patients with acute lung injury due the risk of alveolar overdistension and thus VILI [136]. Since open lung ventilation is an individual approach, only high ventilation pressures will be needed in the most affected patients and low pressures can be used in those who are less affected.

5.1.2 Independent lung ventilation

Independent lung ventilation (ILV) may be an approach in the case of single lung transplantation and PGD because of the differences in compliance between the native lung and allograft to prevent hyperinflation of the native lung and subsequently ventilation-perfusion mismatch [139-142]. Although there is no role for prevention of PGD with ILV, it can be considered in PGD cases when ECLS is not available.

5.2 Red blood cells and plasma transfusions

Both the transfusion of packed red blood cells and plasma are risk factors for development of PGD [12, 14, 21, 110, 143]. In non-transplant patients, transfusion itself may result in transfusion-related acute lung injury (TRALI) with a reported incidence of 0.04% to 0.16% [144]. The exact mechanism of TRALI is unknown [144]. In lung transplantation, a multi-hit model has been hypothesized. Thus, transfusions during lung transplantation should be prevented if possible. The use of a cell-saver during the implantation is advised.

5.3 Fluid transfusion

Since the alveolo-capillary integrity is compromized in PGD, fluid transfusion contributes to capillary leakage of fluid into the alveolus causing alveolar flooding. This results in further impairment of lung function and more dilution of the endogenous surfactant pool. Therefore, if patients develop PGD grade 3, a restrictive fluid protocol is recommended. If a patient is hemodynamically unstable with signs of PGD grade 3, low dose inotropes or

ECLS should be considered instead of judicious fluid administration.

5.4 Surfactant treatment

According to the findings in this thesis, there is no rationale for surfactant treatment of a recipient with severe PGD, comparable to the negative findings of surfactant treatment in ARDS patients [71-73]. We recommend that, if surfactant treatment is considered in a clinical setting, it be administered to the donor in high-risk patients and not to the recipient, as discussed earlier.

5.5 Nitric oxide

Nitric oxide (NO) elevates intracellular cGMP leading to pulmonary vasodilation, less platelet aggregation and leukocyte adhesion and preservation of the endothelial barrier . There is no evidence for the use of inhaled NO in the prevention of PGD [145-148], although prophylaxis with NO has been found to reduce LIRI in experimental setting [149]. Clinical studies demonstrated that NO improves gas exchange and reduces pulmonary artery pressure in established PGD, although no survival benefit has been found [150-152]. In a large ARDS trial, NO treatment improved arterial oxygenation, but did not have an effect on survival [153]. Thus, the use of inhaled NO may be considered as an additional treatment option in lung transplant patients with established PGD, although the beneficial effect is probably transient as in ARDS patients.

5.6 Extra corporeal life support

If patients develop PGD grade 3 that is non-responsive to conventional treatment like mechanical ventilation, extra corporeal life support (ECLS) is the only life-saving option. The incidence of ECLS in PGD patients is around 5% in large volume centers. The earlier ECLS is started, the better the outcome, even though 30-day mortality is significantly higher than the mortality found in patients who did not require ECLS support [154, 155]. If ECLS is started beyond 24 hours after the onset of PGD, reported 30-day mortality is up to 100%, while this is reduced to 30-50% if ECLS is started within 24 hours [39, 154, 155]. Still, 5-year survival (49% versus 61%) and FEV1 (58% versus 83%) is inferior to the values found in patients who did not require ECLS support. If a patient requires ECLS, either venovenous (VV) or venoarterial (VA) ECLS can be chosen. The advantages of VV ECLS include easier cannulation and a reduced risk on systemic embolization, while coronary artery hypoxemia and the incapability to support heart function are drawbacks to this technique. VA ECLS supports the circulation and reduces pulmonary arterial flow allowing controlled reperfusion, while a higher risk on arterial embolization has been described. VV ECLS is at least non-inferior to VA for treatment of PGD and should be the first choice in patients who are hemodynamically stable, since VV ECLS has a lower complication rate [156-158]. If ECLS is started, the lungs should be ventilated with small tidal volumes, a FiO_2 below 30%, and a PEEP value that stabilizes the alveolus. Late institution (>7 days after transplantation) of ECLS in lung transplant recipients for causes other than primary graft dysfunction is associated with extremely high mortality and is not indicated [155, 159].

5.7 Retransplantation

If patients with PGD grade 3 cannot be weaned from ECLS or from the ventilator, pulmonary retransplantation is the last option. Aigner et al reported on 23 PGD patients who underwent retransplantation within 26 ± 27 days with a 30-day mortality rate of 48% as compared to 11% in patients retransplanted because of BOS, which is comparable to other reports [160, 161]. Most patients died due to sepsis with multi-organ failure or a second primary graft dysfunction. Five-year survival of patients retransplanted because of PGD is between 20 and 29%. Since donor organs are scarce, pulmonary retransplantation for PGD is not recommended.

6 | Summary risk factors PGD according to literature

6.1 Recipient

Primary and secondary pulmonary hypertension, sarcoidosis, idiopathic pulmonary fibrosis, female gender, Afro-American race, body-mass index >30 kg/m^2, and pre-transplant extra corporeal life support. Pre-transplant mechanical ventilation in CF patients could be a risk factor.

6.2 Donor

Age (<21 years or >45 years), smoking (>20 pack years), PaO_2/FiO_2 ratio <300-350 mm Hg.

6.3 At harvest

Ischemia, surgical manipulation, suboptimal alveolar and vascular preservation.

6.4 At implantation

Cardiopulmonary bypass, inexperienced surgeon, surgical manipulation, lobar torsion and venous anastomotic obstruction.

6.5 Peri –and postoperative care

Suboptimal mechanical ventilation and red blood cells/plasma/fluid transfusion.

7 | Differential diagnosis of primary graft dysfunction

Hyperacute rejection, left ventricular failure, pneumonia and venous anastomotic obstruction should be considered as alternative diagnoses for PGD in patients with hypoxemia, impaired compliance and pulmonary edema after pulmonary transplantation.

8 | Outcome of primary graft dysfunction

PGD grade 3 leads to an increased mortality rate between 30 and 50%, a longer postoperative ICU and hospital stay, prolonged postoperative mechanical ventilation, more tracheostomy procedures, and significantly higher costs [162]. According to the ISHLT

registry data, PGD accounts for almost one-third of all deaths in the first 90 days after transplantation. Although 1-year survival after exclusion of the early deaths is comparable to PGD negative patients, pulmonary function is significantly reduced after PGD grade 3, as indicated by a significant reduced forced expiratory value in 1 second (FEV1) [27, 111]. Also PGD contributes significantly to chronic transplant dysfunction, the bronchiolitis obliterans syndrome (BOS). As we have confirmed in our experiments, PGD results in activation of the immune system, finally resulting in upregulation of Major Histocompatibility Complex type II positive antigen presenting cells ($MHCII^+$-APC). Since involvement of T-cells and $MHCII^+$-APC may be the link between LIRI and BOS, a reduction in these cells seems important. Interestingly, we have demonstrated that prolonged warm ischemic time results in a chronic fibro-proliferative process, as seen with ARDS. Histologically severe diffuse alveolar damage is seen on HE slides 90 days after reperfusion.

9 | From bench to bedside

In this section we give some recommendations that can help to reduce the incidence of PGD, extrapolated from our experimental findings and/or based on the available literature, as described above.

9.1 General recommendations

1) Medical staff responsible for allocation of the lung, harvesting, implantation and postoperative care should be aware of PGD risk factors and symptoms to minimize the risk of development of PGD and to improve outcome.
2) Continue both the donation after brain death and donation after cardiac death procedures (category III patients according to the Maastricht classification). Keep the warm ischemic time as short as possible in donation after cardiac death procedures.
3) Do not use lungs obtained from Maastricht category II donation after cardiac death patients.
4a) Move from supportive care to prevention of PGD.
4b) Do not allocate a marginal donor to a patient with a high risk for development of PGD; use near-ideal organs for such a patient instead.
4c) Develop an allocation system that incorporates PGD related risk factors of both the donor and recipient.
5) ECLS and pre-transplant mechanical ventilation should not be considered as a contra-indication for transplantation in selected cases. In these patients, PGD risk factors must be minimized but PGD should be expected and treated accordingly.

9.2 Harvesting recommendations

1) Inflate the lungs with an inflation pressure around 30 cm H_2O and with an oxygen concentration of 30%. Alveolar inflation with 100% O_2 should be prevented.
2) To optimize alveolar preservation, barotrauma and injury to the pulmonary parenchyma resulting in an air leak should be prevented.
3) Use antegrade perfusion of the donor lung with perfadex at 4 ^oC with a perfusion pressure of 10-15 mm Hg in a volume of 60-150 ml/kg. Retrograde perfusion can be used

additionally to flush pulmonary emboli.
4) Administer local prostaglandins before the induction of ischemia.
5) If a marginal donor is allocated to a patient with a high risk for PGD, surfactant pretreatment may be considered to optimize the graft. This is, however, still experimental.
6) Ex-vivo lung perfusion can be used to enlarge the donor pool. The combination of EVLP and surfactant and/or open lung ventilation must be investigated to further increase the donor pool.

9.3 Peri- and postoperative recommendations

1) Use cardiopulmonary bypass during the implantation only if there is a hemodynamic or respiratory need. Do not routinely use cardiopulmonary bypass at implantation.
2) Perform lung transplantations only in dedicated and designated high volume hospitals.
3) Atelectasis during the transport and implantation should be prevented. If it is impossible to prevent atelectasis, it can be accepted for a short time with minimal manipulation of the graft.
4) Open lung ventilation is an alternative approach that may reduce the severity of PGD, if started directly after reperfusion.
5) Prevent red blood cells and plasma transfusions during lung transplantation if possible. The use of a cell-saver during the implantation is advised.
6) If patients develop PGD grade 3, a restrictive fluid protocol is recommended. If a patient is hemodynamically unstable with signs of PGD grade 3, low dose inotropes or venoarterial ECLS should be considered instead of judicious fluid administration.
7) There is no role for inhaled nitric oxide in the prevention of PGD.
8) There is no clear indication for surfactant treatment of a recipient with severe PGD.
9) Venovenous ECLS is at least non-inferior to venoarterial ECLS and should be the first choice in patients with PGD who are hemodynamically stable.
10) Use lung rest ventilation settings if a patient is on ECLS.
11) Pulmonary retransplantation for PGD is not recommended.

10 | References

1. Christie JD, Bavaria JE, Palevsky HI, et al. Primary graft failure following lung transplantation. Chest 1998;114:51-60.
2. Khan SU, Salloum J, O'Donovan PB, et al. Acute pulmonary edema after lung transplantation: The pulmonary reimplantation response. Chest 1999;116:187-194.
3. Sleiman C, Mal H, Fournier M, et al. Pulmonary reimplantation response in single-lung transplantation. Eur Respir J 1995;8:5-9.
4. Fisher AJ, Wardle J, Dark JH, Corris PA. Non-immune acute graft injury after lung transplantation and the risk of subsequent bronchiolitis obliterans syndrome (BOS). J Heart Lung Transplant 2002;21:1206-1212.
5. Zenati M, Pham SM, Keenan RJ, Griffith BP. Extracorporeal membrane oxygenation for lung transplant recipients with primary severe donor lung dysfunction. Transpl Int 1996;9:227-230.
6. Arcasoy SM, Fisher A, Hachem RR, Scavuzzo M, Ware LB. Report of the ISHLT Working Group on Primary Lung Graft Dysfunction part V: predictors and outcomes. J Heart Lung Transplant 2005;24:1483-1488.
7. Barr ML, Kawut SM, Whelan TP, et al. Report of the ISHLT Working Group on Primary Lung Graft Dysfunction part IV: Recipient-related risk factors and markers. J Heart Lung Transplant 2005;24:1468-1482.
8. Christie JD, Van Raemdonck D, de Perrot M, et al. Report of the ISHLT Working Group on Primary Lung Graft Dysfunction part I: introduction and methods. J Heart Lung Transplant 2005;24:1451-1453.
9. de Perrot M, Bonser RS, Dark J, et al. Report of the ISHLT Working Group on Primary Lung Graft Dysfunction part III: donor-related risk factors and markers. J Heart Lung Transplant 2005;24:1460-1467.
10. Shargall Y, Guenther G, Ahya VN, Ardehali A, Singhal A, Keshavjee S. Report of the ISHLT Working Group on Primary Lung Graft Dysfunction part VI: treatment. J Heart Lung Transplant 2005;24:1489-1500.
11. Christie JD, Carby M, Bag R, Corris P, Hertz M, Weill D. Report of the ISHLT Working Group on Primary Lung Graft Dysfunction part II: Definition. A consensus statement of the International Society for Heart and Lung Transplantation. J Heart Lung Transplant 2005;24:1454-1459.
12. Liu Y, Liu Y, Su L, Jiang SJ. Recipient-related clinical risk factors for primary graft dysfunction after lung transplantation: a systematic review and meta-analysis. PLoS One 2014;9:e92773.
13. Christie JD, Kotloff RM, Pochettino A, et al. Clinical risk factors for primary graft failure following lung transplantation. Chest 2003;124:1232-1241.
14. Diamond JM, Lee JC, Kawut SM, et al. Clinical risk factors for primary graft dysfunction after lung transplantation. Am J Respir Crit Care Med 2013;187:527-354.
15. Kuntz CL, Hadjiliadis D, Ahya VN, et al. Risk factors for early primary graft dysfunction after lung transplantation: A registry study. Clin Transplant 2009;23:819--830.
16. Samano MN, Fernandes LM, Baranauskas JCB, et al. Risk factors and survival impact of primary graft dysfunction after lung transplantation in a single institution. Transplant Proc 2012;44:2462-2468.
17. Cottini SR, Lerch N, de Perrot M, et al. Risk factors for reperfusion injury after lung transplantation. Intensive Care Med 2006;32:557-563.
18. Ailawadi G, Lau CL, Smith PW, et al. Does reperfusion injury still cause significant mortality after lung transplantation? J Thorac Cardiovasc Surg 2009;137:688-694.
19. King RC, Binns OA, Rodriguez F, et al. Reperfusion injury significantly impacts clinical outcome after pulmonary transplantation. Ann Thorac Surg 2000;69:1681-1685.
20. Thabut G, Vinatier I, Stern JB, et al. Primary graft failure following lung transplantation: Predictive factors of mortality. Chest 2002;121:1876-1882.
21. Fang A, Studer S, Kawut SM, et al. Elevated pulmonary artery pressure is a risk factor for primary graft dysfunction following lung transplantation for idiopathic pulmonary fibrosis. Chest 2011;139:782-787.
22. Whitson BA, Nath DS, Johnson AC, et al. Risk factors for primary graft dysfunction after lung transplantation. J Thorac Cardiovasc Surg 2006;131:73-80.
23. Kramer MR, Marshall SE, Tiroke A, Lewiston NJ, Starnes VA, Theodore J. Clinical significance of hyperbilirubinemia in patients with pulmonary hypertension undergoing heart-lung transplantation. J Heart Lung Transplant 1991;10:317-321.
24. Kawut SM, Horn EM, Berekashvili KK, et al. New predictors of outcome in idiopathic pulmonary arterial hypertension. Am J Cardiol 2005;95:199-203.
25. Oto T, Griffiths AP, Rosenfeldt F, Levvey BJ, Williams TJ, Snell GI. Early outcomes comparing Perfadex, Euro-Collins, and Papworth solutions in lung transplantation. Ann Thorac Surg 2006;82:1842-1848.
26. Yusen RD, Christie JD, Edwards LB, et al. The Registry of the International Society for Heart and Lung

Transplantation: Thirtieth Adult Lung and Heart-Lung Transplant Report-2013; focus theme: Age. J Heart Lung Transplant 2013;32:965-978.
27. Burton CM, Iversen M, Milman N, et al. Outcome of lung transplanted patients with primary graft dysfunction. Eur J Cardiothorac Surg 2007;31:75-82.
28. Jain M, Budinger GRS, Lo A, et al. Leptin promotes fibroproliferative acute respiratory distress syndrome by inhibiting peroxisome proliferator-activated receptor. Am J Respir Crit Care Med 2011;183:1490-1498.
29. Lederer DJ, Kawut SM, Wickersham N, et al. Obesity and primary graft dysfunction after lung transplantation: the Lung Transplant Outcomes Group Obesity Study. Am J Respir Crit Care Med 2011;184: 1055-1061.
30. Bellmeyer A, Martino JM, Chandel NS, Scott Budinger GR, Dean DA, Mutlu GM. Leptin resistance protects mice from hyperoxia-induced acute lung injury. Am J Respir Crit Care Med 2007;175:587-594.
31. Massard G, Shennib H, Metras D, et al. Double-lung transplantation in mechanically ventilated patients with cystic fibrosis.. Ann Thorac Surg 1993;55:1087-1091.
32. Elizur A, Sweet SC, Huddleston CB, et al. Pre-transplant mechanical ventilation increases short-term morbidity and mortality in pediatric patients with cystic fibrosis. J Heart Lung Transplant 2007;26: 127-131.
33. Trulock EP, Edwards LB, Taylor DO, Boucek MM, Keck BM, Hertz MI. Registry of the International Society for Heart and Lung Transplantation: Twenty-second official adult lung and heart-lung transplant report-2005. J Heart Lung Transplant 2005;24:956-967.
34. Flume PA, Egan TM, Westerman JH, et al. Lung transplantation for mechanically ventilated patients. J Heart Lung Transplant 1994;13:15-21;discussion 22-23.
35. Baz MA, Palmer SM, Staples ED, Greer DG, Tapson VF, Davis DD. Lung transplantation after long-term mechanical ventilation: Results and 1-year follow-up. Chest 2001;119:224-227.
36. Meyers BF, Lynch JP, Battafarano RJ, et al. Lung transplantation is warranted for stable, ventilator-dependent recipients. Ann Thorac Surg 2000;70:1675-1678.
37. Vermeijden JW, Zijlstra JG, Erasmus ME, van der Bij W, Verschuuren EA. Lung transplantation for ventilator-dependent respiratory failure. J Heart Lung Transplant 2009;28:347-351.
38. Mason DP, Thuita L, Nowicki ER, Murthy SC, Pettersson GB, Blackstone EH. Should lung transplantation be performed for patients on mechanical respiratory support? The US experience. J Thorac Cardiovasc Surg 2010;139:765-773.
39. Bittner HB, Lehmann S, Rastan A, et al. Outcome of extracorporeal membrane oxygenation as a bridge to lung transplantation and graft recovery. Ann Thorac Surg 2012;94:942-949; author reply 949-50.
40. Toyoda Y, Bhama JK, Shigemura N, et al. Efficacy of extracorporeal membrane oxygenation as a bridge to lung transplantation. J Thorac Cardiovasc Surg 2012;145:1065-1070.
41. Shigemura N, Toyoda Y, Bhama JK, et al. Donor smoking history and age in lung transplantation: A revisit. Transplantation 2012;95:513-518.
42. Narayanan M, Owers-Bradley J, Beardsmore CS, et al. Alveolarization continues during childhood and adolescence: new evidence from helium-3 magnetic resonance. Am J Respir Crit Care Med 2012;185: 186-191.
43. Bonser RS, Taylor R, Collett D, et al. Effect of donor smoking on survival after lung transplantation: A cohort study of a prospective registry. Lancet 2012;380:747-755.
44. Arcasoy SM, Kotloff RM. Lung transplantation. N Engl J Med 1999;340:1081-1091.
45. Ware LB, Wang Y, Fang X, et al. Assessment of lungs rejected for transplantation and implications for donor selection. Lancet 2002;360:619-620.
46. De Oliveira NC, Osaki S, Maloney JD, et al. Lung transplantation with donation after cardiac death donors: Long-term follow-up in a single center. J Thorac Cardiovasc Surg 2012;139:1306-1315.
47. De Vleeschauwer S, Van Raemdonck D, Vanaudenaerde B, et al. Early outcome after lung transplantation from non-heart-beating donors is comparable to heart-beating donors. J Heart Lung Transplant 2009;28:380-387.
48. Van De Wauwer C, Verschuuren EAM, van der Bij W, Nossent GD, Erasmus ME. The use of non-heart-beating lung donors category III can increase the donor pool. Eur J Cardiothorac Surg 2011;39:e175-180; discussion e180.
49. Zych B, Popov AF, Amrani M, et al. Lungs from donation after circulatory death donors: An alternative source to brain-dead donors? Midterm results at a single institution. Eur J Cardiothorac Surg 2012;42:542-549.
50. Gomez-de-Antonio D, Campo-Cañaveral JL, Crowley S, et al. Clinical lung transplantation from

uncontrolled non-heart-beating donors revisited. J Heart Lung Transplant 2012;31:349-353.
51. Schiavon M, Falcoz PE, Santelmo N, Massard G. Does the use of extended criteria donors influence early and long-term results of lung transplantation? Interact Cardiovasc Thorac Surg 2012;14:183-187.
52. Sundaresan S, Semenkovich J, Ochoa L, et al. Successful outcome of lung transplantation is not compromised by the use of marginal donor lungs. J Thorac Cardiovasc Surg 1995;109:1075-1079; discussion 1079-1080.
53. Gabbay E, Williams TJ, Griffiths AP, et al. Maximizing the utilization of donor organs offered for lung transplantation. Am J Respir Crit Care Med 1999;160:265-271.
54. Bhorade SM, Vigneswaran W, McCabe MA, Garrity ER. Liberalization of donor criteria may expand the donor pool without adverse consequence in lung transplantation. J Heart Lung Transplant 2000;19:1199-1204.
55. Lardinois D, Banysch M, Korom S, et al. Extended donor lungs: eleven years experience in a consecutive series. Eur J Cardiothorac Surg 2005;27:762-767.
56. Aigner C, Winkler G, Jaksch P, et al. Extended donor criteria for lung transplantation-a clinical reality. Eur J Cardiothorac Surg 2005;27:757-761.
57. Botha P, Trivedi D, Weir CJ, Searl CP, Corris PA, Dark JH, Schueler SVB. Extended donor criteria in lung transplantation: impact on organ allocation. J Thorac Cardiovasc Surg 2006;131:1154-1160.
58. Pierre AF, Sekine Y, Hutcheon MA, Waddell TK, Keshavjee SH. Marginal donor lungs: A reassessment. J Thorac Cardiovasc Surg 2002;123:421-427; discussion, 427-428.
59. Kawut SM, Reyentovich A, Wilt JS, et al. Outcomes of extended donor lung recipients after lung transplantation. Transplantation 2005;79:310-316.
60. Meers C, Van Raemdonck D, Verleden GM, et al. The number of lung transplants can be safely doubled using extended criteria donors; a single-center review. Transpl Int 2010;23:628-635.
61. Shigemura N, Bermudez C, Hattler BG, et al. Impact of graft volume reduction for oversized grafts after lung transplantation on outcome in recipients with end-stage restrictive pulmonary diseases. J Heart Lung Transplant 2009;28:130-134.
62. Ouwens JP, van der Mark TW, van der Bij W, Geertsma A, de Boer WJ, Koëter GH. Size matching in lung transplantation using predicted total lung capacity. Eur Respir J 2002;20:1419-1422.
63. Lloyd KS, Barnard P, Holland VA, Noon GP, Lawrence EC. Pulmonary function after heart-lung transplantation using larger donor organs. Am Rev Respir Dis 1990;142:1026-1029.
64. Eberlein M, Arnaoutakis GJ, Yarmus L, et al. The effect of lung size mismatch on complications and resource utilization after bilateral lung transplantation. J Heart Lung Transplant 2012;31:492-500.
65. Kozower BD, Meyers BF, Smith MA, et al. The impact of the lung allocation score on short-term transplantation outcomes: A multicenter study.. J Thorac Cardiovasc Surg 2008;135:166-171.
66. Horai T, Shigemura N, Gries C, Pet al. Lung transplantation for patients with high lung allocation score: single-center experience. Ann Thorac Surg 2012;93:1592-1597; discussion 1597.
67. Weiss ES, Allen JG, Merlo CA, Conte JV, Shah AS. Lung allocation score predicts survival in lung transplantation patients with pulmonary fibrosis. Ann Thorac Surg 2009;88:1757-1764.
68. Merlo CA, Weiss ES, Orens JB, et al. Impact of U.S. Lung Allocation Score on survival after lung transplantation. J Heart Lung Transplant 2009;28:769-775.
69. Struber M, Cremer J, Harringer W, Hirt SW, Costard-Jackle A, Haverich A. Nebulized synthetic surfactant in reperfusion injury after single lung transplantation. J Thorac Cardiovasc Surg 1995;110:563-564.
70. van der Kaaij NP, Lachmann RA, Bogers AJJC, Lachmann B. Surfactant alterations and treatment of lung transplant ischemia--reperfusion injury. Journal of organ dysfunction 2006;2:221-229.
71. Spragg RG, Lewis JF, Walmrath HD, et al. Effect of recombinant surfactant protein C-based surfactant on the acute respiratory distress syndrome. N Engl J Med 2004;351:884-892.
72. Kesecioglu J, Beale R, Stewart TE, et al. Exogenous natural surfactant for treatment of acute lung injury and the acute respiratory distress syndrome. Am J Respir Crit Care Med 2009;180:989-994.
73. Anzueto A, Baughman RP, Guntupalli KK, et al. Aerosolized surfactant in adults with sepsis-induced acute respiratory distress syndrome. Exosurf Acute Respiratory Distress Syndrome Sepsis Study Group. N Engl J Med 1996;334:1417-1421.
74. Amital A, Shitrit D, Raviv Y, et al. Surfactant as salvage therapy in life threatening primary graft dysfunction in lung transplantation. Eur J Cardiothorac Surg 2009;35:299-303.
75. Kermeen FD, McNeil KD, Fraser JF, et al. Resolution of severe ischemia-reperfusion injury post-lung transplantation after administration of endobronchial surfactant. J Heart Lung Transplant 2007;26:850-856.

76. Struber M, Fischer S, Niedermeyer J, et al. Effects of exogenous surfactant instillation in clinical lung transplantation: a prospective, randomized trial. J Thorac Cardiovasc Surg 2007;133:1620-1625.
77. Haitsma JJ, Papadakos PJ, Lachmann B. Surfactant therapy for acute lung injury/acute respiratory distress syndrome. Curr Opin Crit Care 2004;10:18-22.
78. Griese M. Pulmonary surfactant in health and human lung diseases: State of the art. Eur Respir J 1999;113:1455-1476.
79. Hausen B, Rohde R, Hewitt CW, et al. Exogenous surfactant treatment before and after sixteen hours of ischemia in experimental lung transplantation. J Thorac Cardiovasc Surg 1997;113:1050-1058.
80. Thabut G, Mal H, Cerrina J, et al. Graft ischemic time and outcome of lung transplantation: A multicenter analysis. Am J Respir Crit Care Med 2005;171:786-791.
81. de Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia-reperfusion-induced lung injury. Am J Respir Crit Care Med 2003;167:490-511.
82. Ueno T, Snell GI, Williams TJ, et al. Impact of graft ischemic time on outcomes after bilateral sequential single-lung transplantation. Ann Thorac Surg 1999;67:1577-1582.
83. Warnecke G, Sommer SP, Gohrbandt B, et al. Warm or cold ischemia in animal models of lung ischemia-reperfusion injury: Is there a difference? Thorac Cardiovasc Surg 2004;52:174-179.
84. Haniuda M, Hasegawa S, Shiraishi T, Dresler CM, Cooper JD, Patterson GA. Effects of inflation volume during lung preservation on pulmonary capillary permeability. J Thorac Cardiovasc Surg 1996;112:85-93.
85. Nath DS, Walter AR, Johnson AC, et al. Does Perfadex affect outcomes in clinical lung transplantation? J Heart Lung Transplant 2005;24:2243-2248.
86. Ingemansson R, Massa G, Pandita RK, Sjoberg T, Steen S. Perfadex is superior to Euro-Collins solution regarding 24-hour preservation of vascular function. Ann Thorac Surg 1995;60:1210-1214.
87. Ferraro P, Martin J, Dery J, et al. Late retrograde perfusion of donor lungs does not decrease the severity of primary graft dysfunction. Ann Thorac Surg 2008;86:1123-1129.
88. Venuta F, Rendina EA, Bufi M, et al. Preimplantation retrograde pneumoplegia in clinical lung transplantation. J Thorac Cardiovasc Surg 1999;118:107-114.
89. de Perrot M, Fischer S, Liu M, Jet al. Prostaglandin E1 protects lung transplants from ischemia-reperfusion injury: a shift from pro- to anti-inflammatory cytokines. Transplantation 2001;72:1505-1512.
90. Chiang CH, Wu K, Yu CP, Yan HC, Perng WC, Wu CP. Hypothermia and prostaglandin E(1) produce synergistic attenuation of ischemia-reperfusion lung injury. Am J Respir Crit Care Med 1999;160:1319-1323.
91. Gohrbandt B, Sommer SP, Fischer S, et al. Iloprost to improve surfactant function in porcine pulmonary grafts stored for twenty-four hours in low-potassium dextran solution. J Thorac Cardiovasc Surg 2005; 129:80-86.
92. Kawashima M, Nakamura T, Schneider S, et al. Iloprost ameliorates post-ischemic lung reperfusion injury and maintains an appropriate pulmonary ET-1 balance. J Heart Lung Transplant 2003;22:794-801.
93. Martin DR, Scott DF, Downes GL, Belzer FO. Primary cause of unsuccessful liver and heart preservation: Cold sensitivity of the ATPase system. Ann Surg 1972;175:111-117.
94. Wang LS, Yoshikawa K, Miyoshi S, et al. The effect of ischemic time and temperature on lung preservation in a simple ex vivo rabbit model used for functional assessment. J Thorac Cardiovasc Surg 1989;98:333-342.
95. Munneke AJ, Rakhorst G, Petersen AH, van Oeveren W, Prop J, Erasmus ME. Flush at room temperature followed by storage on ice creates the best lung graft preservation in rats. Transpl Int 2013;26: 751-760.
96. Albes JM, Fischer F, Bando T, Heinemann MK, Scheule A, Wahlers T. Influence of the perfusate temperature on lung preservation: is there an optimum? Eur Surg Res 1997;29:5-11.
97. Van Raemdonck D, Neyrinck A, Cypel M, Keshavjee S. Ex-vivo lung perfusion. Transpl Int 2014;epub ahead of print
98. Wallinder A, Ricksten SE, Silverborn M, et al. Early results in transplantation of initially rejected donor lungs after ex vivo lung perfusion: A case-control study. Eur J Cardiothorac Surg 2014;45:40-44; discussion 44-45.
99. Cypel M, Yeung JC, Liu M, et al. Normothermic ex vivo lung perfusion in clinical lung transplantation. N Engl J Med 2011;364:1431-1440.
100. Zych B, Popov AF, Stavri G, et al. Early outcomes of bilateral sequential single lung transplantation after ex-vivo lung evaluation and reconditioning. J Heart Lung Transplant 2012;31:274-281.
101. Boffini M, Ricci D, Barbero C, et al. Ex vivo lung perfusion increases the pool of lung grafts: Analysis of its potential and real impact on a lung transplant program. Transplant Proc 2013;45:2624-2626.
102. Aigner C, Slama A, Hötzenecker K, et al. Clinical ex vivo lung perfusion-pushing the limits. Am J

Transplant 2012;12:1839-1847.
103. Lindstedt S, Hlebowicz J, Koul B, et al. Comparative outcome of double lung transplantation using conventional donor lungs and non-acceptable donor lungs reconditioned ex vivo. Interact Cardiovasc Thorac Surg 2011;12:162-165.
104. Cypel M, Yeung JC, Machuca T, et al. Experience with the first 50 ex vivo lung perfusions in clinical transplantation. J Thorac Cardiovasc Surg 2012;144:1200-1206.
105. Luft HS, Bunker JP, Enthoven AC. Should operations be regionalized? The empirical relation between surgical volume and mortality. N Engl J Med 1979;301:1364-1369.
106. Birkmeyer JD, Stukel TA, Siewers AE, Goodney PP, Wennberg DE, Lucas FL. Surgeon volume and operative mortality in the United States. N Engl J Med 2003;349:2117-2127.
107. Hosenpud JD, Breen TJ, Edwards EB, Daily OP, Hunsicker LG. The effect of transplant center volume on cardiac transplant outcome. A report of the United Network for Organ Sharing Scientific Registry. JAMA 1994;271:1844-1849.
108. Pettit SJ, Jhund PS, Hawkins NM, et al. How small is too small? A systematic review of center volume and outcome after cardiac transplantation. Circ Cardiovasc Qual Outcomes 2012;5:783-790.
109. Weiss ES, Allen JG, Meguid RA, et al. The impact of center volume on survival in lung transplantation: an analysis of more than 10,000 cases. Ann Thorac Surg 2009;88:1062-1070.
110. Allen JG, Lee MT, Weiss ES, Arnaoutakis GJ, Shah AS, Detrick B. Preoperative recipient cytokine levels are associated with early lung allograft dysfunction. Ann Thorac Surg 2012;93:1843-1849.
111. Christie JD, Sager JS, Kimmel SE, et al. Impact of primary graft failure on outcomes following lung transplantation. Chest 2005;127:161-165.
112. Aigner C, Wisser W, Taghavi S, et al. Institutional experience with extracorporeal membrane oxygenation in lung transplantation. Eur J Cardiothorac Surg 2007;31:468-473.
113. Pereszlenyi A, Lang G, Steltzer H, et al. Bilateral lung transplantation with intra- and postoperatively prolonged ECMO support in patients with pulmonary hypertension. Eur J Cardiothorac Surg 2002; 21:858-863.
114. Bittner HB, Binner C, Lehmann S, Kuntze T, Rastan A, Mohr FW. Replacing cardiopulmonary bypass with extracorporeal membrane oxygenation in lung transplantation operations. Eur J Cardiothorac Surg 2007;31:462-467.
115. Allen BS, Okamoto F, Buckberg GD, et al. Immediate functional recovery after six hours of regional ischemia by careful control of conditions of reperfusion and composition of reperfusate. J Thorac Cardiovasc Surg 1986;92:621-635.
116. Okamoto F, Allen BS, Buckberg GD, Bugyi H, Leaf J. Reperfusion conditions: importance of ensuring gentle versus sudden reperfusion during relief of coronary occlusion. J Thorac Cardiovasc Surg 1986;92:613-620.
117. Schnickel GT, Ross DJ, Beygui R, et al. Modified reperfusion in clinical lung transplantation: the results of 100 consecutive cases. J Thorac Cardiovasc Surg 2006;131:218-223.
118. Hopkinson DN, Bhabra MS, Odom NJ, Bridgewater BJ, Van Doorn CA, Hooper TL. Controlled pressure reperfusion of rat pulmonary grafts yields improved function after twenty-four-hours' cold storage in University of Wisconsin solution. J Heart Lung Transplant 1996;15:283-290.
119. Bhabra MS, Hopkinson DN, Shaw TE, Hooper TL. Critical importance of the first 10 minutes of lung graft reperfusion after hypothermic storage. Ann Thorac Surg 1996;61:1631-1635.
120. Bhabra MS, Hopkinson DN, Shaw TE, Onwu N, Hooper TL. Controlled reperfusion protects lung grafts during a transient early increase in permeability. Ann Thorac Surg 1998;65:187-192.
121. Shah AS, Michler RE, Downey RJ, Leibowitz DW, Homma S, Smith CR. Management strategies for pulmonary vein thrombosis following single lung transplantation. J Card Surg 1995;10:169-178.
122. Sarsam MA, Yonan NA, Beton D, McMaster D, Deiraniya AK. Early pulmonary vein thrombosis after single lung transplantation. J Heart Lung Transplant 1993;12:17-19.
123. Collins J, Kuhlman JE, Love RB. Acute, life-threatening complications of lung transplantation. Radiographics 1998;18:21-43; discussion 43-47.
124. Grazia TJ, Hodges TN, Cleveland Jr JC, Sheridan BC, Zamora MR. Lobar torsion complicating bilateral lung transplantation. J Heart Lung Transplant 2003;22:102-106.
125. Dreyfuss D, Saumon G. Ventilator-induced lung injury: lessons from experimental studies. Am J Respir Crit Care Med 1998;157:294-323.
126. Hudson LD. Progress in understanding ventilator-induced lung injury. Jama 1999;282:77-78.

127. Dos Santos CC, Slutsky AS. Invited review: Mechanisms of ventilator-induced lung injury: A perspective. J Appl Physiol 2000;89:1645-1655.
128. Petrucci N. Ventilator-induced lung injury. Jama 2000;283:1003-1004.
129. Verbrugge SJ, Lachmann B, Kesecioglu J. Lung protective ventilatory strategies in acute lung injury and acute respiratory distress syndrome: From experimental findings to clinical application. Clin Physiol Funct Imaging 2007;27:67-90.
130. de Perrot M, Imai Y, Volgyesi GA, et al. Effect of ventilator-induced lung injury on the development of reperfusion injury in a rat lung transplant model. J Thorac Cardiovasc Surg 2002;124:1137-1144.
131. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network. N Engl J Med 2000;342:1301-1308.
132. Richard JC, Maggiore SM, Jonson B, Mancebo J, Lemaire F, Brochard L. Influence of tidal volume on alveolar recruitment. Respective role of PEEP and a recruitment maneuver. Am J Respir Crit Care Med 2001;163:1609-1613.
133. van Kaam AH, de Jaegere A, Haitsma JJ, Van Aalderen WM, Kok JH, Lachmann B. Positive pressure ventilation with the open lung concept optimizes gas exchange and reduces ventilator-induced lung injury in newborn piglets. Pediatr Res 2003;53:245-253.
134. Miranda DR, Gommers D, Papadakos PJ, Lachmann B. Mechanical ventilation affects pulmonary inflammation in cardiac surgery patients: The role of the open-lung concept. J Cardiothorac Vasc Anesth 2007;21:279-284.
135. Lachmann B. Open up the lung and keep the lung open. Intensive Care Med 1992;18:319-321.
136. Briel M, Meade M, Mercat A, et al. Higher vs lower positive end-expiratory pressure in patients with acute lung injury and acute respiratory distress syndrome: Systematic review and meta-analysis. JAMA 2010; 303:865-873.
137. Mercat A, Richard JCM, Vielle B, et al. Positive end-expiratory pressure setting in adults with acute lung injury and acute respiratory distress syndrome: A randomized controlled trial. JAMA 2008;299:646-655.
138. Brower RG, Lanken PN, MacIntyre N, et al. Higher versus lower positive end-expiratory pressures in patients with the acute respiratory distress syndrome. N Engl J Med 2004;351:327-336.
139. Pilcher DV, Auzinger GM, Mitra B, et al. Predictors of independent lung ventilation: An analysis of 170 single-lung transplantations. J Thorac Cardiovasc Surg 2007;133:1071-1077.
140. Gavazzeni V, Iapichino G, Mascheroni D, et al. Prolonged independent lung respiratory treatment after single lung transplantation in pulmonary emphysema. Chest 1993;103:96-100.
141. de Perrot M, Quadri SM, Imai Y, Keshavjee S. Independent ventilation of the graft and native lungs in vivo after rat lung transplantation. Ann Thorac Surg 2005;79:2169-2171.
142. Officer TM, Wheeler DR, Frost AE, Rodarte JR. Respiratory control during independent lung ventilation. Chest 2001;120:678-681.
143. Felten ML, Sinaceur M, Treilhaud M, et al. (2012) Factors associated with early graft dysfunction in cystic fibrosis patients receiving primary bilateral lung transplantation. Eur J Cardiothorac Surg 2012;41:686-690.
144. Webert KE, Blajchman MA. Transfusion-related acute lung injury. Transfus Med Rev 2003;17: 252-262.
145. Meade MO, Granton JT, Matte-Martyn A, et al. A randomized trial of inhaled nitric oxide to prevent ischemia-reperfusion injury after lung transplantation. Am J Respir Crit Care Med 2003;167:1483-1489.
146. Ardehali A, Laks H, Levine M, et al. A prospective trial of inhaled nitric oxide in clinical lung transplantation. Transplantation 2001;72:112-115.
147. Cornfield DN, Milla CE, Haddad IY, Barbato JE, Park SJ. Safety of inhaled nitric oxide after lung transplantation. J Heart Lung Transplant 2003;22:903-907.
148. Botha P, Jeyakanthan M, Rao JN, et al. Inhaled nitric oxide for modulation of ischemia-reperfusion injury in lung transplantation. J Heart Lung Transplant 2007;26:1199-1205.
149. Gómez CB, del Valle HF, Bertolotti A, et al. Effects of short-term inhaled nitric oxide on interleukin-8 release after single-lung transplantation in pigs. J Heart Lung Transplant 2005;24:714-722.
150. Date H, Triantafillou AN, Trulock EP, Pohl MS, Cooper JD, Patterson GA. Inhaled nitric oxide reduces human lung allograft dysfunction. J Thorac Cardiovasc Surg 1996;111:913-919.
151. Adatia I, Lillehei C, Arnold JH, et al. Inhaled nitric oxide in the treatment of postoperative graft dysfunction after lung transplantation. Ann Thorac Surg 1994;57:1311-1318.
152. Macdonald P, Mundy J, Rogers P, et al. Successful treatment of life-threatening acute reperfusion injury after lung transplantation with inhaled nitric oxide. J Thorac Cardiovasc Surg 1995;110:861-863.

153. Taylor RW, Zimmerman JL, Dellinger RP, et al. Low-dose inhaled nitric oxide in patients with acute lung injury: A randomized controlled trial. JAMA 2004;291:1603-1609.
154. Wigfield CH, Lindsey JD, Steffens TG, Edwards NM, Love RB. Early institution of extracorporeal membrane oxygenation for primary graft dysfunction after lung transplantation improves outcome. J Heart Lung Transplant 2007;26:331-338.
155. Marasco SF, Vale M, Preovolos A, et al. Institution of extracorporeal membrane oxygenation late after lung transplantation - a futile exercise? Clin Transplant 2012;26:E71-77.
156. Bermudez CA, Adusumilli PS, McCurry KR, et al. Extracorporeal membrane oxygenation for primary graft dysfunction after lung transplantation: Long-term survival. Ann Thorac Surg 2009;87:854-860.
157. Hartwig MG, Walczak R, Lin SS, Davis RD. Improved survival but marginal allograft function in patients treated with extracorporeal membrane oxygenation after lung transplantation. Ann Thorac Surg 2012; 93:366-371.
158. Hartwig MG, Appel JZ, Cantu E, et al. Improved results treating lung allograft failure with venovenous extracorporeal membrane oxygenation. Ann Thorac Surg 2005;80:1872-1879.
159. Glassman LR, Keenan RJ, Fabrizio MC, et al. Extracorporeal membrane oxygenation as an adjunct treatment for primary graft failure in adult lung transplant recipients. J Thorac Cardiovasc Surg 1995;110:723-726; discussion 726-727.
160. Aigner C, Jaksch P, Taghavi S, et al. Pulmonary retransplantation: Is it worth the effort? A long-term analysis of 46 cases. J Heart Lung Transplant 2008;27:60-65.
161. Strueber M, Fischer S, Gottlieb J, et al. Long-term outcome after pulmonary retransplantation. J Thorac Cardiovasc Surg 2006;132:407-412.
162. Lee JC, Christie JD. Primary graft dysfunction. Proc Am Thorac Soc 2009;6:39-46.

Summary and conclusions

1 | Experimental model

We have developed a reproducible, technically less demanding, relatively cheap experimental model, in which symptoms comparable to PGD symptoms can be studied up to months after reperfusion (Chapter 3, 6, 7, 8, 9, 10, 11).

2 | Relation between LIRI and PGD

Warm ischemia and reperfusion resulted in our experimental model in a mortality rate of 20-30%, impaired left lung compliance, decreased PaO_2/FiO_2 ratio, conversion of highly surface active large aggregate surfactant subtype into poor surface active small aggregate surfactant subtype, pulmonary capillary leakage, diffuse alveolar damage on HE slides and MMP-2 and MMP-9 production, which are all features of the exudative phase of PGD. Importantly, LIRI resulted in progressive deterioration of lung function and architecture, leading to extensive immunopathological and functional abnormalities up to 3 months after reperfusion. Thus, warm ischemia and reperfusion in our model resulted in pulmonary injury that is comparable to PGD after clinical lung transplantation and ARDS. LIRI seems a major risk factor for PGD in the absence of other influencing factors, such as alloimmunity (Chapter 3).

3 | Detailed characterization of PGD parameters

3.1 Pulmonary function

LIRI resulted in a reduced PaO_2/FiO_2 ratio up to seven days after reperfusion and hypercapnia on day 1. Importantly, the PaO_2/FiO_2 ratio does not fully reflect the function of the left

ischemic lung in our studies, since this ratio depended on the function of both the ischemic left and non-ischemic right lung. However, pulmonary compliance was severely impaired up to 3 months after reperfusion (Chapter 3).

3.2 Inflammation

Neutrophil infiltration visible in BALf and lung parenchyma lasted for 3 days after reperfusion. Helper and cytotoxic T-cells infiltrated the ischemic lung shortly after reperfusion and were found up to 7 days after reperfusion in our autologous model. The presence of macrophages in BALf and lung parenchyma of ischemic animals 3, 7, and 90 days after reperfusion may be due to their fibroblast regulation function. (Chapter 3). LIRI causes more MHCII expression on antigen presenting cells, which is present from day 2 after reperfusion (Chapter 8, 10, 11).

3.3 Histology on HE slides

LIRI resulted in an exudative phase up to 3 days after reperfusion progressing into a fibroproliferative state in line with clinical ARDS and PGD. This diffuse alveolar damage consists of septal and alveolar edema, inflammation, atelectasis, intra-alveolar hemorrhage, congestion and early signs of fibrosis (Chapter 3, 7, 8, 10, 11).

3.4 Conversion of surfactant large aggregates to small aggregates

Conversion of highly surface active large aggregate surfactant subtype into poor surface active small aggregate surfactant subtype was found in both the left, ischemic lung and right, non-ischemic lung on day 1. Also a decreased level of surfactant large aggregate was measured up to day 30 after LIRI (Chapter 3).

3.5 Capillary permeability

More alveolar serum proteins were found in the ischemic left lung on day 1 to 3 after LIRI. From day seven on, no differences were present (Chapter 3).

4 | Effect of LIRI on the non-ischemic lung

While no major changes were seen on HE slides of the right lung, the inflammatory profile of the right BALf resembled that of the left, although it was less severe. Also, an increased amount of poor surface active small aggregate surfactant subtype was measured in these parts of the lung, demonstrating that the right lung has sustained injury. However, LIRI of the left lung did not result in long-term injury of the right lung (Chapter 3).

5 | Surfactant treatment for PGD

Surfactant pretreatment reduced mortality to 3%, and resulted in a normal lung function and pulmonary compliance already on day 3 postoperatively. It further decreased alveolar capillary leakage and resulted in a normal SA/LA ratio. Finally, we observed a significant increase in the number of macrophages and granulocytes in the surfactant pretreated group,

most prominent on day 1 with levels returning to normal at day 7, which may reflect recycling of the exogenous surfactant. Thus, surfactant pretreatment enhances the recovery of lung function but, even more importantly, may prevent in part the damage caused by LIRI (Chapter 6). Surfactant pretreatment also prevented a fibroproliferative process, since pulmonary compliance was completely normal 3 months after reperfusion and less pulmonary injury was seen on HE slides, although still more macrophages were found in the left ischemic lung (Chapter 7). The timing of surfactant administration used in these studies would permit donor pretreatment in the clinical setting (Chapter 6, 7). However, clinical use may be troubled by the cost of surfactant if used in a dose of 400 mg/kg. Therefore we have tried lower surfactant doses in our model and have investigated if surfactant therapy after reperfusion is still beneficial in selected cases. In our model is 200 mg/kg the lowest surfactant dose that reduces PGD. Surfactant pretreatment is superior to treatment at reperfusion and superior to treatment 24 hours after reperfusion, since surfactant therapy at or after reperfusion had no significant beneficial effect (Chapter 8). Finally, surfactant increased IL-6 and IL-10 in the lung 240 minutes after reperfusion, while it decreased activated caspase-3 expression. Interestingly, surfactant therapy also increased pulmonary inducible nitric oxide synthase expression (Chapter 9).

6 | Alveolar preservation strategies to minimize PGD

Alveolar inflation with 30 cm H_2O during ischemia resulted in improved survival, better pulmonary compliance and arterial oxygenation, less diffuse alveolar damage and a reduced number of helper and cytotoxic T-lymphocytes and $MHCII^+$-antigen presenting cells as compared to inflation with 5 cm H_2O. Furthermore, alveolar inflation with 100% oxygen led to a lower PaO_2 and pulmonary compliance and more pronounced inflammation as compared to the 30% oxygen preserved groups. Thus, the optimal alveolar preservation protocol is a combination of high inflation pressure with a low oxygen concentration. The optimal alveolar inflation protocol reduces the number of T-lymphocytes and MHCII expression on APC after reperfusion. This observation is of particular relevance since these cells are thought to be part of the link between ischemia-reperfusion injury and the subsequent development of BOS (Chapter 10).

7 | Ventilation strategies to reduce PGD

No difference in lung function was found between conventional ventilation according to the ARDS network and extubation within an hour after reperfusion. On the contrary, conventional ventilation according to the ARDS network resulted in more severe inflammation consisting of neutrophils and antigen presenting cells, but also more MHCII expression. After open lung concept ventilation, a normal PaO_2/FiO_2 ratio, less diffuse alveolar damage on HE slides, and an improved pulmonary compliance were found. Also, open lung concept ventilation resulted in less MHCII expression and less alveolar infiltration of neutrophils, antigen presenting cells, and lymphocytes in the left lung. Thus conventional

ventilation according to the ARDS network results in additional lung injury. Open lung concept ventilation, started directly at the start of reperfusion, decreases symptoms of LIRI in our model. It may hereby prevent the development of PGD grade 3 and decreases the risk on long-term organ dysfunction (Chapter 11).

Samenvatting en conclusies

1 | Algemeen

Patiënten met terminaal longfalen, die geen baat meer hebben bij standaard therapie, kunnen in aanmerking komen voor een longtransplantatie. Om op de wachtlijst voor een longtransplantatie te worden geplaatst, moeten patiënten voldoen aan een aantal criteria, welke in de algemene introductie worden toegelicht. Historisch was de duur van de wachttijd een belangrijk criterium om te bepalen wanneer iemand aan de beurt was voor transplantatie. In verband met hoge sterfte op de wachtlijst, is sinds kort de long allocatie score ingevoerd, zodat patiënten die het ziekste zijn de hoogste kans hebben om een long toegewezen te krijgen. Indien dit gebeurt, hebben zij kans op belangrijke verbetering van de kwaliteit van leven en een verlenging van de overleving. Echter, direct na het uitvoeren van de transplantatie, kan de functie van de transplantatie long snel achteruit gaan, hetgeen primaire transplantaat dysfunctie wordt genoemd ("Primary Graft Dysfunction" (PGD)). Bij het uitvoeren van een longtransplantatie wordt de bloedstroom naar de long onderbroken, zodat het transplantaat niet wordt voorzien van zuurstof (ischemie). Zodra de long wordt aangesloten op de bloedvaten van de ontvanger treedt er herstel op van bloed en dus zuurstof voorziening van de long (reperfusie). Ischemie en reperfusie ("lung ischemia-reperfusion injury" (LIRI)) speelt een belangrijke rol in het ontstaan van PGD. Dit proefschrift beschrijft het opzetten van een experimenteel model om de relatie tussen long ischemie-reperfusie schade en PGD te bestuderen en om PGD te karakteriseren. Het model is vervolgens gebruikt om verschillende strategieën, die PGD kunnen voorkomen of behandelen, te onderzoeken. Surfactant bevindt zich in de longblaasjes, verlaagt de oppervlakte spanning en is essentieel om met weinig inspanning te kunnen ademen. Surfactant kan beschadigd raken tijdens een transplantatie, hetgeen een ernstige vermindering van de longfunctie kan betekenen. Studies naar het behoud of het herstel van surfactant vormen een belangrijk

onderdeel van dit proefschrift. De belangrijkste bevindingen worden hierna toegelicht.

2 | Experimenteel model

Het ontwikkelde long ischemie-reperfusie schade model is een bewezen reproduceerbaar, technisch minder veeleisend, relatief goedkoop experimenteel model, waarin symptomen vergelijkbaar met de symptomen van PGD kunnen worden bestudeerd tot maanden na reperfusie (Hoofdstuk 3, 6, 7, 8, 9, 10 en 11).

3 | Relatie tussen ischemie-reperfusie schade en PGD

Ischemie en reperfusie schade van de long bij een temperatuur van 37 °C resulteert in ons model in een sterfte van 15 tot 30%, een afname van de elasticiteit van de long, een daling in de zuurstofspanning in het bloed, inactivatie van surfactant, toename van lekkage van eiwitten vanuit de bloedbaan naar de longblaasjes, welke allen karakteristiek zijn voor PGD. Bovendien is er tot 3 maanden na reperfusie sprake van een belangrijke verstoring van de longfunctie en architectuur op histologische coupes. Dus, warme ischemie en reperfusie schade in dit model veroorzaakt symptomen die vergelijkbaar zijn met de symptomen van PGD. Significante warme ischemie-reperfusie lijkt een belangrijke risicofactor voor PGD te zijn in de afwezigheid van andere beïnvloedende factoren, zoals afstoting en infectie (Hoofdstuk 3).

4 | Gedetailleerde beschrijving van PGD parameters

4.1 Longfunctie

Tot 7 dagen na reperfusie is er sprake van een daling in de zuurstofspanning in het bloed in combinatie met een verhoogde koolstofdioxide spanning op dag 1. Hierbij dient te worden aangetekend dat deze waarden bepaald worden door de ischemische linker en de niet beschadigde rechter long, zodat de gemeten waarde niet een exacte weergave is van de functie van de ischemische long. Echter, de elasticiteit van de linker ischemische long is drie maanden na reperfusie nog ernstig verminderd (Hoofdstuk 3).

4.2 Ontstekingsreactie

Een toename in het aantal witte bloedcellen (neutrofielen en macrofagen) is meetbaar tot 3 dagen na reperfusie in de linker long. Tevens infiltreren lymfocyten de ischemische linker long kort na reperfusie en zijn aantoonbaar tot dag 7 na reperfusie. Macrofagen hebben een belangrijke functie in de regulatie van fibroblasten (betrokken bij verlittekening), hetgeen (deels) hun aanwezigheid tot 90 dagen na reperfusie verklaart (Hoofdstuk 3). Gezien het autologe karakter van ons model, is de expressie van MHCII op antigeen presenterende cellen, zichtbaar vanaf dag 2 na reperfusie, een belangrijke bevinding (Hoofdstuk 8, 10, 11).

4.3 Histologie

Conform het beloop bij PGD resulteert ernstige ischemie-reperfusie schade in een exudatieve fase gevolgd door een fibroproliferatieve staat. Deze diffuse alveolaire beschadiging bestaat uit longvocht, inflammatie, samenvallen van longblaasjes, bloedingen in de longblaasjes, veneuze stuwing en vroege tekenen van verlittekening (Hoofdstuk 3, 7, 8, 10,11).

4.4 Conversie van surfactant subtype

Conversie van een actief oppervlakte verlagend surfactant subtype naar een inactieve vorm werd gevonden in zowel de linker, ischemische long en de rechter, niet-ischemische long op dag 1 na reperfusie. Tot 30 dagen na reperfusie is er sprake van een belangrijke verlaging van het actief oppervlakte verlagende subtype (Hoofdstuk 3).

4.5 Pulmonale capillaire permeabiliteit

Er zijn 1 tot 3 dagen na reperfusie eiwitten in de longblaasjes zichtbaar, die gelekt moeten zijn vanuit de bloedbaan, hetgeen suggestief is voor lekkage van de bekleding van de bloedbaan (het endotheel) en de longblaasjes (epitheel) (Hoofdstuk 3).

5 | Effect van long ischemie-reperfusie schade op de niet-ischemische long

Warme ischemie en reperfusie schade van de linker long leidt tot een vergelijkbaar, maar minder ernstige ontstekingsreactie in de rechter, niet ischemische long en conversie van surfactant, mogelijk duidend op indirecte schade van de rechter long. Op histologische coupes worden echter geen significante afwijkingen gevonden en lange termijn metingen laten ook geen afwijkingen zien wat betreft longfunctie. Dus, de acute maar milde afwijkingen van de rechter long in de eerste week na reperfusie hebben geen effect op de lange termijn longfunctie (Hoofdstuk 3).

6 | Surfactant en PGD

Surfactant behandeling voor de inductie van ischemie verlaagt de mortaliteit in ons model tot 3%, voorkomt de achteruitgang in longfunctie en elasticiteit en reduceert de capillaire lekkage en surfactant conversie. Dus, surfactant voorbehandeling versnelt niet alleen het herstel van de longfunctie, maar, nog veel belangrijker, voorkomt gedeeltelijk long ischemie-reperfusie schade. De keerzijde van surfactant behandeling is een gemeten toename van het aantal witte bloedcellen. Een mogelijke verklaring voor deze toename is de surfactant recycling functie van deze cellen (Hoofdstuk 6). Surfactant voorbehandeling voorkomt het verlittekenings proces op lange termijn, hetgeen ondersteund wordt door histologische coupes en een niet afwijkende long compliantie tot op dag 90, hoewel nog wel meer weefsel macrofagen aanwezig zijn in de ischemische, linker long (Hoofdstuk 7). Aangezien surfactant in deze studies 1 uur voor de inductie van ischemie werd toegediend, zou dit klinisch toepasbaar zijn (Hoofdstuk 6, 7). Echter, aangezien surfactant zeer kostbaar is, is de klinische toepasbaarheid bij een dosering van 400 mg/kg bij het huidige prijsniveau

twijfelachtig. Derhalve hebben wij onderzocht of lagere doseringen hetzelfde effect hebben. Bovendien zou het goedkoper zijn om alleen die patiënten te behandelen die PGD graad 3 ontwikkelen. Daarom is surfactant behandeling direct na reperfusie of 24 uur na reperfusie vergeleken met behandeling voor de inductie van ischemie. In ons model is 200 mg/kg de laagste surfactant dosis die PGD vermindert. Bovendien is surfactant voorbehandeling superieur aan surfactant behandeling direct na reperfusie en 24 uur na reperfusie, aangezien surfactant therapie na reperfusie geen gunstig effect had op de uitkomsten (Hoofdstuk 8). Surfactant speelt ook een modulerende rol in de ontstekingsreactie na ischemie-reperfusie en zou dus ook afstoting na long transplantatie kunnen beïnvloeden. Daarom is het effect van surfactant therapie op een aantal ontstekingsparameters (cytokine en stikstofoxide productie) en celdood onderzocht in Hoofdstuk 9. Behandeling met surfactant induceert de productie van ontstekingsremmende mediatoren en vermindert celdood.

7 | Alveolaire preservatie

Nadat de long bij de donor is uitgenomen is het belangrijk hoe de long op ijs wordt bewaard. Preservatie van de long met een luchtweg druk van 30 cm H_2O bij de start van ischemie resulteert in een afname van long ischemie-reperfusie schade vergeleken met een preservatie van 5 cm H_2O. Als longen worden gepreserveerd met 100% zuurstof leidt dit tot ernstige ischemie-reperfusie schade, die duidelijk toegenomen is in vergelijking met longen die worden gepreserveerd met 30% zuurstof. Het optimale alveolaire preservatie protocol is een combinatie van hoge inflatiedruk met een lage zuurstofconcentratie. Indien dit wordt toegepast, leidt dit tot een verbeterde longfunctie en overleving, een afname van diffuus alveolaire schade, een daling van het aantal witte bloedcellen en vermindering van MHCII expressie. Deze laatste constatering is van bijzonder belang, omdat MHCII expressie na ischemie-reperfusie schade een rol speelt in het ontwikkelen van chronisch transplantaat dysfunctie, oftewel het bronchiolitis obliterans syndroom (Hoofdstuk 10).

8 | Beademing en PGD

Als bij de start van reperfusie de beademing van het transplantaat wordt hervat, is er geen verschil in longfunctie op dag 1 als conventioneel longprotectief beademd wordt of als er binnen een uur na reperfusie detubatie plaatsvindt. Echter, conventionele ventilatie leidt tot een toename van het aantal witte bloedcellen en meer MHCII expressie. Beademing volgens het open long concept vermindert long ischemie-reperfusie schade. Dit wordt gekaraktiseerd door een normale zuurstofspanning, minder diffuus alveolaire schade, een verbeterde long elasticiteit, minder MHCII expressie en afname van de ontstekingsreactie. Dus, conventionele ventilatie leidt tot additionele longschade. Open long concept beademing, mits toegepast direct na reperfusie, vermindert symptomen van ischemie-reperfusie schade in ons model. Vroegtijdige stabilisering van de longblaasjes middels open long concept beademing vermindert de ernst van PGD en vermindert mogelijk het risico op chronisch transplantaat dysfunctie (Hoofdstuk 11).

Appendix 1

Materials and methods

1 | Animals (Chapter 3, 6, 7, 8, 9, 10, 11)

All experimental protocols were approved by the Animal Experiments Committee under the national Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC.

2 | Surgical procedure (Chapter 3, 6, 7, 8, 9, 10, 11)

Animals were anesthetized with 60 mg/kg ketaminhydrochloride (Chapter 3, 6, 7) or 80 mg/kg pentobarbital (60 mg/ml, Chapter 8, 9, 10, 11) intraperitoneally and a gas mixture (1,5-3% isoflurane, 64% NO_2 and 33% O_2), whereafter they were intubated and pressure control ventilated (15 cm H_2O peak inspiratory pressure (PIP), 5 cm H_2O positive endexpiratory pressure (PEEP), frequency 30-50 breaths/minute, 50% O_2, and a ratio of inspiration to expiration time of 1:2) on a Siemens Servo 900C ventilator (Maquet Critical Care AB, Solna, Sweden, Chapter 3, 6, 7) or an Evita XL ventilator (Dräger medical SAS, Lübeck, Germany, Chapter 8, 9, 10, 11). A left dorsolateral thoracotomy in the fourth intercostal space was performed. The left lung was mobilized atraumatically and the mediastinum was dissected around the left lung hilus. Hereafter, animals of the LIRI group underwent 120 (Chapter 3, 6, 7, 9) or 150 (Chapter 8, 10, 11) minutes of warm lung ischemia by clamping the bronchus, pulmonary artery and veins of the left lung in inflated state, using a single non-crushing microvascular clamp. During the operation a moist tissue to minimize evaporative loss covered the left hemithorax. At reperfusion, the lung was recruited by a stepwise increase of PIP and PEEP (maximum respectively 50 and 18 cm H_2O) until the lung was visually expanded. Recruitment was also performed in sham-operated animals. The thorax was closed and the animals received 5 ml of 5% glucose intraperitoneally and 0.1 mg/kg of buprenorphinhydrochloride (0.3 mg/ml) intramuscularly and were weaned from

the ventilator. Throughout the whole experiment, body temperature was kept within normal range by the use of a heating pad, placed underneath each animal. All animals recovered with additional oxygen during the first 12 hours, received tap water ad libitum and standard laboratory pellets, and were inspected daily.
At the end of the experiment animals were anesthetized with 20-40 mg/kg pentobarbital intraperitoneally (60 mg/ml) and a gas mixture (3% isoflurane, 64% NO_2 and 33% O_2). After weighing the animals, a polyethylene catheter (0.8 mm outer diameter) was inserted into the carotid artery and a metal cannula was inserted into the trachea. Thereafter, anaesthesia was continued with 20 mg/kg pentobarbital intraperitoneally and 0.7 mg/kg pancuronium bromide or 0.6 mg/kg rocuronium bromide intramuscularly. The animals were subsequently ventilated for 5 minutes (13 cm H_2O PIP, 3 cm H_2O PEEP, frequency 30 breaths/minute and a FiO_2 of 1.00). Blood gas values were recorded in 0.3 ml heparinized blood taken from the carotid artery (ABL555 gas analyser, Radiometer, Copenhagen, Denmark). Animals were exsanguinated and euthanized by an overdose of pentobarbital (200 mg/kg), administered intravenously.

3 | Alveolar arterial oxygen difference (Chapter 10)

The AAOD was calculated as the gradient between the partial pressure of oxygen in the alveolar space and the arterial blood and by the following formula: $(713 x FiO_2)–(PaCO_2/0.8)–(PaO_2)$.

4 | Static compliance (Chapter 3, 6, 7, 8, 10, 11)

The thorax and diaphragm were opened to eliminate the influence of chest wall compliance and abdominal pressure and a static pressure-volume curve (PVC) of the left and right lung together and left lung separately was recorded. The PVC of the individual left lung was conducted by clamping the contralateral hilum. Maximal compliance (Cmax) was determined as the steepest part of the lung deflation curve. Maximal lung volume (Vmax), corrected for body weight, was recorded at a pressure of 35 cm H_2O.

5 | Bronchoalveolar lavage (Chapter 3, 6, 7, 8, 10, 11)

Left and right lung were lavaged separately five times with 0.9% sodium chloride containing 1.5 mM $CaCl_2$ at Vmax. Individual lung bronchoalveolar lavage fluid (BALf) analysis was accomplished by clamping the contralateral hilum. Total recovered volume of BALf was noted. Cell suspensions were centrifuged at 400g and 4 °C for 10 minutes to pellet the cells. Supernatant of BALf was taken and stored at -20 °C for surfactant analysis and the amount of alveolar serum protein, if applicable. After lysis of red blood cells with erythrocyte lysis buffer, the suspension was washed with murine Fluorescence-Activated Cell Sorter (FACS) buffer (MFB) (phosphate buffered saline (PBS), 0.05% weight/volume (w/v) sodium azide and 5% w/v bovine serum albumin (BSA)), centrifuged and resuspended in MFB. Cells were counted with a Bürker-Turk cell counter (Erma, Tokyo, Japan).

6 | Other cell collection (Chapter 3, 7, 11)

Left lung (Chapter 3, 7, 11), right lung (Chapter 3, 7), thoracic lymph nodes (TLN, Chapter 3, 7), and spleen (Chapter 3) were dissected, smashed, mashed through a filter, and suspended in NaCl. Cell suspensions were centrifuged at 400g and 4 °C for 10 minutes to pellet the cells. Red blood cells were lysed with erythrocyte lysis buffer, whereafter the suspension was washed with MFB, centrifuged and resuspended in MFB. Cells were counted with a Bürker-Turk cell counter (Erma, Tokyo, Japan).

7 | Flowcytometry (Chapter 3, 6, 7, 8, 10, 11)

Pelleted cells (max $1*10^6$ cells per well) were incubated on ice with 2% volume/volume (v/v) normal rat serum (NRS) in MFB for 15 minutes to prevent non-specific binding of Fc-receptors with primary antibodies. Hereafter, cells were washed, centrifuged and surface-stained for 30 minutes at 4 °C in the dark with the following primary mouse anti rat antibodies and 2% NRS: 1/20 CD3-AlexaFluor647 (1F4)[a], 1/80 phycoerythrin (PE) labelled CD8 (OX8[b]), 1/160 fluorescein-isothiocyanate (FITC) labelled CD4 (OX38[a]), 1/80 CD45RA-PE (OX33[a]), 1/200 HIS48-fluorescein-isothiocyanate[b], 1/20 CD68-AlexaFluor647 (ED1)[a], and 1/160 Major-Histocompatibility-Complex (MHC) Class II-fluorescein-isothiocyanate (OX6)[a]. Antibodies were obtained commercially from Serotec[a] (Kidlington, United Kingdom) and BD[b] (Franklin Lakes, New Jersey, USA).
Cellular differentiation was calculated based on morphology (Side Scatter (SSC) for granularity, Forward Scatter (FSC) for size), auto fluorescence and specific positive antibody staining. Cells were identified as follows: lymphocytes low FSC, low SSC, no auto fluorescence, and expressing either $CD45RA^+$ (B-lymphocytes), $CD3^+$ (T-lymphocytes), $CD3^+CD4^+$ (helper T-lymphocytes), and $CD3^+CD8^+$ (cytotoxic T-lymphocytes); neutrophils low FSC, intermediate SSC and $HIS48^+$; macrophages as auto fluorescent, high SSC and FSC and $CD68^+$; antigen presenting cells as auto fluorescent, high SSC and FSC, $CD68^+$ and $MHCII^+$. Data were acquired on a FACS Calibur flowcytometer (BD Biosciences, New Jersey, USA) and were analysed using CellQuest (BD Biosciences) and FlowJo software (Tree Star, Oregon, USA).

8 | SA/LA ratio (Chapter 3, 6, 7)

Supernatant of BALf was centrifuged at 4 °C for 15 minutes at 40.000g to separate the surface-active surfactant pellet (large aggregate (LA)) from the non-surface active supernatant fraction (small aggregate (SA)). LA was resuspended in 2ml NaCl, whereafter the phosphorus concentration of LA and SA was determined by phospholipid extraction, followed by phosphorus analysis.
The supernatant of the BALf was centrifuged at 4 °C for 15 minutes at 40,000 g to separate the surface-active surfactant pellet (large aggregates (LA)) from the non-surface active

supernatant fraction (small aggregates (SA)). LA was resuspended in 2 ml NaCl, whereafter phosphorus concentration of LA and the supernatant (SA) was determined by phospholipid extraction, followed by phosphorus analysis. Finally, the ratio inactive SA to active LA surfactant was calculated.

9 | Protein concentration (Chapter 3, 6, 7)

The centrifuged supernatant was also used to determine alveolar protein concentration by using a Beckmann DU 7400 photospectrometer with a wavelength set at 595 nm (Beckmann, Fullerton, California, USA), as described by Bradford (Bio-Rad protein assay, Munich, Germany). Bovine serum albumin (Sigma, St Louis, MO, USA) was used as a standard.

10 | Matrix-metallo-proteinase activity (Chapter 3)

To determine the activity of MMP-2 and MMP-9, gelatin zymography was performed on BALf of the left lung (n=6 per group, randomly assigned). Zymography was conducted on 10% SDS-polyacrylamide gels containing 1% w/v porcine skin gelatin (Sigma-Aldrich, St. Louis, Missouri, USA). The samples were 1:1 mixed with SDS-PAGE sample buffer (0.25M Tris HCl, pH 6.8, 2% w/v SDS, 20% v/v glycerol, 0.01% v/v bromofenol blue), heated for 3 minutes at 55 ^{o}C and subjected to standard electrophoretic analysis at room temperature using the protean II system (Bio-Rad, Hercules, California, USA). After electrophoresis, gels were washed two times for 15 minutes with 2.5% Triton X-100 buffer to renature MMPs by removal of SDS. Hereafter, gels were incubated with development buffer (5mM $CaCl_2$, 50mM Tris HCl, pH 8.8, 0.02% w/v NaN_3, aquadest) for 20 hours and proteins were fixated for 15 minutes using 45% v/v methanol and 10% v/v acetic acid. Gelatinolytic activity was visualized as clear zones after staining with 0.1% w/v Coomassie Brilliant Blue R-250 in 45% v/v methanol and 10% v/v acetic acid and subsequent destaining in the same solution without Coomassie Brilliant Blue. Gels were scanned (Kodak image station 440cf; Kodak, Rochester, New York, USA) and quantified (Kodak image analysis software). A control sample was used in all gels to be able to compare the various blots. After measuring the band intensity of all blots, values were multiplied by a correction factor, determined by the values of the control sample.

11 | Western Blotting (Chapter 9)

Total lung homogenates were prepared with tissue lysis buffer and a protease inhibitor cocktail (Sigma-Aldrich Co, St Louis, Mo). From 10 to 30 mg protein was separated by either 10% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and analysed for inducible nitric oxide (iNOS) expression (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) and cleaved caspase-3 expression (Santa Cruz), respectively, by immunoblot analysis. Beta-Actin (Santa Cruz) was used as loading control. Immunoreactivity was detected by enhanced chemiluminescence (ECL; Amersham plc, Little Chalfont, UK).

12 | Cytokines (Chapter 8, 9, 10)

IL-6 and IL-10 cytokine levels were measured in total lung homogenates (Chapter 9) and IL-6, MIP-2 and TNF-alpha (Chapter 10, 11) were measured in BALf by enzyme-linked immunosorbent assay according to manufacturer protocol.

13 | Histology (Chapter 3, 7, 8, 10, 11)

After removal of the heart and lungs, the lungs were fixed at a pressure of 10 cm H_2O in 4% paraformaldehyde for 24 hours and embedded in paraffin wax. Sections were cut and stained with hematoxylin and eosin (HE). A histopathologist (MdB), blinded for the treatment, performed histological examination on the following parameters: intra-alveolar and septal edema, hyaline membrane formation, inflammation (classified as histiocytic, lymphocytic, granulocytic, and mixed), fibrosis, atelectasis, intra-alveolar hemorrhage, and overall classification. Each parameter was scored as (0) absent, (1) mild/scattered, (2) moderate/occasional, or (3) severe/frequent. Sections were overall classified as N) normal, if no abnormalities were seen, E) exudative, if pulmonary edema and/or hyaline membranes were present, F) fibroproliferative, if activated fibroblasts and/or proliferating alveolar type II cells were found, and R) resolving, if injury was on return to normal. The extent of injury was characterized as normal, patchy (<20% lung section), focal (20%-50% lung section), extensive (50%-75% lung section), or severe (75-100% of lung section). The pulmonary severity score is the sum of the individual scores of the eight categories, resulting in a possible score ranging from 0 for normal lungs to 24 for the most injured lungs. Slides were scored on a Leica DMLB light microscope and photographs were taken using a Leica DC500 camera (Leica Microsystems AG, Germany).

14 | Histology (Chapter 9)

Lung tissue specimens were fixed in formalin, dehydrated, cleared, and embedded in paraffin. Specimens were cut into 8-mm serial sections and stained with hematoxylin and eosin.

Appendix 2

Abbreviations

ALI	Acute Lung Injury
APC	Antigen Presenting Cell
ARDS	Acute Respiratory Distress Syndrome
ATII	Alveolar type II cells
ATP	Adenosine Triphosphate
BAL	Broncho Alveolar Lavage
BALf	Broncho Alveolar Lavage Fluid
BLT	Bilateral Lung Transplantation
BOS	Bronchiolitis Obliterans Syndrome
BSA	Bovine Serum Albumine
CF	Cystic Fibrosis
Cmax	Maximal Compliance of the expiration curve
CMV	Cytomegalovirus
COPD	Chronic Obstructive Pulmonary Disease
CPB	Cardio-Pulmonary Bypass
CV	Conventional Ventilation (according to ARDS network)
DBD	Donation after Brain Death
DCD	Donation after Cardiac Death
DPPC	DiPalmitoyl-PhosphatidylCholine
ECLS	Extra Corporeal Life Support
EVLP	Ex-Vivo Lung Perfusion
FACS	Fluorescence Activated Cell Sorter
FiO_2	Fraction of inspired Oxygen
FITC	Fluorescein-IsoThioCyanate
FSC	Forward Scatter
HE	Hematoxylin and Eosin
IL	InterLeukin
iNOS	Inducible Nitric Oxide Synthase

ISHLT	International Society of Heart and Lung Transplantation
LA	Large Aggregate surfactant subtype
LAS	Lung Allocation Score
LIRI	Lung Ischemia-Reperfusion Injury
MFB	Murine FACS Buffer
MHCII	Major Histocompatibility Complex II
MMP	Matrix MetalloProteinase
NO	Nitric Oxide
NRS	Normal Rat Serum
OLV	Open Lung Ventilation
PGD	Primary graft dysfunction
P_{ALV}	Alveolar Pressure
P_{AW}	Airway Pressure
P_{PL}	Pleural Pressure
P_{TA}	Transairway Pressure
P_{TP}	Transpulmonary Pressure
PaO_2	Arterial Oxygen Pressure
PBS	Phosphate Buffered Saline
PE	PhycoErythrin
PE-Cy5	PhycoErythrin-Cychrome 5
PEEP	Positive End Expiratory Pressure
PIP	Peak Inspiratory Pressure
PPH	Primary Pulmonary Hypertension
PVC	Pressure Volume Curve
ROS	Reactive Oxygen Species
SA	Small Aggregate surfactant subtype
SLT	Single Lung Transplantation
SPs	Surfactant associated Proteins
SSC	Side SCatter
TLN	Thoracic Lymph Nodes
TLR4	Toll-Like Receptor 4
TNF-α	Tumor Necrosis Factor-α
VA ECLS	VenoArterial ECLS
VILI	Ventilator Induced Lung Injury
V/V	Volume/Volume
VV ECLS	VenoVenous ECLS
Vmax	Maximal Lung Volume
W/V	Weight/Volume

Appendix 3

Contributing authors

M.A. den Bakker MD PhD. Department of Pathology Erasmus MC Rotterdam, the Netherlands; Department of Pathology Maasstad Hospital Rotterdam, the Netherlands.

A.J.J.C. Bogers MD PhD (Promoter). Department of Cardio-Thoracic Surgery, Erasmus MC Rotterdam, the Netherlands.

R.W.F. de Bruin PhD (Co-promoter). Laboratory for Experimental Surgery, Erasmus MC Rotterdam, the Netherlands.

P.M Cobelens PhD. Laboratory for Psychoneuroimmunology, UMC Utrecht, the Netherlands.

J.J. Haitsma MD PhD. Department of Critical Care Medicine, University of Toronto, Canada.

C.J. Heijnen MD PhD. Department of Immunology, UMC Utrecht, the Netherlands.

A. Kavelaars PhD. Laboratory for Psychoneuroimmunology, UMC Utrecht, the Netherlands.

J. Kesecioglu MD PhD. Department of Intensive Care, UMC Utrecht, the Netherlands.

J. Kluin MD PhD (Co-promoter). Department of Cardio-Thoracic Surgery, UMC Utrecht, the Netherlands.

B. Lachmann MD PhD. Department of Anesthesiology, Erasmus MC Rotterdam, the Netherlands. Currently at work at the department of Anesthesia and Intensive Care Medicine, Charité, Campus Virchow-Klinikum, Humboldt-University, Berlin, Germany.

R.A. Lachmann MD PhD. Department of Anesthesiology, Erasmus MC Rotterdam, the Netherlands. Currently freelancer, the World.

B.N. Lambrecht MD PhD. Department of Pulmonary Diseases, Erasmus MC Rotterdam, the Netherlands. Currently department director of VIB, life sciences research institute Flanders, Belgium.

B.P. Van Putte, MD PhD. Department of Cardio-Thoracic Surgery, St. Antonius Hospital Nieuwegein, the Netherlands.

Curriculum Vitae

Niels Peter van der Kaaij werd geboren op vrijdag 13 januari 1978 in Alphen aan de Rijn. Hij groeide op in de omgeving van Leiden en Leiderdorp. In 1996 werd het Gymnasium diploma behaald aan het Stedelijk Gymnasium Leiden. Na een teleurstellende loting voor de studie Geneeskunde, ging hij Economie studeren aan de Erasmus Universiteit Rotterdam en werd de propedeuse Economie behaald. In 1998 werd hij alsnog ingeloot voor Geneeskunde, zodat in datzelfde jaar met de studie werd gestart. Gedurende deze periode werkte hij als "Forgeron" op de afdeling spoedeisende hulp van het Ikazia ziekenhuis Rotterdam. Tevens maakte hij tussen 1999 en 2003 deel uit van het eerste heren hockeyteam van hockeyclub Klein-Zwitserland, dat uitkwam in de Hoofdklasse. Zijn wetenschappelijke afstudeerstage werd verricht op het experimentele chirurgische laboratorium van het Erasmus MC Rotterdam en mondde uit in een promotietraject onder leiding van professor Bogers van de Cardiothoracale Chirurgie. In 2006 werd de opleiding tot arts hervat met de start van de co-schappen. In april 2008 werd Cum-Laude het artsexamen behaald. Na een AGNIO periode op de afdeling Cardiothoracale Chirurgie van het UMC Utrecht, werd in maart 2009 begonnen met de opleiding tot Cardiothoracaal Chirurg in het UMC Utrecht (opleider prof.dr. L.A. van Herwerden). De verplichte tweejarige vooropleiding Heelkunde werd gevolgd in het Diakonessenhuis Utrecht onder supervisie van Dr. Clevers, waarna de resterende opleiding tot Cardiothoracaal Chirurg in 2011 werd vervolgd in het UMC Utrecht. Naar verwachting zal in 2015 de opleiding worden volbracht. Niels woont samen met Keke d'Ancona. Zij hebben samen 3 zoons: Meint, Olle en Steven.

PhD portfolio

PhD student	N.P. van der Kaaij
Erasmus MC Department	Cardiothoracic Surgery
Research School	COEUR
PhD Period	2003-2014
Promoter	Prof.dr. A.J.J.C. Bogers
Supervisors	Prof.dr. A.J.J.C. Bogers

1. PhD training

	Year	Workload (ECTS)
General academic skills		
- Laboratory animal science	2002	3
In-depth courses		
- COEUR pathophysiology of ischemic heart disease	2003	1.5
- COEUR vascular medicine	2003	1.5
- COEUR congenital heart diseases	2004	1.5
- COEUR heart failure research	2004	1.5
- COEUR arrhythmia research methodology	2005	1.5
- PACONU, virtual reality	2009	0.3
- PACONU, basic surgical skills	2009	0.6
- PACONU, anatomy	2009	0.3
- Laparoscopic surgery	2009	0.6
- PACONU, breast cancer	2010	0.3
- PACONU, evidence based medicine	2011	0.3
- PACONU, abdominal sepsis	2011	0.3
- PACONU, varices	2011	0.3
- Advanced trauma life support	2011	1.5
- Medtronic cannula training course	2012	0.6
- Clinically applied cardiac development and morphology	2012	0.6
- Fundamental critical care support course	2012	1.5
- Radiation safety course	2012	1.5
- Off-pump CABG	2013	0.6
- Anastomotic skills lab	2013	0.3
- Fundamentals in cardiac surgery part I	2014	1.5
- National cardio-thoracic surgery education program	2009-2014	1.8
Presentations		
- Dutch transplantation congress, lung ischemia reperfusion injury	2003	0.3
- SEOHS, surfactant pretreatment for lung ischemia reperfusion injury	2003	0.3
- American transplant congress, surfactant pretreatment for lung ischemia reperfusion injury	2004	0.3
- International surfactant congress, surfactant pretreatment for lung ischemia reperfusion injury	2004	0.3
- European association for cardio-thoracic surgery, surfactant pretreatment for lung ischemia reperfusion injury	2004	0.3
- Dutch cardio-thoracic surgery society, surfactant pretreatment for lung ischemia reperfusion injury	2004	0.3
- SEOHS, long term effect of surfactant pretreatment for lung ischemia reperfusion injury	2004	0.3
- SEOHS, HO treatment for lung ischemia reperfusion injury	2004	0.3
- American transplant congress, long term effect of surfactant pretreatment for lung ischemia reperfusion injury	2005	0.3

- Dutch cardio-thoracic surgery society, open lung ventilation for lung ischemia reperfusion injury	2005	0.3
- American thoracic society, long term effect of surfactant pretreatment for lung ischemia reperfusion injury	2005	0.3
- The society of thoracic surgeons, long term effect of surfactant pretreatment for lung ischemia reperfusion injury	2005	0.3
- American thoracic society, open lung ventilation for lung ischemia reperfusion injury	2006	0.3
- Dutch cardio-thoracic surgery society, optimal alveolar preservation in lung transplantation	2006	0.3
- Lung transplantation symposium, prevention and treatment of lung ischemia reperfusion injury	2008	0.3
- European association for cardio-thoracic surgery, long term effect of surfactant pretreatment for lung ischemia reperfusion injury	2008	0.3
- International society for heart & lung transplantation, open lung ventilation for lung ischemia reperfusion injury	2009	0.3
- International society for heart & lung transplantation, optimal alveolar preservation in lung transplantation	2009	0.3
International conferences		
- American transplant congress, Boston, USA	2004	1.5
- International surfactant congress, Berlin, Germany	2004	0.9
- European association for cardio-thoracic surgery, Leipzig, Germany	2004	1.5
- American thoracic society, San Diego, USA	2005	1.5
- European association for cardio-thoracic surgery, Lissabon, Portugal	2008	1.5
- International society for heart & lung transplantation, Paris, France	2009	1.5
National conferences		
- Dutch transplantation congress	2003	0.3
- SEOHS, Amsterdam	2003, 2004	0.6
- Erasmus MC science, Rotterdam	2005	0.3
- Lung transplantation, Utrecht	2008	0.3
- Oor symposium, Utrecht	2012	0.3
- Pulmonary surgery for NSCLC N2 disease, Utrecht	2013	0.3
- Safety in cardiac surgery, Utrecht	2013	0.3
- DOO, Utrecht	2013	0.6
- Dutch cardio-thoracic surgery society	2004-2014	2.4

2. Teaching activities

	Year	**Workload (ECTS)**
Lecturing		
- Thoracic Surgery, ICU and OR nurses	2012-2014	0.8
- Cardiac surgery, ICU and OR nurses	2012-2014	0.3
- Thorax trauma, ICU nurses	2012-2014	0.2
- Atrial fibrillation, anesthesiology trainees	2014	0.1
- Thymectomy, nurses & physicians	2012-2014	0.2
- Lung transplantation, physicians	2013-2014	0.2
Supervising practicals and excursions		
- Cardio-thoracic anatomy, medical students	2008-2012	1.0

3. Awards		
	Year	**Workload (ECTS)**
- Professor P.J. Klopper award	2003	
- Travel award American transplant congress, Novartis transplantation advisory board	2004	
- Young investigator award, international surfactant congress, Berlin, Germany	2004	
- Oral presentation award, Dutch cardio-thoracic society	2004	
- Prof. Dr. J.C. Birkenhäger award, Erasmus MC Rotterdam	2005	
4. Other		
- Reviewer Annals of Thoracic Surgery	2004-2014	8.4
- Reviewer PLOS one	2013-2014	0.9
- Reviewer American Journal of Respiratory Cell and Molecular Biology	2008-2014	0.6
- Reviewer European Journal of Pharmacology	2008	0.3
- Reviewer Journal of Leukocyte Biology	2014	0.3

Publications

1 | Publications this thesis

- van der Kaaij NP, et al. "Alveolar preservation with high inflation pressure and intermediate oxygen concentration reduces ischemia reperfusion injury of the lung". The Journal of Heart and Lung Transplantation 2012;31:531-537
- van Putte BP, Cobelens PM, van der Kaaij NP, et al. Exogenous surfactant attenuates ischemia-reperfusion injury in the lung by altering inflammatory and apoptotic factors. Journal of Thorac and Cardiovascular Surgery 2009;137:824-828
- van der Kaaij NP, et al. Surfactant pretreatment decreases long-term lung damage after lung ischemia-reperfusion injury. European Journal of Cardio-thoracic Surgery 2009;35:304-312
- van der Kaaij NP, Bogers AJJC. Invited commentary on: "Alveolar Macrophage Secretory Products Effect Type 2 Pneumocytes Undergoing Hypoxia/Reoxygenation". Annals of Thoracic Surgery 2008;86:1779-1780
- van der Kaaij NP, et al. Ischemia of the lung causes extensive long-term pulmonary injury: an experimental study. Respiratory Research 2008;9:28
- van der Kaaij NP, Bogers AJJC. Invited commentary on: "Respiratory viral infection in obliterative airway disease following orthotopic tracheal transplantation". Annals of Thoracic Surgery 2006;82:1050-1051
- Van der Kaaij NP, et al. "Surfactant alterations and treatment in lung transplant

ischemia-reperfusion injury". Journal Organ Dysfunction 2006;2:221-229

- Van der Kaaij NP, et al. "Surfactant pretreatment ameliorates ischemia-reperfusion-injury of the lung". European Journal of Cardio-thoracic Surgery 2005;27:774-782

2 | Other publications

- van der Kaaij NP, Kluin J. Invited commentary on: "Risk factors for early mortality and morbidity after pneumonectomy: a reappraisal". Annals of Thoracic Surgery 2009;88:1743-1744
- van der Kaaij NP, Bogers AJJC. Invited commentary on: "Cytokines link Toll-like receptor 4 signalling to cardiac dysfunction after global myocardial ischemia". Annals of Thoracic Surgery 2008;85:1685
- Van der Kaaij NP, et al. "Ischemia-reperfusion-injury of the lung: Role of surfactant". Yearbook of intensive Care and Emergency Medicine, Springer-Verlag, Berlin Heidelberg 2005;49-62
- Ten Raa S, Oosterling SJ, van der Kaaij NP, et al. "Surgery induces enhanced implantation of disseminated tumor cells, but does not increase growth of tumor cell clusters". Journal of Surgical Oncology 2005;92:124

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