

RESEARCH ARTICLE

Transbronchial administration of adenoviral-mediated interleukin-10 gene to the donor improves function in a pig lung transplant model

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Interleukin-10 (IL-10) gene transfection of donor lungs prior to transplantation is an attractive strategy to reduce ischemia–reperfusion induced lung injury. However, experimental data with gene therapy in large animal models of lung transplantation are generally lacking. We have developed a simple clinically applicable technique for adenoviral-mediated gene delivery of human IL-10 to the lung of large animals that provides homogenous gene expression after 12–24 h of transfection. Using this technique of gene delivery, we have studied the dynamics of adenoviral gene delivery to the lung in the setting of lung transplantation. Although there is a persistent inflammatory response to the adenoviral vector, we achieved significant expression of human IL-10 in lung tissue before lung retrieval to obviate the

deleterious impact of the adenoviral vector on the donor lung. The administration of adenoviral-mediated human IL-10 to the donor lung reduced ischemia–reperfusion injury and improved graft function after lung transplantation in this pig lung transplantation model. Transfection of adenoviral-mediated human IL-10 to the donor lung prevented the release of inflammatory cytokines such as IL-6 in lung tissue and plasma. We have demonstrated that IL-10 gene therapy has significant potential to prevent or treat the inflammatory response associated with ischemia–reperfusion injury in lung transplantation. In the future, IL-10 gene therapy could also be used for immunomodulation or tolerance induction.

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Introduction

Lung transplantation has enjoyed increasing success and has become the mainstay of therapy for patients with a variety of end-stage lung diseases. Over the past 10 years, the number of recipients on the waiting list has been progressively increasing and now far exceeds the number of organs available.¹ Consequently, the median waiting time for lung transplantation has nearly doubled in the United States, and some centers in Europe have reported that up to 50% of patients awaiting lung transplantation die on the waiting list.²

The persistent shortage of lung donors has led to increasing interest in re-evaluating the existing lung donor pool. Traditionally, the rigid selection criteria that were developed in the early clinical experience limited clinicians to using 10% or less of the donor pool. Over the years, the donor selection criteria have been expanded to the use of 'nonideal' lung donors.^{3–6} However, this strategy can contribute to an increased risk of immediate graft dysfunction that in turn may lead to enhanced

rejection and impaired long-term graft function.^{3,7–9} Hence, the development of new strategies to repair and improve the quality of donor lungs could have a tremendous impact on the number of transplants performed as well as on the outcome after transplantation.

Genetic modification of donor organs using gene therapeutic approaches is one such novel and promising strategy. The utilization of gene therapy in the transplantation setting is facilitated by the fact that the immunosuppressive therapy used to prevent graft rejection allows more effective and prolonged transfection.¹⁰ This strategy, however, is hindered by the poor transfection rate obtainable during the period of cold temperature required to preserve the organ, and by the drawback of unnecessarily transfecting other organs if the gene is delivered systemically instead of locally.^{11,12} To overcome these problems, in previous experiments we delivered the gene to the donor lung through the transtracheal route before retrieving and cooling the lungs to 4°C.¹³ This technique appears to be superior to other techniques of gene delivery and avoids unnecessary transfection of other organs such as the heart, liver, or kidneys.^{12,13}

Cytokines have been shown to play a critical role in modulating inflammatory processes and in enhancing cellular infiltration in transplanted organs. In human lung transplantation, we and others have observed that

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cytokines are detectable in lung tissue during the transplant process and that the level of interleukin-8 (IL-8) negatively correlated with lung function and outcome.^{14,15} Hence, we hypothesized that upregulation of a potent anti-inflammatory cytokine such as Interleukin-10 (IL-10) in the donor lung before graft retrieval could potentially limit ongoing lung inflammation and prevent further injury related to ischemia and reperfusion.

The transtracheal administration of adenoviral-mediated human IL-10 gene to donor rat lungs 12–24 h prior to lung retrieval reduces ischemia–reperfusion injury and improves post-transplant graft function in an isogenic rat single lung transplant model.^{16,17} The beneficial impact of IL-10 on ischemia–reperfusion injury has also been demonstrated by other investigators working in the field of lung transplantation.^{18,19} However, these experiments were all performed in small animal models, and the applicability to large animals remains to be demonstrated before any potential application in humans appears feasible.

In this study, we have developed a simple and reproducible method to deliver gene to the lungs of large animals, and to obtain significant gene expression without causing significant adverse reactions related to the adenoviral vector after up to 24 h of transfection. We have further demonstrated that the administration of IL-10 gene therapy can prevent ischemia–reperfusion-induced lung injury in a large animal when transfection is performed 24 h prior to donor lung retrieval.

Materials and methods

Animals

Yorkshire male domestic pigs (24–41 kg) were used for all study phases. Animals were purchased from 'Remens Fur Ranch' (Kitchener, Ontario, Canada). All animals received care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research, the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996, U.S. Government Printing Office, Washington, DC 20402-9325), and the Guide to the Care and Use of Experimental Animals formulated by the Canadian Council on Animal Care. The Animal Care Committee of the Toronto General Hospital Research Institute approved the Experimental protocol. All experiments in this study were performed in a blinded manner.

Generation of recombinant adenovirus expressing human IL-10

Second generation (E1, E3 deleted) adenoviral vectors (serotype 5) containing human IL-10 gene with a Rous Sarcoma Virus (RSV) promoter (AdRSVhIL-10) or empty vectors (AdBGL2) were constructed at the Gene Transfer Vector Core of the University of Iowa College of Medicine (Iowa City, IA, USA). The human IL-10 adenoviral construct is referred to as 'AdhIL-10' and the Ad5BGL2 is referred to as the 'empty vector'.

Human IL-10 cDNA was obtained by polymerase chain reaction (PCR) with 5' and 3' flanking primers (5'-hIL-10*Bam*HI, 5'-CGCGGATCCCATGCACAGCT-CAGC-*ACTG*-3'; 3'-hIL-10*Bam*HI, 5'-CGCGGATCCGCCACCCT-GATGTCTCAGT-3'), using the clone pSR α hIL-10 as

template (kindly provided by E Field, University of Iowa). The PCR product was cloned, using the *Bam*HI restriction site tails added to the oligonucleotide sequences, in a shuttle plasmid (pAdRSV4). This shuttle plasmid contains the RSV promoter, the simian virus 40 (SV-40)-poly(A) signal, and the genomic adenoviral sequences from 0–1 and 9–16 map U of human adenovirus type 5. Recombinant adenovirus expressing IL-10 was generated by homologous recombination between AdhIL-10 and human adenovirus serotype 5 derivative *dI309*, using standard methods.²⁰ AdBGL2 has the same viral backbone as AdRSVhIL-10.

Transfection procedure

All pigs received 1 g of methylprednisolone (Solu-medrol[®], Pharmacia & Upjohn Inc., Mississauga, Ontario, Canada) intramuscularly (i.m.) 30 min before premedication with ketamine (Ketalan[®] Bimeda-MTC Pharmaceuticals, Cambridge, Ontario, Canada) 40 mg/kg i.m. Induction of general anesthesia was achieved with 2.5% sodium pentobarbital (Pentothal[®], MTC Pharmaceuticals, Cambridge, Canada) 5 mg/kg intravenous (i.v.), and general anesthesia was maintained with Propofol (Diprivan[®], Novopharm Limited, Toronto, Canada) 5 mg/kg/h i.v. and Fentanyl Citrate[®] (Abbott Laboratories Limited, Toronto, Canada) 25 μ g/kg/h i.v. Animals were then intubated with a number 8 endotracheal tube (Portex[®], Sims, Markham, Canada) inserted through the mouth or through a tracheostomy according to whether they were awakened or kept anesthetized between the transfection and the retrieval procedures, respectively. Ventilation was performed with a volume-controlled ventilator (Servo Ventilator, Siemens-Eléma Ab, Sweden). Mechanical ventilation was performed with an FiO₂ of 100% during the transfection procedure and 40% thereafter. Respiratory rate was set at 16–18 breaths/min, tidal volume at 10 ml/kg, and positive end expiratory pressure (PEEP) at 5 cm H₂O. Pancuronium Bromide[®] (Pancuronium Bromide, Sabex Inc., Boucherville, Canada) 100 μ g/kg i.v. was used for neuromuscular relaxation.

After the induction of anesthesia, the transfection procedure was started. An adult flexible fiberoptic bronchoscope (Olympus[®] BF type 1T 20, Japan) was inserted through the endotracheal tube. The vector (AdhIL-10 or AdBGL2) was diluted in 4 ml of normal saline. A catheter was inserted through the bronchoscope channel used to deliver 3 ml into each segmental bronchus. This was followed by 3 ml of saline solution to flush the catheter. A total of about 25 ml of normal saline per lung was injected. The control group received the same amount of normal saline, but no vector was injected. The whole procedure took approximately 30 min.

Donor procedure

Pigs were sedated with ketamine (40 mg/kg i.m.), anesthetized with pentobarbital (6–8 mg/kg i.v.), and maintained with propofol (5–8 mg/kg/h i.v.), and fentanyl citrate (2–20 μ g/kg/h i.v.). Animals were ventilated with a volume-controlled ventilator (Servo Ventilator, Siemens-Eléma Ab, Sweden) at a tidal volume of 10 ml/kg, PEEP of 5 cm H₂O, with a respiratory rate of 16–18 breaths/min, and an FiO₂ of 100%. A median sternotomy was performed, the thymus was removed,

the pericardium opened, and the superior and inferior vena cavae were encircled with silk ties. A bolus of Heparin (Hepalean[®], Leo Pharma Inc., Ajax, Canada) 15 000 U was injected i.v. A 21 French cannula was inserted into the main pulmonary artery (PA) and secured with a 4-0 prolene (Ethicon, Peterborough, Canada) purse-string suture. To minimize pulmonary atelectasis, a volume recruitment manoeuvre with a sustained airway pressure of 25 cm H₂O was applied for 20 s. A bolus of 500 µg of Prostaglandin E₁ (Alprostadil[®] Pharmacia & Upjohn Inc., Mississauga, Ontario, Canada) was then injected into the main PA. Once the systemic blood pressure started to drop, the superior and inferior vena cavae were ligated, the left atrial appendage was transected, and the PA was flushed with 60 ml/kg of low-potassium dextran glucose preservation solution (LPDG) solution (Perfadex[®]; Vitrolife, Uppsala, Sweden) at 4°C from a height of 30 cm above the heart. Ventilation was continued throughout the extraction of the heart–lung block. The trachea was clamped with the lungs inflated with a sustained airway pressure of 15 cm H₂O. After removal of the heart–lung block, a retrograde flush through the left atrium with 15 ml/kg of LPDG solution was performed on the back table. The heart–lung block was then placed in a plastic bag containing 500 ml of LPDG, double bagged, then stored at 4°C in a cold room (Constant Temperature Control Ltd, Weston, Canada). The room was monitored with a temperature-recording device (Series AR100 Recorder Controller, Honeywell, Minneapolis, MI, USA) to confirm the reliability of the storage conditions. All lungs remained well inflated over the 18 or 24 h storage periods.

Transplantation procedure

Recipient pigs were sedated and anesthetized as in the donor animals. However, a bronchial blocker (Univent tube, Phycon-Fuji Systems Corp., Tokyo, Japan) was used for single lung ventilation. After induction, 1 g of Solu-medrol[®] i.v. was given. Vascular catheters were inserted into the femoral artery and vein. A PA catheter (Swan-Ganz, American Edwards Laboratories, Añasco, Puerto Rico) was then inserted through the left internal jugular vein. The arterial line and the PA catheter were attached to a multichannel recorder (Hewlett Packard, Mississauga, Canada) to continuously monitor the pressure. Ringer's Lactate and NaCl 0.9% was used for volume replacement at a rate of 100 ml/h. A left thoracotomy was performed through the fourth intercostal space. The pulmonary hilum was dissected and the left azygous vein was carefully elevated from the left atrium and ligated. The inferior pulmonary ligament was divided. Both the right and left main pulmonary arteries were carefully dissected taking care to avoid injury to either the vagus or the phrenic nerves. The right PA was encircled with an umbilical tape proximal to the first branch of the right upper lobe. A bolus of heparin (3000 U i.v.) was then given, followed by administration of an additional 1000 U every 2 h. A vascular clamp was placed proximally on the left main PA and it was divided distally. The left pulmonary veins were ligated and divided. Then, the bronchus was divided and the bronchial blocker was inflated in the left main bronchus. The tidal volume was reduced to 5–8 ml/kg and the respiratory rate adjusted to keep the PCO₂ between 35–40 mmHg and the PO₂ over 95 mmHg (Ciba-Corning

blood gas system model 278, Ciba-Corning, Markham, Canada) during one lung ventilation. The donor left lung was then prepared on the back-table.

Once the donor lung was ready, the bronchial anastomosis was performed first with a running 4-0 prolene suture on the posterior wall and interrupted 4-0 prolene sutures on the anterior wall. The PA anastomosis was performed next with a continuous 6-0 prolene suture interrupted in two places. The atrial anastomosis was then performed with a continuous everting horizontal mattress suture using 6-0 prolene interrupted in two places. After re-inflation of the transplanted lung to a pressure of 20–25 cm H₂O, the PA clamp was removed gradually and the lung was de-aired through the left atrial anastomosis. The period of warm ischemia was relatively constant between each animal (mean: 62 ± 8 min). The lungs were then ventilated with 100% oxygen, 5 cm H₂O PEEP, and respiratory rate adjusted to keep the PCO₂ between 35 and 40 mmHg. Volume recruitment manoeuvres with a sustained airway pressure of 20 cm H₂O for 30 s were performed every 30 min after reperfusion. An infusion of Norepinephrine[®] (Norepinephrine Bitartrate, Sabex Inc., Boucherville, Canada) was titrated in the range of 0.07–1 mg/ml/h during reperfusion to maintain a systolic blood pressure over 80 mmHg.

Study phases

Phase I – optimization of gene transfection. The goal of the first phase of the study was to develop and optimize a simple and reproducible technique of gene delivery in a large animal. We examined the timing of transfection as well as the dose of AdhIL-10 to use to obtain measurable levels of human IL-10 expression before lung retrieval. Expression of human IL-10 was detected by enzyme-linked immunosorbent assay (ELISA) on tissue homogenates as well as by immunohistochemistry on lung section.

Phase II – detection of adverse reactions after the transplantation procedure secondary to the application of adenoviral vector. The goal of second phase of the study was to detect any adverse reactions due to the administration of adenoviral vector, and to determine whether transfection of the anti-inflammatory cytokine, IL-10, could potentially prevent these reactions. Animals were thus randomly divided into three groups ($n = 5$ per group): Group 1 received 4×10^{10} pfu of AdhIL-10 into the left lung (AdhIL-10 group), group 2 received 4×10^{10} pfu of Ad5BGL2 into the left lung (Empty vector group), and group 3 received normal saline solution into the left lung (Control group). The lungs were transfected for 12 h in the donor and then extracted and stored in LPDG (Perfadex[®]) for 18 h before transplantation. After the transplantation procedure, the lungs were reperfused for 2 h. All experiments and analyses in this study were performed in a blinded fashion.

Phase III – examination of the potential therapeutic impact of human IL-10 gene therapy in ischemia–reperfusion-induced lung injury. The goal of the third phase of the study was to examine the impact of IL-10 gene therapy during ischemia–reperfusion injury in a large animal model of lung transplantation. Animals

were, thus, divided into two groups: Group 1 ($n=4$) received 4×10^{10} pfu of AdhIL-10 into the left lung (AdhIL-10 group), and Group 2 ($n=3$) received normal saline solution into the left lung (Control group). At 24 h after transfection, the lungs were excised from the donor and stored in LPDG for 24 h before transplantation. After the transplantation procedure, the lungs were reperfused for 2 h.

Measurement of lung graft function

Blood gas analyses were performed every hour during the reperfusion period. Blood gas samples were taken 10 min after the right PA was occluded with a tourniquet in order to measure function of the transplanted lung only.²¹ Oxygenation of the arterial blood (PaO_2) during single lung ventilation of the transplanted lung is considered to be the gold standard to evaluate the pulmonary graft function. Peak airway pressures (PawP) were continuously measured throughout the reperfusion period. The wet-to-dry (W/D) lung weight ratio was calculated at the end of the reperfusion period. A lung biopsy was weighed, and then placed in an oven at 85°C for 24 h. After this drying procedure, the sample was reweighed, and the ratio of the lung weight before and after drying was calculated. The W/D weight ratio reflects the degree of lung water in the lung at the end of the reperfusion period.

Tissue and plasma samples

Lung tissue biopsies were taken at the end of the cold ischemic time (CIT, the end of the cold storage period), at the end of the warm ischemic time (WIT, the end of the implantation procedure), and then hourly during the reperfusion period. Half of the biopsy was fixed in 10% buffered formalin and embedded in paraffin, whereas the other half was immediately snap frozen in liquid nitrogen and preserved at -70°C . Blood samples were also taken hourly during reperfusion, centrifuged at 5000 rpm for 10 min and the plasma was immediately snap frozen in liquid nitrogen and stored at -70°C .

Tissue preparation and ELISA for cytokines

Tissues were homogenized and incubated at 4°C in cell lysis buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.6% octylphenoxy-polyethoxy-ethanol (Nonidet P-40), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), Aporotinin and Leupeptine. Homogenates were then sonicated and centrifuged at 14 000 rpm for 10 min at 4°C . Supernatants and plasma samples were assayed in duplicate using the specific Cytoscreen[®] immunoassay for swine TNF- α , IFN- γ , interleukin-2 (IL-2), IL-8, and IL-10 (Biosource International, Camarillo, CA, USA), and the Quantikine[®] immunoassay for human IL-10 and swine interleukin-6 (IL-6) (R&D Systems, Minneapolis, MN, USA). The optical density of each well was read at 450 or 540 nm according to the manufacturer's instruction with an NM-600 microplate reader (Dynatech Laboratories, Chantilly, VA, USA). The final concentration was calculated by converting the OD readings against a standard curve. The protein content was determined by the method of Bradford.²² The ELISA assay for human IL-10 did not crossreact with endogenous swine IL-10 as confirmed in assays on lungs that were not transfected with hAdhIL-10. The absence of

crossreactivity was also confirmed by assaying the respective standards provided with each kit (swine and human) with the opposite kit (human and swine).

Immunocytochemistry for human IL-10

Formalin-fixed, paraffin-embedded tissue sections ($5 \mu\text{m}$) were mounted on positively charged microscope slides. Tissue sections were then incubated for 12 h at 60°C , deparaffinized in xylene, and rehydrated through decreasing concentrations of alcohol. All sections, including negative controls slides, were treated before the application of the primary antibody with a protein blocker for endogenous peroxidase and biotin. After a brief wash in phosphate-buffered saline-bovine serum albumin (PBS-BSA) the specific primary polyclonal goat anti-human IL-10 antibody (R&D Systems, Minneapolis, MN, USA) was applied at a 1:50 dilution. Negative control slides were prepared using two methods: (1) primary antibody was omitted and replaced by PBS-BSA, and (2) primary antibody was omitted and replaced by a generic non-immun goat serum at the same concentration as the working dilution of the primary antibody. All slides were incubated overnight at 4°C .

The detection procedure was performed with the Elite Vector Stain ABC system (Vector Laboratories, Burlingame, CA, USA) as recommended by the manufacturer. Color visualization was performed using 3,3'-diaminobenzidine (DAB) as the chromogen substrate (Sigma, St Louis, MO, USA). The counterstain of preference was hematoxylin for nuclear detail.

Statistical analysis

All data are expressed as mean \pm s.d. Statistical analysis of the physiologic data was performed using Student's *t*-tests or one-way analysis of variance (ANOVA) when necessary. For differences in peak airway pressures in transplanted lungs over the 2 h reperfusion period, the Friedman repeated measures ANOVA on ranks was used. Differences in cytokine measurement at each time point were analyzed by repeated measures ANOVA (Friedman test). $P < 0.05$ was considered statistically significant. When statistical significance was reached, it was followed by a *post hoc* analysis using the Student-Newman-Keul method. The StatView[®] software package version 5.0.1 (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses.

Results

Phase I – optimization of gene transfection

Using a commonly available clinical fiberoptic bronchoscope, we developed a simple and reproducible method to deliver a transgene to each segment of the lung. We then determined the optimal dose of AdhIL-10 to use in a large animal. This was an essential part of the experiment as one cannot simply extrapolate the dose of AdhIL-10 vector on a weight for weight basis between rats and pigs. Hence, we performed a dose-escalation study to determine the minimal amount of vector to use to obtain significant expression of human IL-10 after 12 h of transfection. We progressively administered doses ranging from 5×10^9 , which was effective in the rat,¹⁶ to 1×10^{10} , 2×10^{10} and 4×10^{10} pfu of AdhIL-10. A period

of transfection of 12 h was chosen based on previous results in our rat model.¹⁷

Doses lower than 4×10^{10} pfu of AdhIL-10 did not provide measurable amounts of human IL-10 in the pig lung tissue. However, when transfection was carried out with 4×10^{10} pfu of AdhIL-10 administered to the left lung only (which is the lung used for transplantation in our model), a significant level of human IL-10 was detected in lung tissue after 12 h of transfection (Figure 1). Furthermore, as expected, transfection with a dose of 4×10^{10} pfu for a period of 24 h achieved an even higher transfection level. The distribution of human IL-10 after 12 and 24 h of transfection was homogenous across the different segments of the left lung (Figure 2). No expression of human IL-10 was detected in the right lung when only the left lung was transfected. Immunostaining of the left lung for human IL-10 demonstrated expression in epithelial cells of large airways and to a lesser degree in alveolar cells, macrophages, and interstitial cells (Figure 3). Importantly, all animals remained stable throughout the transfection period of 12 and 24 h and tolerated the procedure without any adverse effects.

Phase II – the inflammatory response induced by the empty adenoviral vector can be suppressed by the transgene product (human IL-10)

Based on the results of phase I, we used a vector dose of 4×10^{10} pfu administered to the donor pig left lung and a transfection period of 12 h. A transfection period of 12 h was chosen since it was associated with significant expression of human IL-10 and it was in a clinically relevant timeframe for the setting of lung transplantation. Animals were randomly allocated into one of the three groups (Control, Empty Vector, and AdhIL-10). All animals were intubated and ventilated during the transfection period, and remained stable during the entire period with no evidence of adverse effects. The PaO₂/FiO₂ ratio remained above 400 mmHg during the 12 h transfection period in all three groups (Figure 4). The PaO₂ on an FiO₂ of 100% at the time of retrieval was also similar between groups: 500 ± 37 , 494 ± 15 , and

471 ± 35 mmHg in the Control, Empty Vector, and AdhIL-10 group, respectively ($P = 0.3$).

After a period of 18 h of cold ischemia followed by transplantation and 2 h of reperfusion, the group transfected with the AdhIL-10 maintained excellent lung function with a PaO₂ of 523 ± 71 mmHg at the end of reperfusion. The control group had a PaO₂ of 452 ± 69 at the end of reperfusion. In contrast, the Empty vector group had a significantly worse lung function with a PaO₂ of 252 ± 91 mmHg at the end of reperfusion. The W/D lung weight ratio and the PawP were also significantly worse in the Empty vector group than in the Control group and AdhIL-10 group (Figure 5). The difference between the AdhIL-10 and control group did not achieve statistical significance. This finding likely reflects the injurious impact of the adenoviral vector on lung function. However, it also shows that the addition of an anti-inflammatory transgene such as IL-10 can obviate this deleterious impact as long as sufficient time is allowed to obtain significant levels of transgene expression (IL-10) before the period of ischemia. The inflammatory reaction observed in lung parenchyma of animals treated with the empty vector only disappeared when IL-10 was transfected along with the vector (Figure 6).

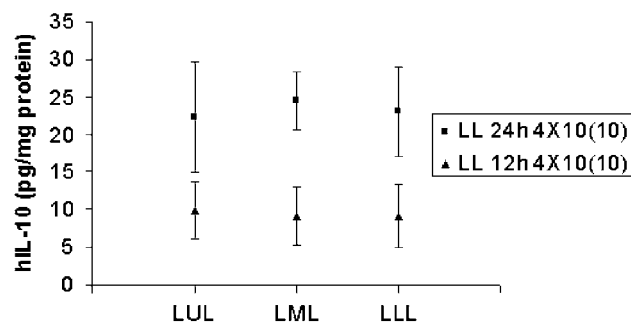


Figure 2 The distribution of human IL-10 was homogeneous after 12 and 24 h of transfection with 4×10^{10} pfu of AdhIL-10 administered to the left lung. The levels were similar in the left upper lobe (LUL), left middle lobe (LML), and left lower lobe (LLL) of the left lung.

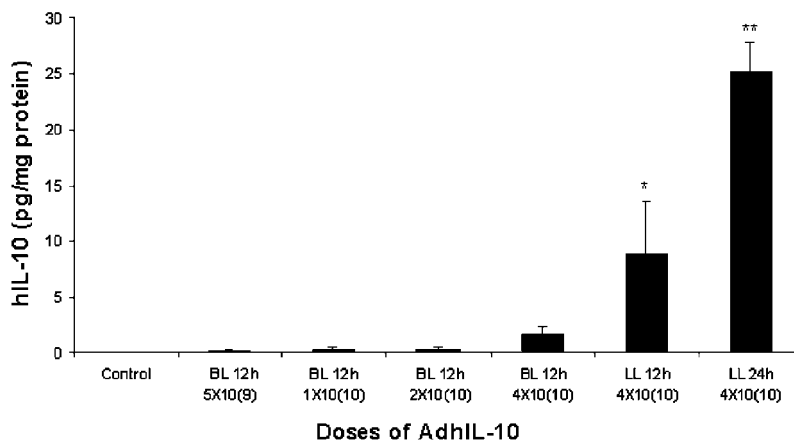


Figure 1 Dose-escalation study to determine the minimal amount of adenoviral-mediated IL-10 gene (AdhIL-10) to administer in order to obtain significant expression of human IL-10 after 12 h of transfection. A dose of 5×10^9 pfu ($n = 2$), 1×10^{10} pfu ($n = 2$), 2×10^{10} pfu ($n = 2$), and 4×10^{10} pfu ($n = 3$) administered to both lungs did not give a measurable amount of human IL-10 after 12 h of transfection. A dose of 4×10^{10} pfu administered to the left lung produced significant level of human IL-10 after 12 h of transfection ($n = 3$). The level of human IL-10 was even greater if the transfection period was extended to 24 h ($n = 3$). ** $P < 0.01$ and * $P < 0.05$ when compared to Control. BL: both lungs; LL: left lung.

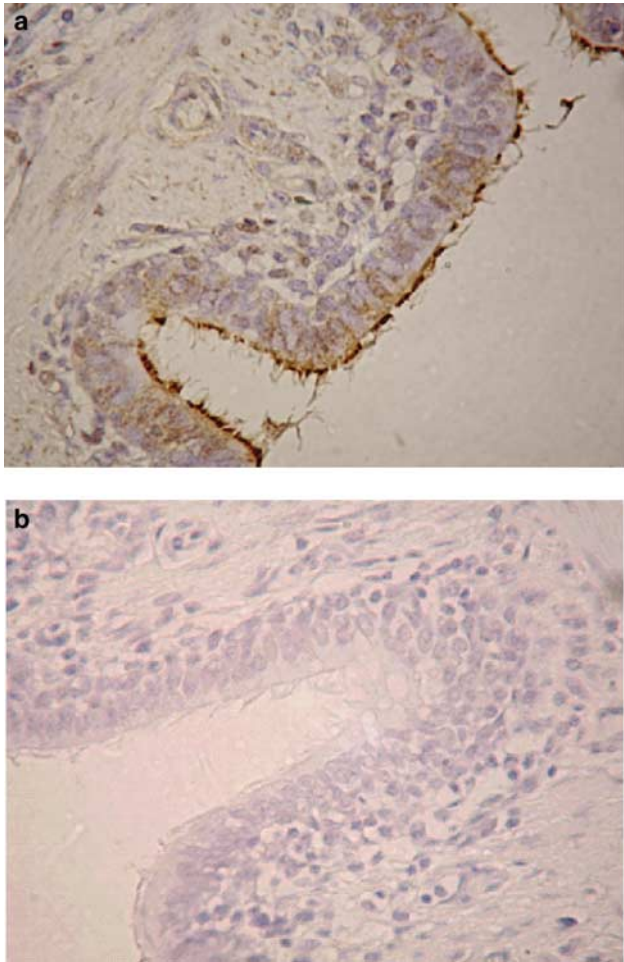


Figure 3 (a) Immunostaining for human IL-10 in pig lung tissue after 12 h of transfection demonstrates expression of the transgene in epithelial cells of large airways as well as to a lesser degree in macrophages, and interstitial cells. (b) Immunostaining with the control antibody shows no staining.

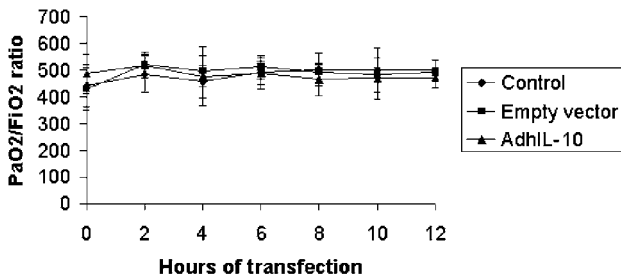


Figure 4 All donor animals remained stable with no evidence of adverse effect throughout the 12 h transfection period. The mean PaO₂/FiO₂ ratio remained between 400 and 500 mmHg in the Control group as well as in the group transfected with the empty vector and in the group transfected with human IL-10 (AdhIL-10). This finding demonstrates the absence of significant toxicity from the adenoviral vectors on lung function during the transfection period.

Phase III – transfection of IL-10 improves lung function and reduces ischemia–reperfusion-induced lung injury
The difference between the control group and the group transfected with AdhIL-10 was not significantly different

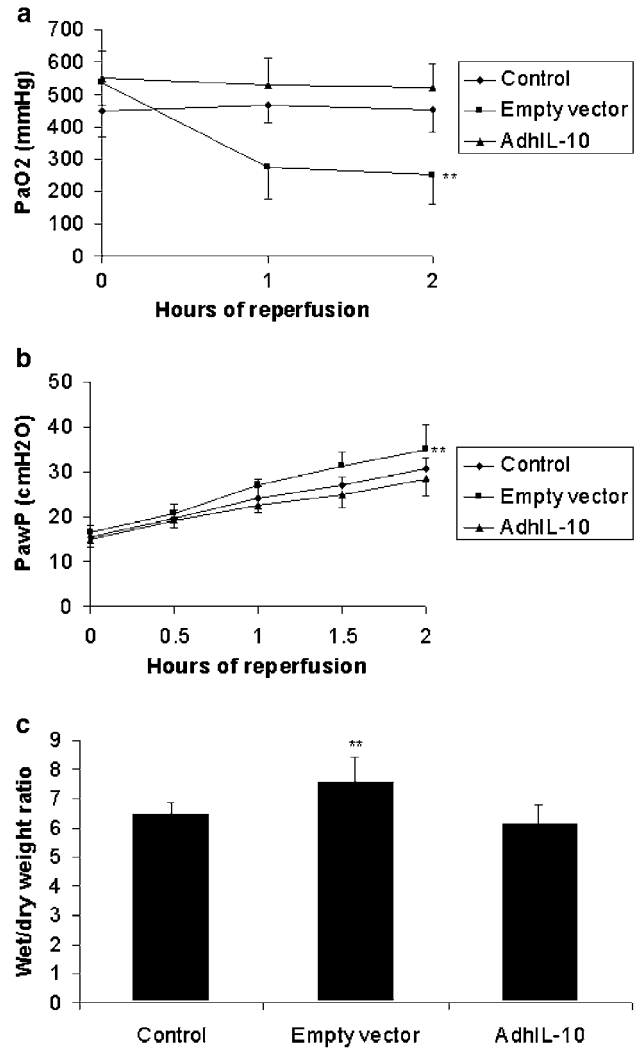


Figure 5 Transplanted lung function after 12 h donor transfection followed by 18 h cold ischemia and 2 h reperfusion. Three groups ($n=5$ /group) were compared to determine the impact of the empty vector on lung function after reperfusion. One group was transfected with human IL-10 (AdhIL-10), one group with an empty vector (Empty vector), and one group with normal saline (Control). Despite the absence of toxicity of the empty vector on lung function during the transfection period (Figure 4), the Empty vector group presented with significantly worse lung function after reperfusion when compared to the Control group and the AdhIL-10 group. The oxygenation of the transplanted lung (PaO₂) was significantly lower (a), the peak airway pressure (PawP) was significantly higher (b), and the wet/dry lung weight ratio, which is a marker of lung edema, was significantly worse in the Empty vector group (c). ** $P<0.01$ when compared to the Control group and AdhIL-10 group.

in phase II because the ischemic time (18 h) was too short and not injurious enough to be able to demonstrate the benefit of the intervention. Hence, a longer period of ischemia was used to investigate whether the administration of AdhIL-10 had a protective effect on the transplanted lung during ischemia–reperfusion injury. For this purpose, we increased the cold ischemic time from 18 to 24 h, and used a longer period of transfection, that is, 24 h, in order to have greater amount of human IL-10 in the lung tissue. A dose of 4×10^{10} pfu of AdhIL-10 administered to the donor left lung was again used for transfection.

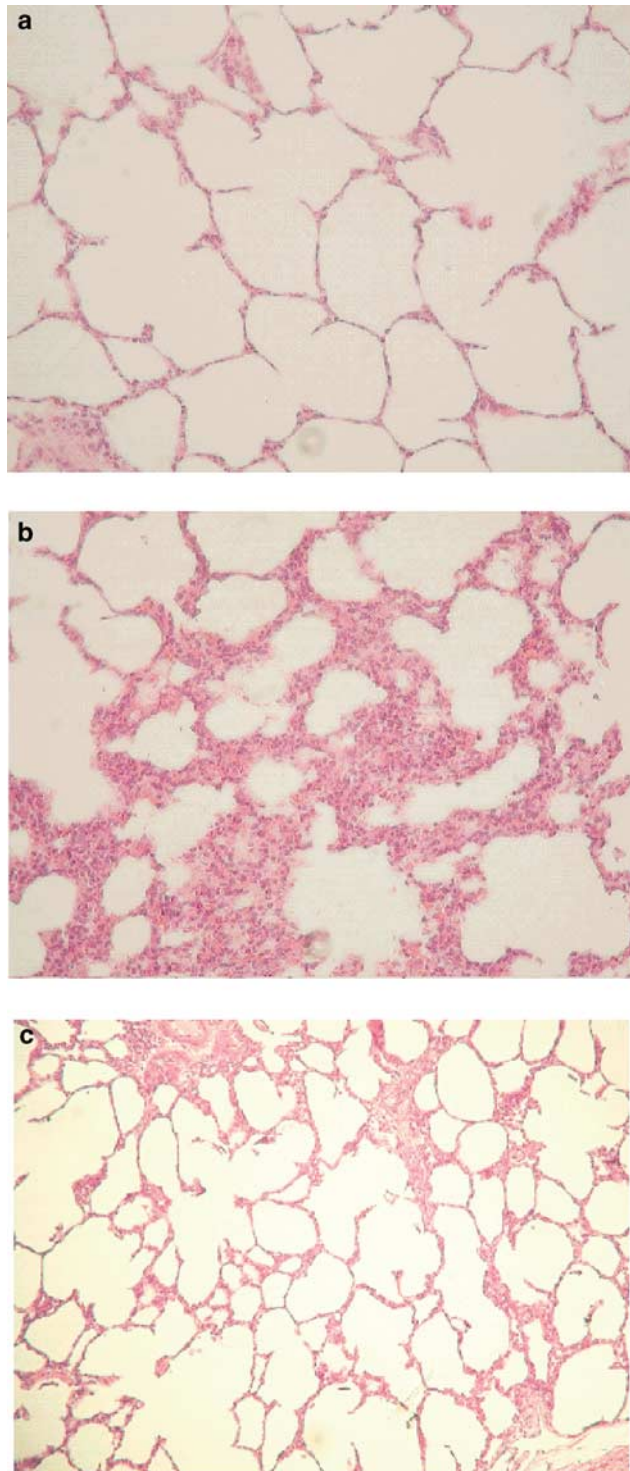


Figure 6 Hematoxylin and eosin staining of control (a), empty vector, (b) and AdhIL10 (c) lung graft 2 h after reperfusion. The inflammatory reaction observed in lung parenchyma of animals treated with the empty vector was not seen when IL-10 was transfected along with the vector.

Lung function in the animals transplanted with donor lungs that had been transfected with AdhIL-10 24 h prior to retrieval followed by 24 h of cold ischemic storage was significantly better than the Control group (Figure 7). Indeed, the mean PaO₂ remained close to 400 mmHg throughout the reperfusion period in the AdhIL-10

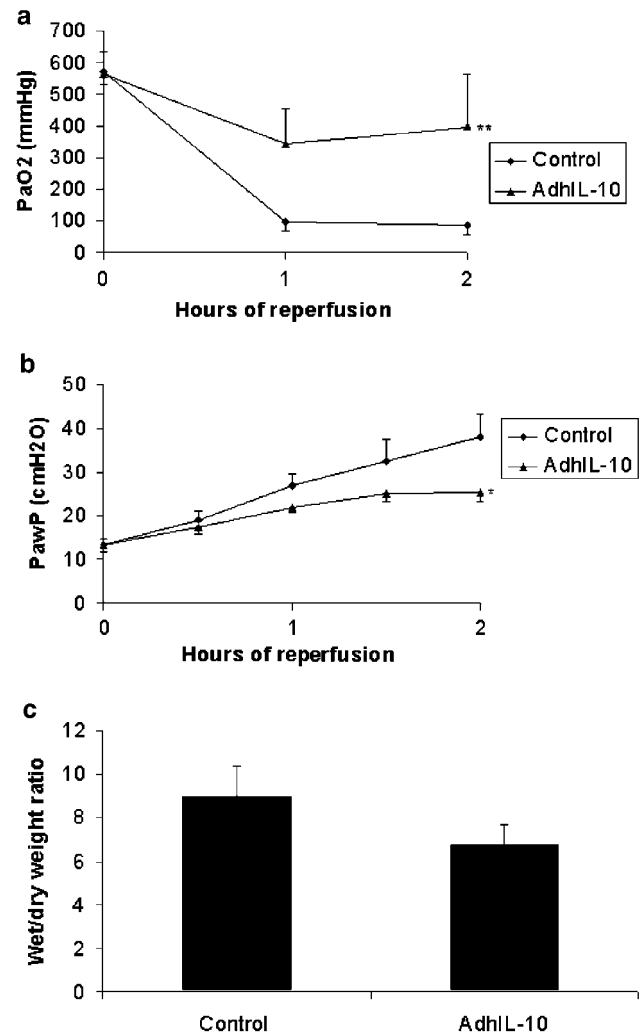


Figure 7 Two groups were compared to determine the impact of human IL-10 on ischemia–reperfusion injury after a period of 24 h of cold ischemia and 2 h of reperfusion. One group was transfected with human IL-10 (AdhIL-10, n = 4) and one group with normal saline (Control, n = 3). The group transfected with human IL-10 had significantly better lung function during the reperfusion period than the Control group. The oxygenation of the transplanted lung (PaO₂) was significantly higher (a), the peak airway pressure (PawP) was significantly lower (b), and the wet/dry lung weight ratio tended to be better in the AdhIL-10 group (c). **P < 0.01 and *P < 0.05 when compared to the Control group.

group, whereas it dropped to less than 100 mmHg in the Control group. Similarly, the PawP was significantly better in the group of animals transfected with AdhIL-10 than in the Control group at the end of the reperfusion period. The W/D lung weight ratio also tended to be lower in the AdhIL-10 group than in the Control group at the end of the 2 h reperfusion, but this difference did not reach statistical significance (P = 0.2).

Cytokine release in recipient transplanted lung tissue and plasma

Swine TNF- α , IFN- γ , IL-2, IL-6, IL-8, IL-10, and human IL-10 were assayed in lung tissue homogenates and in the plasma during the reperfusion period. As expected, human IL-10 was detected only in lung tissue from the groups transfected with AdhIL-10 (Figure 8). IL-6 was

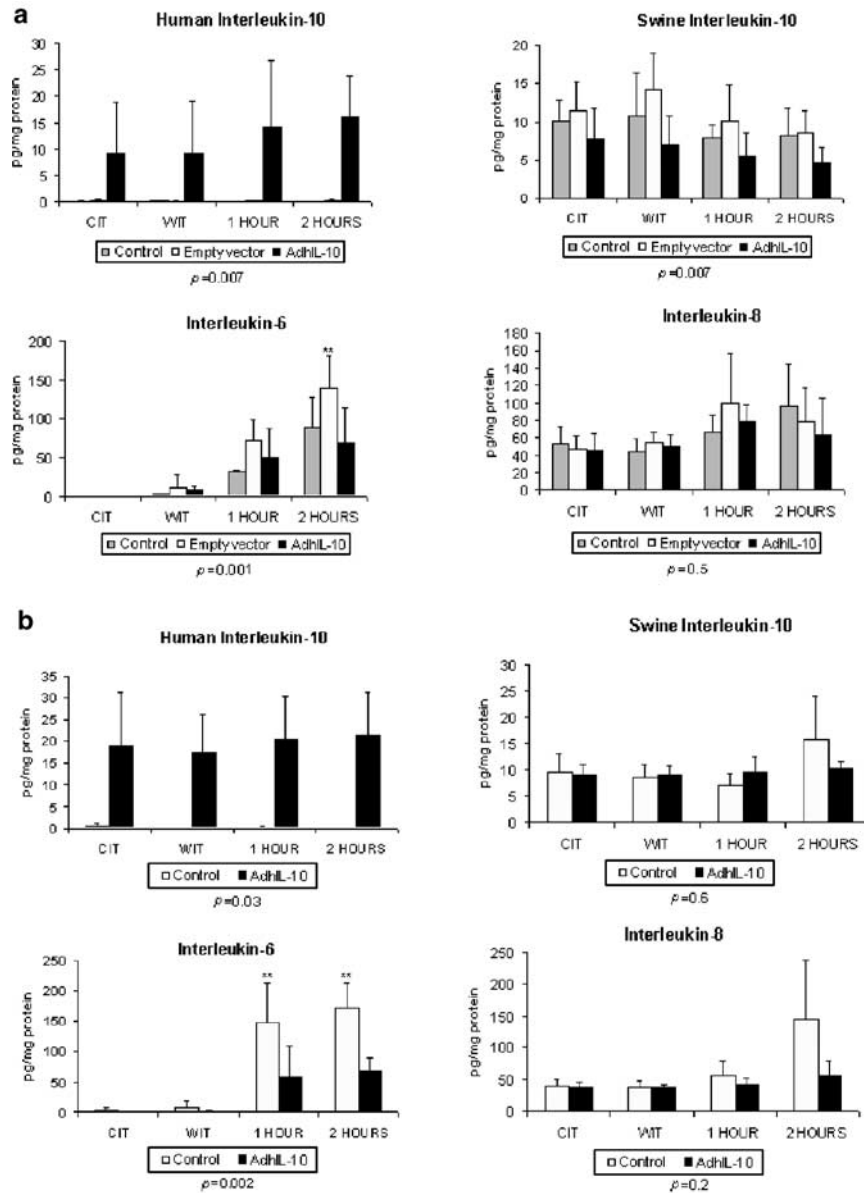


Figure 8 ELISA was used to measure cytokines in lung tissue at the end of the cold ischemic time (CIT), at the end of the warm ischemic time (WIT), and after 1 and 2 h of reperfusion. (a) Groups transfected with human IL-10 (AdhIL-10), the empty vector (Empty vector), and normal saline (Control) after 12 h of transfection and 18 h of cold ischemia. (b) Groups transfected with human IL-10 (AdhIL-10) and with normal saline (Control) after 24 h of transfection and 24 h of cold ischemia. Human IL-10 was detected only in lung tissue of animals transfected with AdhIL-10 (a and b). Swine IL-10, however, did not significantly vary between groups, although it tended to increase in the control group after 24 h of ischemia (b). IL-6 was progressively released during reperfusion after 18 (a) and 24 h of cold ischemia (b). After 18 h of cold ischemia, IL-6 reached significantly higher levels in the group transfected with the empty vector than in the control group and in the group transfected with AdhIL-10 (a). After 24 h of cold ischemia, the group transfected with human IL-10 had significantly lower levels of IL-6 than the control group (b). These findings demonstrate the anti-inflammatory effect of human IL-10 on the activation of the innate immune system by both the adenoviral vector and the ischemic period. IL-8 tended to remain lower in the group transfected with human IL-10 than in the Control group after 24 h of cold ischemia, but the difference did not reach statistical significance (b).

released after reperfusion and its level gradually increased in lung tissue during the reperfusion period, both after 18 and 24 h of cold ischemia (Figure 8). Transfection of the empty vector was associated with significantly greater release of IL-6, whereas transfection with AdhIL-10 was associated with significantly lower release of IL-6 when compared to the control groups (Figure 8). Similar findings were observed in the plasma with significantly lower levels of IL-6 in animals

transfected with AdhIL-10 (55 ± 34 versus 167 ± 104 pg/ml in the Control group after 24 h of cold ischemia, $P=0.03$), and higher levels in animals transfected with an empty vector (231 ± 98 versus 59 ± 46 pg/ml in the Control group after 18 h of cold ischemia, $P=0.003$) at the end of the reperfusion period. There was no significant difference between groups in lung tissue and plasma levels of IL-2, TNF- α , and IFN- γ (data not shown).

Discussion

Ischemia–reperfusion injury remains a significant limitation in clinical lung transplantation. Genetic modification of donor organs to improve their ability to withstand ischemia–reperfusion injury is an attractive strategy to address this problem. We have recently demonstrated that the transtracheal administration of adenoviral-mediated human IL-10 to donor lungs 24 h before lung retrieval reduces ischemia–reperfusion injury and improves post-transplant graft function in a rat single lung transplant model.¹⁶ Using the same rat model, we have also demonstrated that the transfection period could be safely reduced from 24 to 12 h, still achieving a similar improvement in lung function after transplantation in a more relevant timeframe clinically.¹⁷

IL-10 is a potent anti-inflammatory cytokine that has a protective action in the mechanisms of ischemia–reperfusion injury. IL-10 is induced by the production of reactive oxygen species and the release of pro-inflammatory cytokines during the period of reperfusion.^{23,24} Inhibition of endogenous IL-10 has been shown to enhance the inflammatory response and to exacerbate the injury induced by reperfusion, whereas the administration of exogenous IL-10 before reperfusion decreases the release of inflammatory mediators and reduces the lesions of reperfusion.^{19,24,25}

T cells could be an important source of production of IL-10 during reperfusion.²⁶ T cells have been shown to infiltrate ischemic organs rapidly after reperfusion and to be involved in the mechanisms of ischemia–reperfusion injury.^{27,28} Th1 cells release pro-inflammatory cytokines such as interferon- γ or IL-12 and exacerbate the lesions of reperfusion, whereas Th2 cells tend to release anti-inflammatory cytokines such as IL-10 and appear to have a protective role during reperfusion.^{28,29} The exogenous administration of IL-10 could induce a shift from Th1 to Th2 cells during the early phase of reperfusion that may reduce ischemia–reperfusion injury of transplanted organs. The shift towards Th2 cells could eventually mitigate the development of acute graft rejection and prolong graft survival in the long term by a mechanism of tolerance induction.^{30,31}

Since the concept of exogenous gene delivery of human IL-10 to the donor to improve post-transplant lung function proved feasible in rats, we moved forward to a large animal model to develop a technique that might ultimately be applied to humans in the setting of clinical lung transplantation. This technique could improve lung preservation, and eventually help to expand the utilization of lung donors, and allow reliable prolongation of the ischemic time beyond 8 h. Improvement in the quality of donor lungs with IL-10 gene therapy could potentially have a tremendous impact on the number of transplants performed and on the outcome after transplantation.

The first objective of the study was to develop a technique of gene delivery to the lungs that was simple and reproducible. Using this technique, we then looked for evidence of adverse reactions associated with the application of adenoviral vectors, in particular during the reperfusion period. Finally, our goal was to confirm the potential therapeutic impact of adenoviral mediated IL-10 gene transfer in this large animal model of ischemia–reperfusion-induced lung injury.

The trans-tracheal route has been shown to be the optimal method to deliver adenoviral vectors to the donor lung.^{12,13} This method allows more effective gene expression in the lungs than any other currently available method of gene delivery and avoids transfection of organs other than the lungs. However, to date there have been few reports of gene delivery to the lungs in large animal models; most of them were either ineffective or were used in the setting of cystic fibrosis and involved complex techniques of gene delivery.^{14,32,33} The current study demonstrates that direct administration of an adenoviral vector diluted with normal saline into each segment of lung through a bronchoscope is efficient, and allows homogenous expression of transgene (IL-10) in the different segments of the lung after 12 h of transfection.

It is well appreciated from previous animal and human experience that the dose of vector to administer cannot be simply extrapolated on a weight for weight basis between rats and pigs. We therefore performed a dose-escalation study to determine the minimal amount of vector to use to obtain significant expression of human IL-10 after 12 h of transfection. We started with a dose of 5×10^9 pfu, which is the dose we used in the rats, and progressively increased up to a dose of 4×10^{10} pfu of AdhIL-10 administered to the left lung only. This dose was found to be sufficient to obtain significant expression of human IL-10, while not causing any adverse reactions during the transfection period. A similar dose has been used by other groups to obtain transgene expression in non-human primate gene therapy studies.^{34,35}

In the second phase of the study, we noted that transtracheal administration of an empty adenoviral vector to the donor lung 12 h before lung retrieval was actually associated with significant deterioration in lung function during the reperfusion period after transplantation. This finding was observed despite the absence of any adverse reaction before lung retrieval in donors transfected with the empty adenoviral vector. Hence, the deterioration in lung function observed after reperfusion is likely caused by the cumulative double hit injury of the adenoviral vector and the cold ischemic period. This in fact is shown by elevated levels of the inflammatory cytokine IL-6 seen in the empty vector group. Indeed, IL-6 has been shown to be a key regulator of the acute phase response to adenoviral vectors and a marker of ischemia–reperfusion injury in both animal and human studies.^{36–39} Hence, the IL-6 response that we observed in our animals was probably due to the synergistic activation of the innate immune system by both the administration of the adenoviral vector and the ischemia–reperfusion injury.

The transfection of human IL-10 with the adenoviral vector prevented the deleterious effect of the adenoviral vector itself on ischemia–reperfusion injury and was associated with better lung function than the empty vector group. Hence, as previously demonstrated in the rats, sufficient expression of human IL-10 before the period of cold ischemia has a beneficial effect on both the acute phase response to adenoviral vectors and ischemia–reperfusion-induced lung injury.^{16,17} This finding highlights the importance of adequate expression of the transgene prior to lung retrieval in order to not only improve lung function, but also to avoid toxicity from

the adenoviral vector in the recipient – a critical issue if gene therapy is going to be applied clinically in lung transplantation. In the future, the development of more efficient and less inflammatory vectors such as gut-less adenovirus should limit this problem.

When the ischemic period was extended to 24 h in the third phase of the study, the function of the transplanted lung dropped considerably in the control group (as expected with a significantly greater ischemic injury), whereas lung function remained excellent in the group that was transfected with AdhIL-10. This experiment confirms that the administration of human IL-10 by an adenoviral vector can reduce ischemia–reperfusion injury and improve graft function after lung transplantation in a large animal. This improved lung function was also associated with less release of the pro-inflammatory cytokines IL-6 and IL-8. IL-6 and IL-8 progressively increased after reperfusion in the control group, whereas they remained low at the end of the 2 h reperfusion in the group treated with AdhIL-10.

In conclusion, we have demonstrated that transtracheal administration of AdhIL-10 to the lung through a bronchoscope is simple, feasible, and reproducible. This method of gene delivery allows significant and homogeneous expression of human IL-10 after 12 and 24 h of transfection in all segments of the lungs to be transplanted. The amount of human IL-10 obtained after 12 h of transfection can prevent the potentially deleterious impact that adenoviral vectors can have on transplanted lung function after a period of ischemia. In addition, we have confirmed in a large animal model that the administration of adenovirus-mediated human IL-10 to donor lung can reduce ischemia–reperfusion injury and improve graft function after lung transplantation. This study provides additional foundation for phase I clinical trials of gene therapy in lung transplantation. In the future, the development of new vectors should help to decrease the initial inflammatory response induced by current generation adenoviral vectors and potentially reduce the period required between donor transfection and lung retrieval. Such developments will ultimately improve the efficacy of adenoviral mediated gene therapy and hopefully will lead to improved outcomes after lung transplantation.

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