Recovery of extracorporeal lungs using cross-circulation with injured recipient swine

Panpan Chen, MD,[a,b] Julie Van Hassel, MD,[a,b] Meghan R. Pinezich, PhD,[a] Mohamed Diane, MD,[a] Maria R. Hudock, BS,[a] Sarah R. Kaslow, MD, MPH,[a,b] Olimpia P. Gavaudan, BS,[a] Kenmond Fung, MS, CCP,[c] Mandy L. Kain, DVM,[d] Hermogenes Lopez II, CCP,[c] Anjali Saqi, MD, MBA,[e] Brandon A. Guenthart, MD,[f] Ahmed E. Hozain, MD,[b] Alexander Romanov, DVM,[d] Matthew Bacchetta, MD, MBA, MA,[g,h] and Gordana Vunjak-Novakovic, PhD[a]

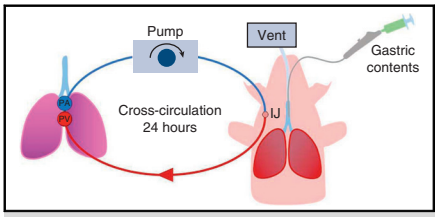
ABSTRACT

Objective: Lung transplantation remains limited by the shortage of healthy organs. Cross-circulation with a healthy swine recipient provides a durable physiologic environment to recover injured donor lungs. In a clinical application, a recipient awaiting lung transplantation could be placed on cross-circulation to recover damaged donor lungs, enabling eventual transplantation. Our objective was to assess the ability of recipient swine with respiratory compromise to tolerate cross-circulation and support recovery of donor lungs subjected to extended cold ischemia.

Methods: Swine donor lungs (n = 6) were stored at 4 °C for 24 hours while recipient swine (n = 6) underwent gastric aspiration injury before cross-circulation. Longitudinal multiscale analyses (blood gas, bronchoscopy, radiography, histopathology, cytokine quantification) were performed to evaluate recipient swine and extracorporeal lungs on cross-circulation.

Results: Recipient swine lung injury resulted in sustained, impaired oxygenation (arterial oxygen tension/inspired oxygen fraction ratio 205 ± 39 mm Hg vs 454 ± 111 mm Hg at baseline). Radiographic, bronchoscopic, and histologic assessments demonstrated bilateral infiltrates, airway cytokine elevation, and significantly worsened lung injury scores. Recipient swine provided sufficient metabolic support for extracorporeal lungs to demonstrate robust functional improvement (0 hours, arterial oxygen tension/inspired oxygen fraction ratio 138 ± 28.2 mm Hg; 24 hours, 539 ± 156 mm Hg). Multiscale analyses demonstrated improved gross appearance, aeration, and cellular regeneration in extracorporeal lungs by 24 hours.

Conclusions: We demonstrate that acutely injured recipient swine tolerate cross-circulation and enable recovery of donor lungs subjected to extended cold storage. This proof-of-concept study supports feasibility of cross-circulation for recipients with isolated lung disease who are candidates for this clinical application. (J Thorac Cardiovasc Surg 2024;167:e106-30)



Cross-circulation using injured recipient swine for extracorporeal lung recovery.

CENTRAL MESSAGE

Recipient swine with impaired respiratory function safely tolerate cross-circulation while enabling recovery of extracorporeal lungs subjected to extended cold ischemia.

PERSPECTIVE

Lung transplantation is limited by donor organ availability. Cross-circulation could allow patients with isolated lung disease to recover damaged donor lungs, enabling transplantation. Here, cross-circulation of donor lungs subjected to extended cold ischemia with recipient swine with impaired lung function recovered donor lungs, providing proof-of-feasibility for this method to expand the donor pool.

From the [a]Department of Biomedical Engineering and [d]Institute of Comparative Medicine, Columbia University, New York, NY; Departments of [b]Surgery, [c]Clinical Perfusion, and [e]Pathology and Cell Biology, Columbia University Medical Center, New York, NY; [f]Department of Cardiothoracic Surgery, Stanford University Medical Center, Stanford, Calif; [g]Department of Cardiac Surgery, Vanderbilt University Medical Center, Nashville, Tenn; and [h]Department of Biomedical Engineering, Vanderbilt University, Nashville, Tenn.

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Address for reprints: Gordana Vunjak-Novakovic, PhD, Department of Biomedical Engineering, Columbia University, 622 W 168th St, VC 12-230, New York, NY 10032 (E-mail: Gv2131@columbia.edu).

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Abbreviations and Acronyms

BAL = bronchoalveolar lavage
CIT = cold ischemia time
ECMO = extracorporeal membrane oxygenation
EVLP = ex vivo lung perfusion
Fio_2 = inspired oxygen fraction
IL = interleukin
Pao_2 = arterial oxygen tension

▶ Video clip is available online.

Lung transplantation remains the only life-saving treatment for patients with end-stage lung disease. However, the use of donor lungs remains at 20% to 25% due to unsuitability of most lungs for transplantation.[1] As a result, the number of patients in need of lungs far exceeds the available donor pool, contributing to significant waitlist mortality.[2]

Efforts to expand the donor pool include the use of ex vivo lung perfusion (EVLP), which allows for normothermic support of extracorporeal lungs up to 12 hours and recovery of marginal-quality lungs.[3,4] However, EVLP provides a limited period of homeostasis that is insufficient to enable bioengineering strategies to recover severely damaged lungs and lacks multisystem organ crosstalk capabilities to provide prolonged support.[5,6] Our group previously reported a swine model of cross-circulation that provides a durable, physiologic milieu for the maintenance and recovery of both swine and human lungs, for up to 4 days.[7-10] Cross-circulation allows for recovery of lungs severely damaged by gastric aspiration and enables bioengineering interventions such as therapeutic stem cell delivery.[10-12]

Cross-circulation has demonstrated a robust ability to recover lungs damaged by various etiologies. However, the question remains whether a similar level of recovery is achievable when the recipient swine has impaired respiratory function. A clinical application of cross-circulation of donor lungs and recipients with isolated lung disease could allow recovery of declined organs ex vivo for subsequent transplantation into the same recipient. Extracorporeal membrane oxygenation (ECMO) as a bridge to transplant has been described as a method of maximizing pretransplant conditioning.[13] Particularly for the subset of patients already on ECMO, donor lungs may be spliced into the extracorporeal circuit without additional invasiveness for the recipient. However, the key difference in this clinical configuration is the reliance on a recipient with diseased lungs for support of the donor extracorporeal organ. This raises the clinical question of whether a recipient with impaired gas exchange can safely tolerate cross-circulation and provide sufficient metabolic support for recovery of damaged donor lungs.

Motivated by this question, we used our previously developed model of acute lung injury via standardized gastric acid aspiration to induce recipient swine respiratory compromise and more specifically, impaired oxygenation.[11] To this end, we examined the ability of injured recipient swine to manage the additional metabolic demand of extracorporeal lungs (5% of total metabolism)[14] and enable organ recovery. We elected to subject extracorporeal lungs to 24 hours of static cold ischemia time (CIT) at 4 °C. The effect of ischemia time on subsequent function is debated, with some previous studies correlating CIT with early graft function and degree of ischemia–reperfusion injury.[15,16] Extension of the acceptable preservation period may help overcome logistical challenges of urgent versus semielective lung transplantation, as well as geographical constraints of organ allocation.

In this proof-of-concept study, we developed a model with high physiologic demand from an acutely injured recipient swine and extracorporeal lungs exposed to 24 hours of cold ischemia. Such an approach supports evidence of feasibility, particularly for less-demanding clinical contexts and allows for robust evaluation of extracorporeal lung recovery on a functional, histopathologic, and cellular level throughout 24 hours of cross-circulation. We hypothesized that cross-circulation with injured recipient swine is both safe and feasible and enables multiscale regeneration of severely damaged extracorporeal lungs.

METHODS

Study Design

In this proof-of-concept study, we investigated the feasibility of cross-circulation using injured recipient swine to recover extracorporeal lungs subjected to 24 hours of cold ischemia at 4 °C (Figure 1, *A*).

Animals

Twelve Yorkshire swine (n = 6 donor–recipient pairs, 50.4 ± 8.2 kg) were used for this study, under a protocol approved by the Institutional Animal Care and Use Committee at Columbia University. Animal care and procedures were conducted in accordance with the US National Research Council of the National Academies Guide for the Care and Use of Laboratory Animals, 8th Edition.

Recipient Swine Lung Injury

Gastric contents were collected as previously described,[11] standardized to pH 2, stored at −80 °C, and thawed immediately before use. Animals were intubated and ventilated for the duration of the study. A total of 1 mL kg^{-1} of gastric contents was delivered to the bilateral lungs by first introducing a 3.8-mm flexible bronchoscope (aScope 4; Ambu) into the right mainstem bronchus 2 cm distal to the carina, and then into the left mainstem bronchus. Lung-protective ventilation was delivered according to previously described protocols.[17] Arterial blood gas analysis was performed every 3 hours using an Epoc point of care blood analysis system (Epocal). If the arterial oxygen tension (Pao_2)/inspired oxygen fraction (Fio_2) ratio on any blood gas analysis was >200 mm Hg, the recipient swine was re-dosed with 20 mL of gastric contents to maintain lung injury (Table E1).

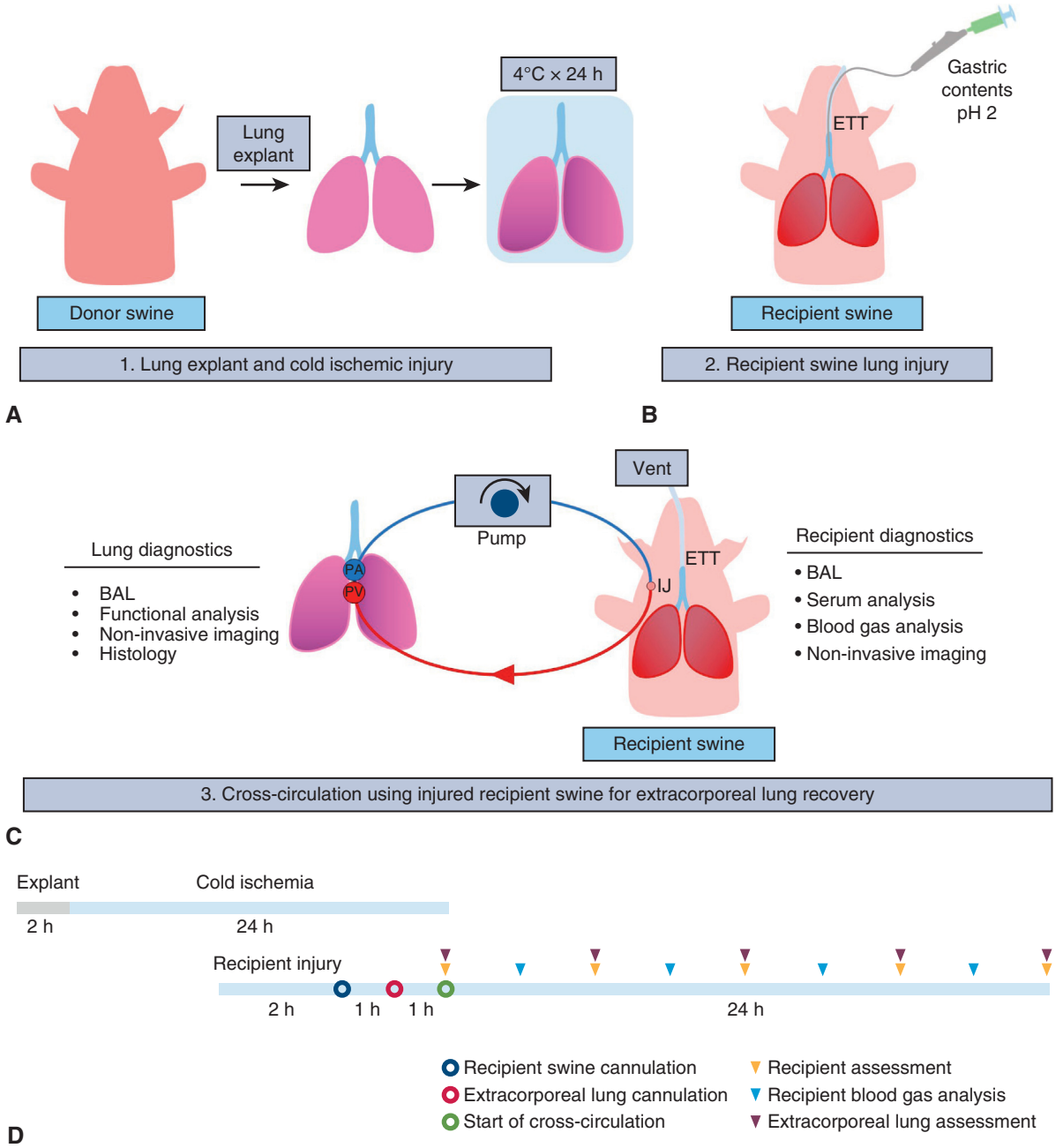


FIGURE 1. Experimental overview and timeline. A, Donor swine lungs were procured and stored at 4 °C for 24 hours. B, Recipient swine were injured via bronchoscopic delivery of gastric contents (standardized to pH 2) to the bilateral lungs. C, Cross-circulation was initiated between injured recipient swine and extracorporeal lungs after 24 hours of cold storage. Continuous assessments of recipient swine and extracorporeal lung were performed. D, Experimental timeline of donor lung procurement and cold storage, recipient swine lung injury, and assessment of recipient swine and extracorporeal lungs during cross-circulation. *ETT*, Endotracheal tube; *BAL*, bronchoalveolar lavage; *IJ*, internal jugular.

Donor Lung Procurement

Donor swine lungs were procured in standard fashion as previously described.[10,11] Lungs were flushed antegrade in situ with a cold low-potassium dextran solution (Perfadex; Vitrolife). Detailed methods are described in Appendix E2.

Lung Storage for Extended Cold Ischemia

Lungs were placed in a sterile isolation bag containing 500 mL of cold Perfadex and a secondary bag with 1 L of cold normal saline, and stored on ice at 4 °C for 24 hours.

Lung Cannulation

The aortic arch was sutured to the left atrial cuff as a vascular biobridge (Appendix E1) and lungs were cannulated as previously described.[10] Detailed methods are described in Appendix E2.[10]

Recipient Swine Cannulation

Cefazolin (30 mg kg^{-1}; WG Critical Care) was administered before skin incision. Bilateral neck cutdowns were performed to expose the internal jugular veins (Figure E1, *C*). A total of 15,000 U of heparin (Sagent Pharmaceuticals) was administered immediately before 16-F cannulas were placed using Seldinger technique (Figure E1, *D*).

Cross-Circulation

Calcium gluconate (1 g; Fresenius Kabi) was administered immediately before cross-circulation between recipient swine and extracorporeal lungs. Recipient swine received 1 g of methylprednisolone (Pfizer) every 8 hours and mycophenolate (500 mg; Genentech) every 12 hours. Extracorporeal lungs were ventilated within 10 minutes of initiating cross-circulation with the following settings: respiratory rate, 10 to 12 breaths/min; tidal volume, 6 to 8 mL kg^{-1}; positive end-expiratory pressure, 5 cmH_2O; inspired oxygen fraction, 50%. Alveolar recruitment maneuvers were performed by increasing positive end-expiratory pressure (10 cmH_2O), inspiratory hold maneuvers, and manual recruitment maneuvers. Detailed methods are described in Appendix E2.

Recipient Swine and Extracorporeal Lung Analysis

Multimodal analyses of recipient swine and extracorporeal lung function were performed throughout cross-circulation. Detailed methods are described in Appendix E2.

RESULTS

Experimental Design

Extracorporeal lungs were placed on ice for 24 hours to induce a cold ischemia injury (Figure 1, *A*). Meanwhile, recipient swine underwent lung injury via bronchoscopic delivery of gastric contents to the bilateral lungs (Figure 1, *B*). Cross-circulation was initiated between injured recipient swine and extracorporeal lungs after 24 hours of cold storage (Figure 1, *C*). Throughout 24 hours of cross-circulation, multiscale assessments of recipient swine and extracorporeal lung were performed at scheduled intervals (Figure 1, *D*).

Validation of Recipient Swine Lung Injury

Chest radiographs demonstrated bilateral infiltrates and consolidations throughout 24 hours of cross-circulation (Figure 2, *A*, and Figure E2, *B*). Recipient swine demonstrated impaired Pao_2/Fio_2 ratio (205 ± 39 mm Hg vs 454 ± 111 mm Hg at baseline, paired *t*-test $P = .006$; Figure 2, *B*) as well as decreased lung compliance (19.2 ± 5.0 mL cmH_2O^{-1} vs 31.4 ± 4.8 mL cmH_2O^{-1} at baseline, paired *t* test $P = .008$; Figure 2, *C*) and elevated peak inspiratory pressures (24.0 ± 5.5 cmH_2O vs 19.5 ± 3.0 cmH_2O at baseline, paired *t* test $P = .024$; Figure 2, *D*) throughout the duration of the study. A systemic inflammatory response was characterized by increased neutrophilia compared with baseline (52.5 ± 8.0% vs 33.7 ± 5.8% at baseline, paired *t* test $P = .002$; Figure 2, *E*). Bronchoscopic airway evaluation revealed increased erythema and edema (Figure 2, *F*). Bronchoalveolar lavage (BAL) fluid analysis revealed elevated levels of inflammatory cytokines (interleukin [IL]-1α, IL-1Ra, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18) throughout cross-circulation, as well as increased cellularity and total protein content (Figure 2, *G-I*, Table E2). Histologic analysis demonstrated a severe inflammatory response characterized by areas of alveolar congestion and edema with neutrophilic infiltration (Figure 2, *J*). Immunohistochemical analysis of recipient swine lungs demonstrated significantly elevated apoptotic cell counts on terminal deoxynucleotidyl transferase dUTP nick end labeling staining and increased CD163 + macrophage infiltration (Figure 2, *K*, and Figure E2, *D-F*). Blinded pathologic assessment was performed to generate a composite lung injury score, which was significantly greater in injured lungs compared with normal lungs (Figure 2, *L*). Additional immunofluorescence analysis of endothelial and epithelial markers revealed loss of expression of gap junction alpha-1 protein (Connexin 43) and aquaporin 5, an integral membrane protein critical for water transport (Figure 2, *M-P*).[18]

Recipient Swine Safety During 24 Hours of Cross-Circulation

Throughout 24 hours of cross-circulation, recipient swine remained hemodynamically stable with normal pH on blood gas analysis (Table 1). There was no significant difference in serum lactate at 24 hours compared with the start of cross-circulation (1.1 ± 0.3 mM vs 2.2 ± 3.2 mM, paired *t* test, $P = .469$). Hemolytic markers (lactate dehydrogenase, plasma free hemoglobin, D-dimer, fibrinogen) did not significantly increase throughout cross-circulation (Figure 3, *A*). P-selectin, an indicator of endothelial injury, and serum apoptotic marker M30 did not increase significantly over 24 hours (Figure 3, *B*). There were no significant elevations in recipient serum inflammatory cytokines (interferon-γ, IL-1α, IL-1Ra, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-18, tumor necrosis factor-α) at 24 hours versus the start of cross-circulation, despite the reperfusion of extracorporeal lungs (Figure 3, *C*, and Table E3).

Functional Assessment of Extracorporeal Lungs

After 24 hours of CIT, initial Pao_2/Fio_2 ratio of extracorporeal lungs was significantly impaired but improved after 6 hours of cross-circulation (138 ± 28 mm Hg vs 511 ± 105 mm Hg, paired *t* test, $P < .001$) and remained stable until the 24-hour end point (Figure 4, *A*). Lungs initially exhibited poor compliance and elevated peak inspiratory pressure, which improved by 24 hours of cross-circulation (Figure 4, *B* and C). Differences between pulmonary artery and pulmonary vein hemogases demonstrated improvement in both oxygenation and ventilatory capacities (Figure 4,

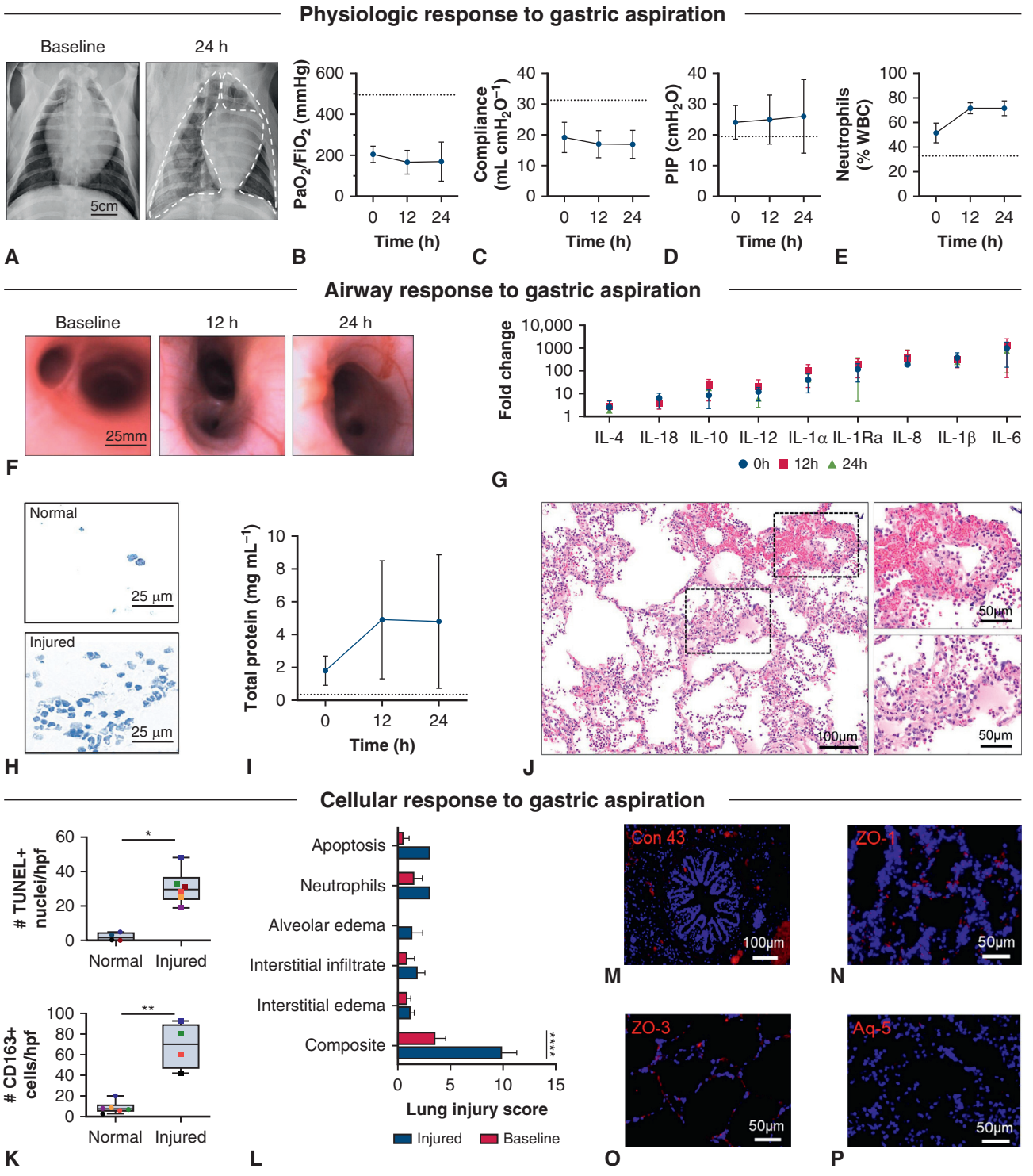


FIGURE 2. Recipient swine response to lung injury. Recipient physiologic response after gastric aspiration: A, Radiograph of the chest at baseline before injury and at 24 hours of cross-circulation (*dotted white lines* represent areas of diffuse consolidation and infiltrates); B, arterial oxygen tension (Pao_2)/inspired oxygen fraction (Fio_2) ratio at 0, 12, and 24 hours of cross-circulation (*dotted line* indicates baseline values before lung injury); C, dynamic compliance; D, peak inspiratory pressure (*PIP*); and E, neutrophils (% white blood cells [*WBC*]). Airway response to gastric aspiration: F, bronchoscopic analysis of airways at baseline prior to injury, and at 12 and 24 hours of cross-circulation; G, fold change in bronchoalveolar lavage (BAL) fluid cytokine levels at 0, 12, and 24 hours of cross-circulation compared with normal lung; H, Kwik-Diff staining of BAL fluid smears from normal lungs and after injury at 12 hours of cross-circulation; I, total protein in BAL fluid; and J, hematoxylin and eosin staining of injured lungs at 24 hours, with insets demonstrating alveolar

TABLE 1. Recipient safety during 24 hours of cross-circulation

Time (h)	0	6	12	18	24
Hemodynamics					
Heart rate, bpm	88 ± 22	76 ± 6.6	66 ± 20	61 ± 11	68 ± 31
Systolic BP, mm Hg	114 ± 11	123 ± 7.3	125 ± 19	127 ± 20	124 ± 13
Spo_2, %	96 ± 1.7	96 ± 2.6	97 ± 2.3	94 ± 3.3	90 ± 17
Temperature, °C	37 ± 0.9	37 ± 0.3	38 ± 0.5	38 ± 0.4	38 ± 0.6
Hemogas analysis					
pH	7.37 ± 0.07	7.40 ± 0.02	7.41 ± 0.04	7.43 ± 0.04	7.34 ± 0.14
p_{o2}, mm Hg	136 ± 67	139 ± 70	164 ± 76	156 ± 80	133 ± 72
p_{co2}, mm Hg	50 ± 8.1	50 ± 8.8	50 ± 6.5	48 ± 4.1	60 ± 13
HCO_3, mM	28 ± 0.6	31 ± 3.8	32 ± 1.7	32 ± 2.8	32 ± 4.2
Biochemical analysis					
Platelets, 10^9 L^{-1}	247 ± 52	243 ± 62	203 ± 60	213 ± 46	205 ± 48
Hgb/Hct, g dL^{-1}/%	10/30 ± 1.3/4.1	7.6/23 ± 0.5/2.2	7.4/22 ± 0.9/2.2	7.0/21 ± 0.9/2.7	6.7/20 ± 1.1/3.4
AST/ALT, U L^{-1}	28/34 ± 8.2/4.4	50/32 ± 15/9.6	45/31 ± 11/8.2	40/31 ± 9.9/6.1	38/29 ± 11/6.0
Creatinine, mg dL^{-1}	1.0 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.4	1.1 ± 0.4
Lactate, mM	1.1 ± 0.3	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.2	2.2 ± 3.2
Hemolytic markers					
LDH, U l^{-1}	349 ± 51	446 ± 134	411 ± 74	427 ± 60	421 ± 75
ACT, s	305 ± 91	303 ± 39	296 ± 63	332 ± 39	299 ± 53
Free Hgb, mg dL^{-1}	5.2 ± 1.8	6.0 ± 2.6	5.2 ± 1.8	5.4 ± 0.9	5.7 ± 1.6

All values represent mean ± standard deviation. *BP*, Blood pressure; *Spo_2*, oxygen saturation; *po_2*, oxygen tension; *pco_2*, carbon dioxide tension; *HCO_3*, bicarbonate; *Hgb*, hemoglobin; *Hct*, hematocrit; *AST*, aspartate transaminase; *ALT*, alanine transaminase; *LDH*, lactate dehydrogenase; *ACT*, activated clotting time.

D-F). After 24 hours, lungs demonstrated intact vasoresponsiveness and bronchoresponsiveness after administration of intravenous epinephrine and nebulized methacholine, respectively (Figure 4, *G-H*).

Multiscale Recovery of Extracorporeal Lungs

Extracorporeal lungs initially exhibited focal areas of congestion and consolidation, which significantly improved by 24 hours (Figure 5, *A*). Similarly, radiographs initially demonstrated diffuse consolidations and air bronchograms, which largely resolved by 24 hours. On thermal imaging, overall perfusion of extracorporeal lungs was improved by 12 hours of cross-circulation. Bronchoscopic evaluation revealed significant airway inflammation which initially worsened with reperfusion, but resolved by 24 hours (Figure 5, *B*). Histologic analysis demonstrated interstitial and alveolar edema, alveolar congestion, and neutrophilic infiltration, which largely resolved by 24 hours (Figure 5, *C*). Myeloperoxidase activity, an indicator of neutrophil activation, decreased over 24 hours compared with the start of cross-circulation (Figure 5, *H*). Blinded pathologic assessment of extracorporeal lungs demonstrated significantly decreased lung injury score at 24 hours (Figure 5, *I*). Analysis of BAL fluid demonstrated decreased protein content and inflammatory infiltrate (Figure 5, *E-F*). Differences between 0 and 24 hours of cross-circulation demonstrated mixed trends across inflammatory cytokines in extracorporeal lung BAL fluid, with the greatest positive fold-change in IL-6 (Figure 5, *J*, and Table E4). Lung weight decreased throughout 24 hours, consistent with resolution of edema (Figure 5, *G*). At the experimental end point, radiography with radiocontrast dye demonstrated intact pulmonary vasculature throughout all lobes and peripheral regions (Figure 5, *D*).

Cellular Recovery of Extracorporeal Lungs

Alcian blue staining demonstrated marginating neutrophils in the epithelium and loss of mucus-containing goblet cells at 12 hours, which recovered to a degree similar to normal lung by 24 hours (Figure 6, *A*). Pentachrome and trichrome staining demonstrated initially preserved ciliated epithelium, which was lost at 12 hours in addition to neutrophil migration into the airway space. At 24 hours, ciliated columnar epithelium was recovered, with similar appearance to normal lungs seen (Figure 6, *B* and *C*). Damaged extracorporeal lungs demonstrated initial high counts of

congestion, neutrophilic infiltrate, and alveolar edema. Cellular response to lung injury: K, quantification of apoptotic cells via terminal deoxynucleotidyl transferase dUTP nick end labeling (*TUNEL*) counts ($P \leq .05$) and macrophage infiltration via CD163 + cell count per high powered field (hpf) ($P \leq .01$); L, lung injury score obtained at 24 hours, and immunofluorescent staining of M, alpha-1 protein (connexin 43 [Con 43]); N, zonula occludens-1 (ZO-1); O, zonula occludens-3 (ZO-3); and P, aquaporin-5 (Aq-5). Box and whisker plots: *lower and upper borders* represent lower and upper quartiles, *middle* represents median, *lower and upper whiskers* represent minimum and maximum values. *IL*, Interleukin; *CD*, cluster of differentiation.

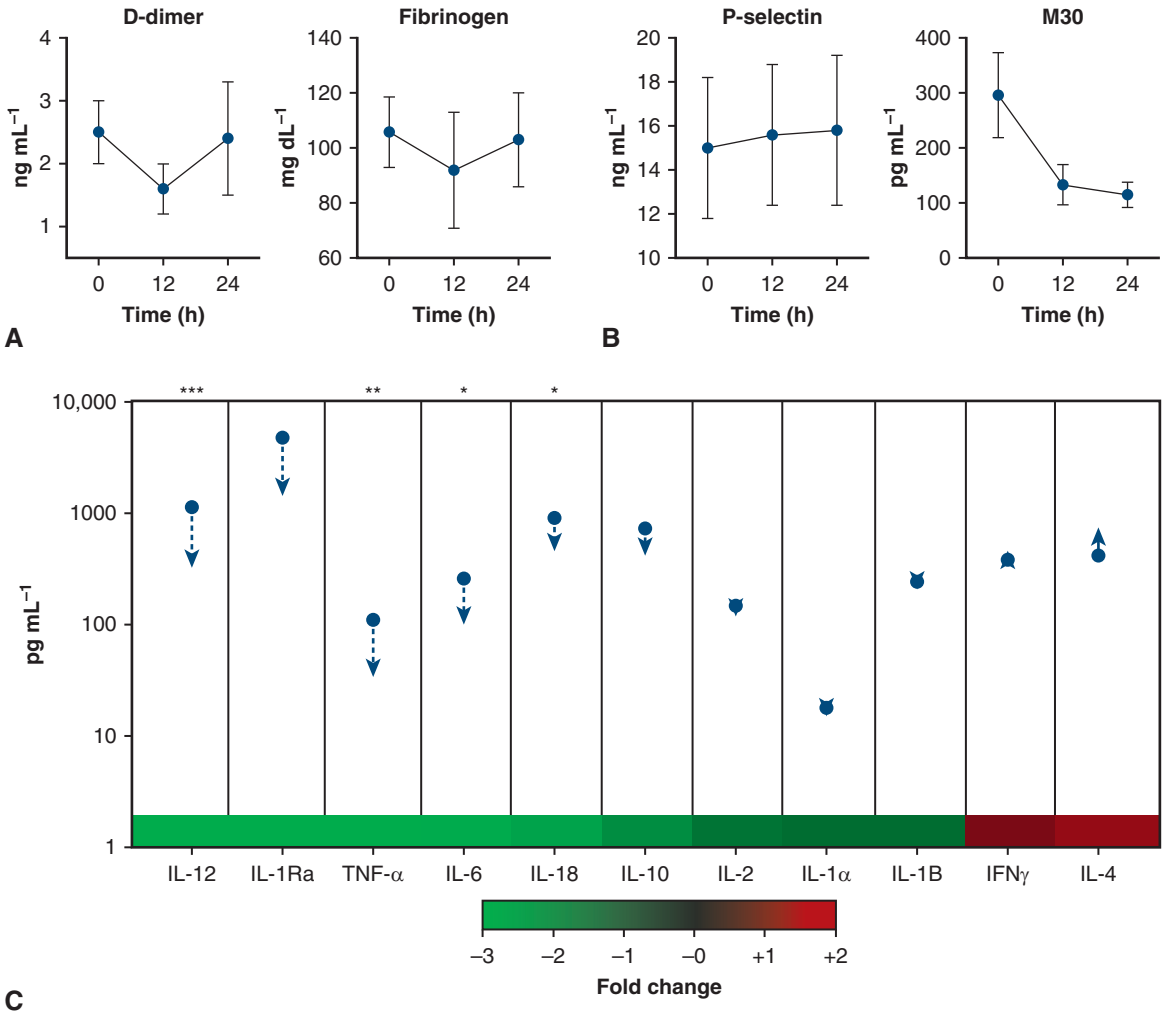


FIGURE 3. Recipient coagulation and inflammatory profile. Hemolytic markers: A, D-dimer and fibrinogen, activation markers; B, P-selectin and M30. C, Quantification of inflammatory cytokines in recipient serum over 24 hours of cross-circulation. IL-12, ***$P \leq .001$; TNF-α, **$P \leq .01$; IL-6, *$P \leq .05$; IL-18, *$P \leq .05$. *Circles* represent cross-circulation 0-hour time point, and the *arrow* represents direction of change after 24 hours of cross-circulation. *IL*, Interleukin; *TNF-α*, tumor necrosis factor-α.

terminal deoxynucleotidyl transferase dUTP nick end labeling + apoptotic cells, which significantly decreased by 24 hours (Figure 6, *D*, and Figure E4). CD163 + macrophage and neutrophil count also significantly decreased at 24 hours (Figure 6, *E* and *F*). Metabolic activity did not change significantly throughout 24 hours (Figure 6, *G*).

Extracorporeal recovery of lung epithelium and endothelium was assessed in the context of ischemia–reperfusion injury. Expression of epithelial tight junction protein zonula occludens-3 was not fully recovered until 24 hours (Figure 6, *H*). Aquaporin-5 demonstrated a similar timescale of recovery at 24 hours (Figure 6, *I*). Assessment of the pulmonary microvasculature demonstrated loss of tight junction protein zonula occludens-1 expression, which recovered to normal levels by 24 hours (Figure 6, *J*). Endothelial integrity at 24 hours was further assessed by expression of connexin 43 and pulmonary microvasculature marker CD31 (Figure 6, *K* and *L*). After 24 hours, pulmonary endothelial viability was assessed by uptake of acetylated LDL (Figure 6, *M*).

DISCUSSION

We demonstrate that recipient swine with impaired gas exchange can safely tolerate cross-circulation and enable extracorporeal lung recovery (Figure 7). Gastric aspiration induced an isolated acute lung injury characterized by oxygenation deficit, impaired lung mechanics, and significant airway inflammation. However, recipient swine remained hemodynamically stable throughout cross-circulation and provided sufficient metabolic support for recovery of extracorporeal lungs after extended cold storage and ensuing ischemia–reperfusion injury. We also characterized the recovery of extracorporeal lungs after 24 hours of CIT. Although lungs showed robust

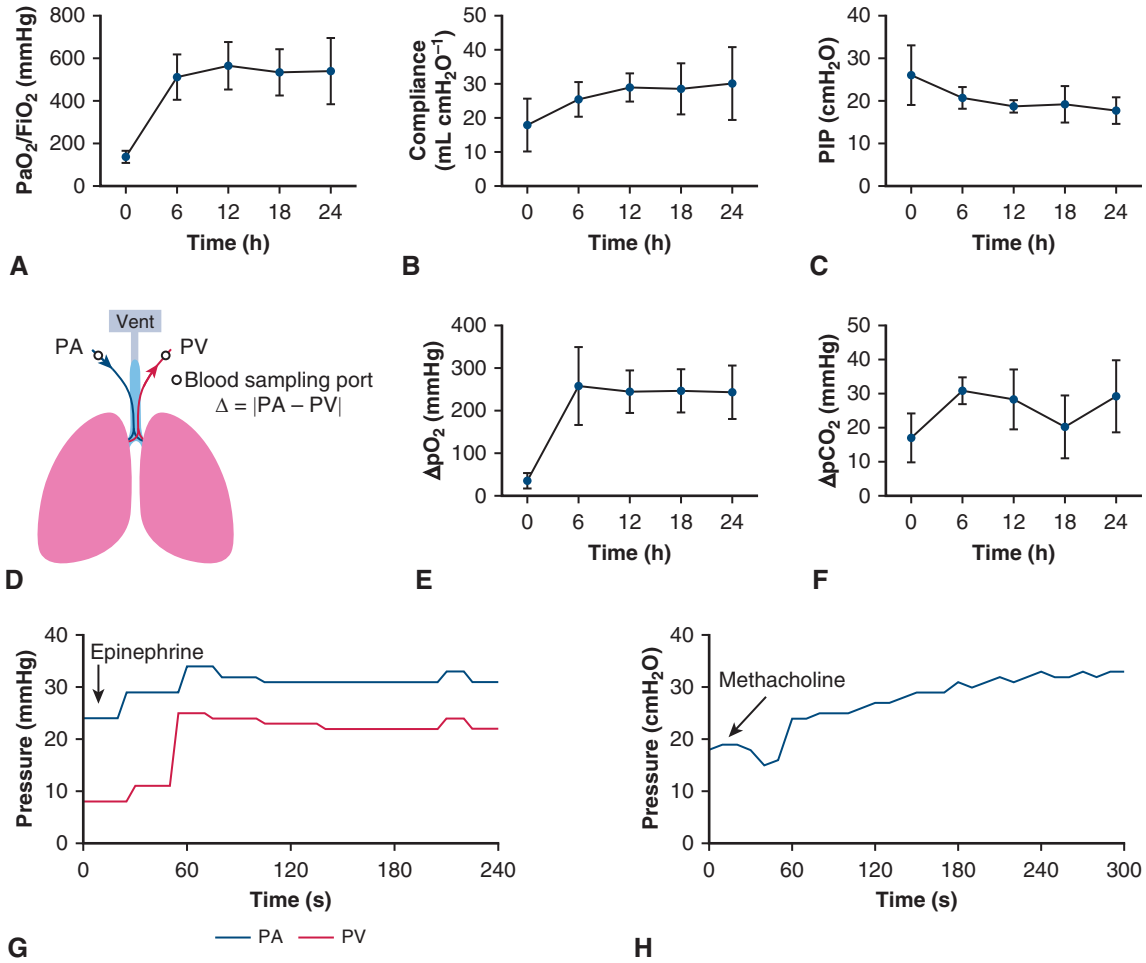


FIGURE 4. Functional recovery of donor extracorporeal lungs after 24 hours' cold ischemia. A, Arterial oxygen tension (Pao_2)/inspired oxygen fraction (Fio_2) ratio throughout 24 hours of cross-circulation. B, Dynamic compliance and C, peak inspiratory pressures (*PIP*) throughout 24 hours of cross-circulation. D, Depiction of pulmonary artery (*PA*) and pulmonary vein (*PV*) sampling and E, Δpo_2 and F, Δpco_2 throughout 24 hours of cross-circulation. G, Vasoresponsiveness following intravenous administration of epinephrine into pulmonary artery after 24 hours of cross-circulation. H, Bronchoresponsiveness following nebulized administration of methacholine after 24 hours of cross-circulation. Δpo_2, Change in oxygen tension; Δpco_2, change in carbon dioxide tension.

improvement in gas exchange functionality by 6 hours of cross-circulation, full histologic and cellular recovery was not demonstrated until 24 hours.

Our use of a gastric aspiration injury model in recipient swine represents an acute, rather than chronic, lung injury. Although lung transplantation has been described for acute pathologies such as refractory acute respiratory distress syndrome, the vast majority of recipients are those with chronic end-stage lung disease.[19,20] However, in this study, we use aspiration injury not as representation of lung pathologies that require transplant but rather to specifically recapitulate the gas exchange impairment and hypoxia that are common features across patients awaiting transplant.[20] In addition, we induced a sustained inflammatory response via repeated delivery of gastric contents at any time the Pao_2/Fio_2 ratio was >200 mm Hg to maintain injury and sustain recipient hypoxia. Our target threshold Pao_2/Fio_2 ratio of 200 mm Hg after recipient swine lung injury is intended to model the degree of hypoxia exhibited by patients with end-stage lung disease.[20]

Acute lung injury, although rarely an indication for transplant, carries significant morbidity and mortality.[21] After gastric aspiration, BAL analysis in recipient swine demonstrated significantly elevated levels of IL-6 and IL-8 (Figure 2, *G*), which are known cytokine mediators of the initial inflammatory response in acute lung injury and confer significant risk of mortality.[22] In addition, the cellular apoptosis, epithelial injury, and loss of alveolar-epithelial barrier integrity (Figure 2) seen in recipient swine have also been associated with poor clinical outcomes.[23,24] Although this inflammatory response is more robust in acute lung injury, it also remains a contributor to poor

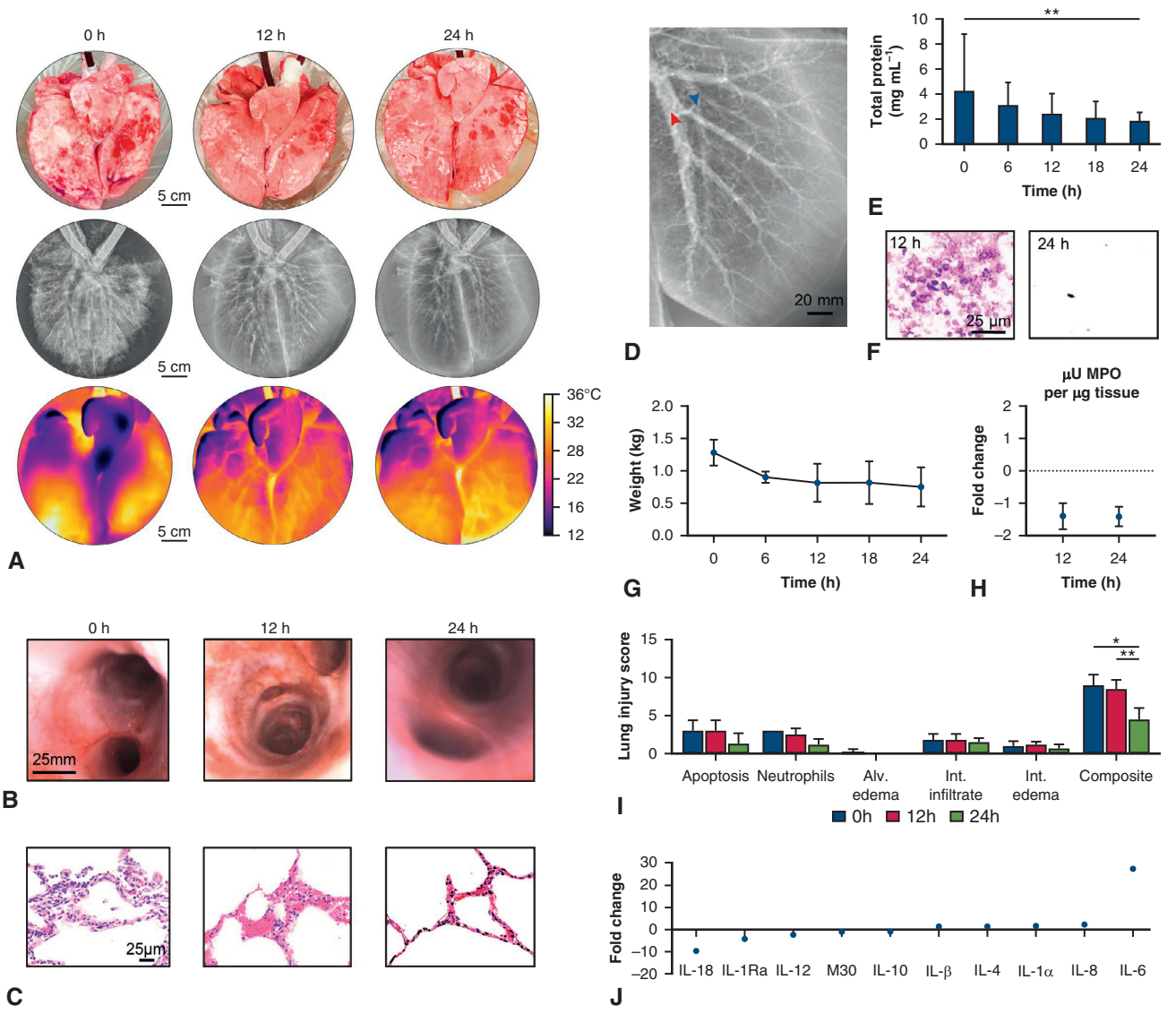


FIGURE 5. Multiscale recovery of donor extracorporeal lungs after 24 hours cold ischemia. Extracorporeal lung appearance on A, gross photography, radiography, and thermography throughout 24 hours of cross-circulation. B, Bronchoscopic evaluation of airways through 24 hours of cross-circulation. C, Hematoxylin and eosin staining depicting decreased neutrophilic infiltration and alveolar congestion by 24 hours of cross-circulation. D, Vascular integrity on radiograph with radiocontrast dye. *Red arrowhead*, vein; *blue arrowhead*, artery. E, Quantification of protein content in BAL fluid over 24 hours' circulation ($P \leq .01$) and F, Kwik-Diff staining of BAL fluid smears at 12 and 24 hours of cross-circulation. G, Lung weight over 24 hours of cross-circulation. H, Myeloperoxidase (*MPO*) activity in extracorporeal lung tissue at 12 and 24 hours, compared with start of cross-circulation, as represented by *dotted line*. I, Lung injury scores throughout 24 hours of cross-circulation (*$P \leq .05$, **$P \leq .01$). J, Quantification of inflammatory cytokines in extracorporeal lung BAL fluid at 24 hours, compared with the start of cross-circulation. *Alv.*, Alveolar; *Int.*, interstitial; *IL*, interleukin.

clinical outcomes in chronic lung disease.[25] Despite the morbidity and mortality associated with acute lung injury, the success of cross-circulation with acutely injured recipient swine provides encouraging evidence that this approach may be extended to stable recipients with long-standing chronic disease. Our group previously described the potential of this application for cross-circulation in patients with isolated lung disease awaiting donor organs, in order to allocate lungs that would otherwise be declined.[11] This proof-of-concept study demonstrates that recipient swine with acute impairment of gas exchange capability can safely tolerate cross-circulation and recover extracorporeal lungs, as well as provides important evidence of feasibility for future clinical translation.

We also robustly characterized extracorporeal lung recovery following 24 hours of CIT. Although transplantation

of donor lungs with CIT >8 hours has been associated with increased mortality, the exact duration of acceptable CIT has been debated for decades and clinical practices are now rapidly changing.[15,16,26] Recent work by Ali and colleagues[27,28] has also demonstrated favorable outcomes of 10 °C static lung storage, even when extended to preservation times of up to 15 hours. However, 4 °C remains the current clinical standard for cold storage. Previous work with swine models of lung transplantation have extended 4 °C CIT to 24 hours; however, donor lung function was only assessed up to a period of 2 to 7 hours after reperfusion.[29,30] Our findings demonstrate that although extracorporeal lung gas exchange capabilities recover by 6 hours of cross-circulation (Figure 4, *A*, *E*, and *F*), full multiscale recovery is not demonstrated until 24 hours of reperfusion. Bronchoscopic evaluation at 12 hours demonstrated persistent inflammation that was substantially improved by 24 hours, and BAL fluid analysis similarly revealed significant cellular infiltrates that were not cleared until 24 hours (Figure 5, *B* and *F*). Lung injury severity scores were similar between 0 and 12 hours, but reflected a significant improvement by 24 hours (Figure 5, *I*).

Extracorporeal lungs demonstrated a mixed cytokine profile at 24 hours, with proinflammatory cytokine IL-6 exhibiting the greatest increase compared to baseline (Figure 5, *J*), consistent with previous reports in extracorporeal lungs supported by EVLP.[31] Such elevation without concomitant detrimental effects on function and histological recovery suggest that these proinflammatory biomarkers may be implicated in immune cell recruitment and cell turnover.[32] Consistently, extracorporeal lungs demonstrated significant cellular apoptosis along with macrophage and neutrophil infiltration after reperfusion, which substantially abated by 24 hours (Figure 5, *D-F*). On histologic examination, at 12 hours lung airways demonstrated loss of mucus-containing goblet cells and ciliated columnar epithelium that regenerated by 24 hours to resemble normal lung (Figure 6, *A-C*). Epithelial and endothelial integrity demonstrated a similar timeline of recovery by 24 hours of cross-circulation (Figure 6, *H-L*). Taken together, these findings highlight the value of cross-circulation for robust recovery of extracorporeal lungs beyond the initial few hours after reperfusion.

Importantly for the recipient swine, the natural course of extracorporeal lung ischemia–reperfusion injury in this configuration is safe and tolerable, even in the setting of simultaneous recipient acute lung injury. Recipient swine remained hemodynamically stable, with normal lactate levels throughout 24 hours on cross-circulation (Table 1). Recipient swine serum cytokines demonstrated only a modest increase in levels of interferon-γ and IL-4, and there were no elevations in serum endothelial injury marker P-selectin or apoptotic marker M30 (Figure 3, *B* and *C*). Ischemia–reperfusion injury is a significant contributor to early graft dysfunction and morbidity and mortality after transplant.[33] However, extracorporeal lung reperfusion after extended CIT using cross-circulation offers the opportunity for well-tolerated lung recovery before transplantation.

Our finding that extracorporeal lungs subjected to extended cold ischemia can be recovered by cross-circulation with injured recipient swine naturally begs the question of why these organs cannot be directly transplanted into the recipient, circumventing the need for cross-circulation. Particularly for severely injured donor lungs, cross-circulation offers the opportunity to safely perform therapeutic interventions before subjecting the recipient to the morbidity and stress of transplantation. For example, frequent manual recruitment maneuvers (Video 1) and therapeutic bronchoscopy can be performed without negatively impacting recipient gas exchange by de-recruitment during suctioning or requiring invasive procedures. At the start of cross-circulation, we noted that aggressive toilet bronchoscopy was necessary to clear significant airway edema, which may not be well-tolerated by a recipient immediately after transplantation. We acknowledge, however, that the use of postoperative ECMO would ameliorate many of these challenges. We envision this clinical application of cross-circulation to be used not as a replacement for perioperative ECMO but rather as an adjunctive tool for rehabilitation of severely injured donor lungs that require more intensive therapeutic interventions and manual handling that is more feasible ex vivo.

There are several limitations to the present study that merit further study. First, candidates for this clinical application of cross-circulation are likely to have chronic lung disease. Although we hypothesize that the capability of acutely injured swine to handle the additional demand of extracorporeal lungs can be extrapolated to chronic disease recipients, future work should investigate additional challenges that may be posed by chronic disease models.[34,35] Second, this study addresses CIT as a barrier to donor lung acceptability, but not other common causes of donor lung damage such as gastric aspiration. We extended the CIT to 24 hours to maximize the demands for recovery, but additional studies will need to validate the ability of injured recipients to recover severely damaged lungs from other etiologies. Third, lungs recovered on cross-circulation were not transplanted. Although the duration of cross-circulation support was intended to sufficiently capture the course of reperfusion and recovery, future studies will investigate early and late graft function after transplantation. Fourth, in this study we aimed to achieve a degree of lung injury that sufficiently modeled the degree of hypoxia exhibited by patients with end-stage lung disease, but not to a degree so severe that recipient swine were unable to survive the experimental period. Future

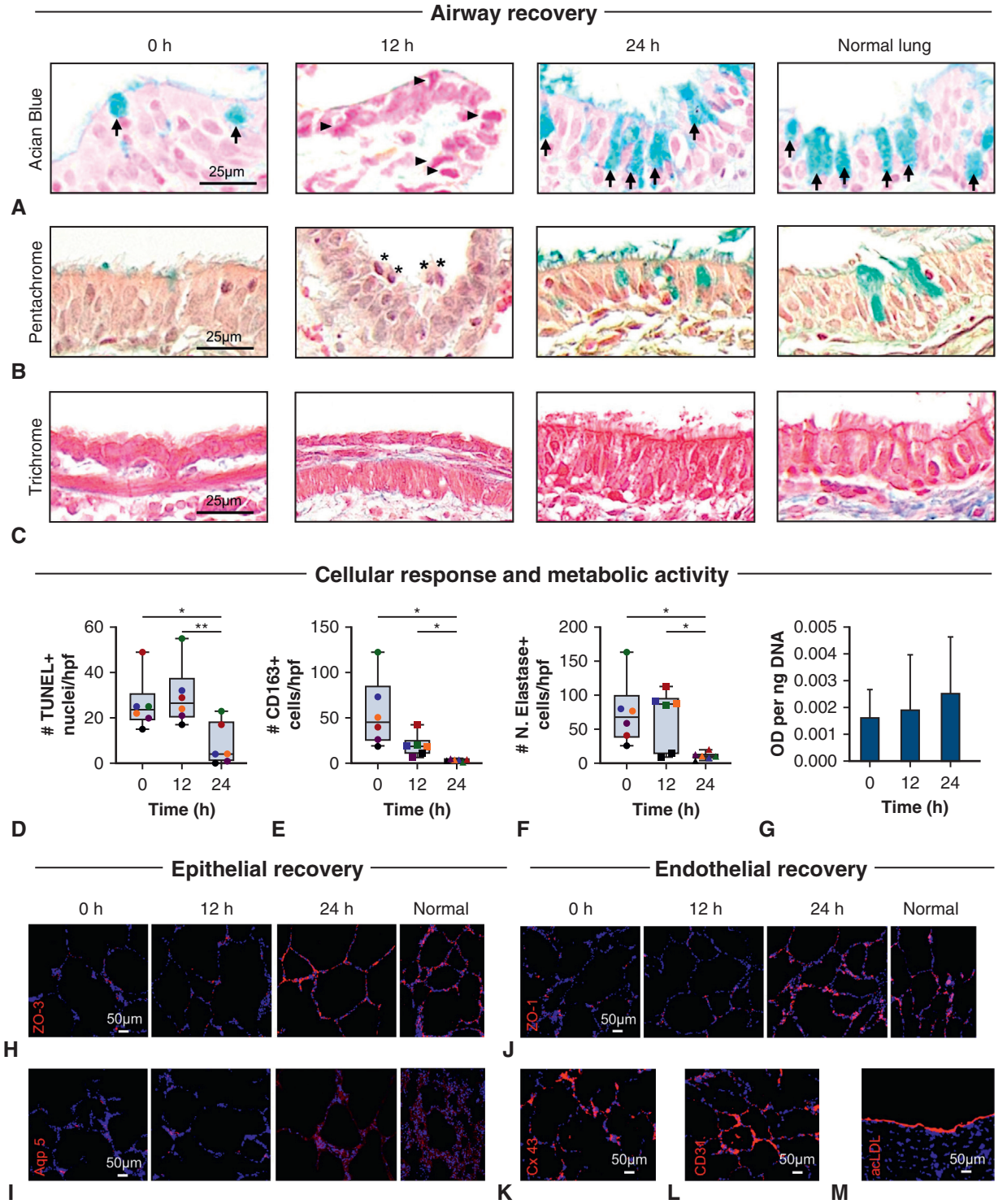


FIGURE 6. Cellular recovery of donor extracorporeal lungs after 24 hours cold ischemia. Histologic analysis of airway recovery: A, Alcian blue staining demonstrating loss of mucus-containing goblet cells (*arrows*) at 12 hours with marginating neutrophils (*arrowheads*), followed by goblet cell recovery at 24 hours (*arrows*) resembling normal lung, B, pentachrome and C, trichrome staining demonstrating loss of columnar ciliated epithelium with neutrophilic infiltration into the airway at 12 hours (*asterisks*), followed by recovery of ciliated epithelium at 24 hours similar to normal lung. D, Quantification of terminal deoxynucleotidyl transferase dUTP nick end labeling (*TUNEL*) + cells throughout cross-circulation (*$P \leq .05$, **$P \leq .01$). E, Quantification of macrophages with CD163 + cell counts (*$P \leq .05$). F, Neutrophil quantification over 24 hours of cross-circulation (*$P \leq .05$). G, Metabolic activity throughout cross-circulation. Immunofluorescence staining demonstrating recovery of H, zonula occludens-3 (ZO-3), I, aquaporin-5 (Aqp 5), and J, zonula occludens-1

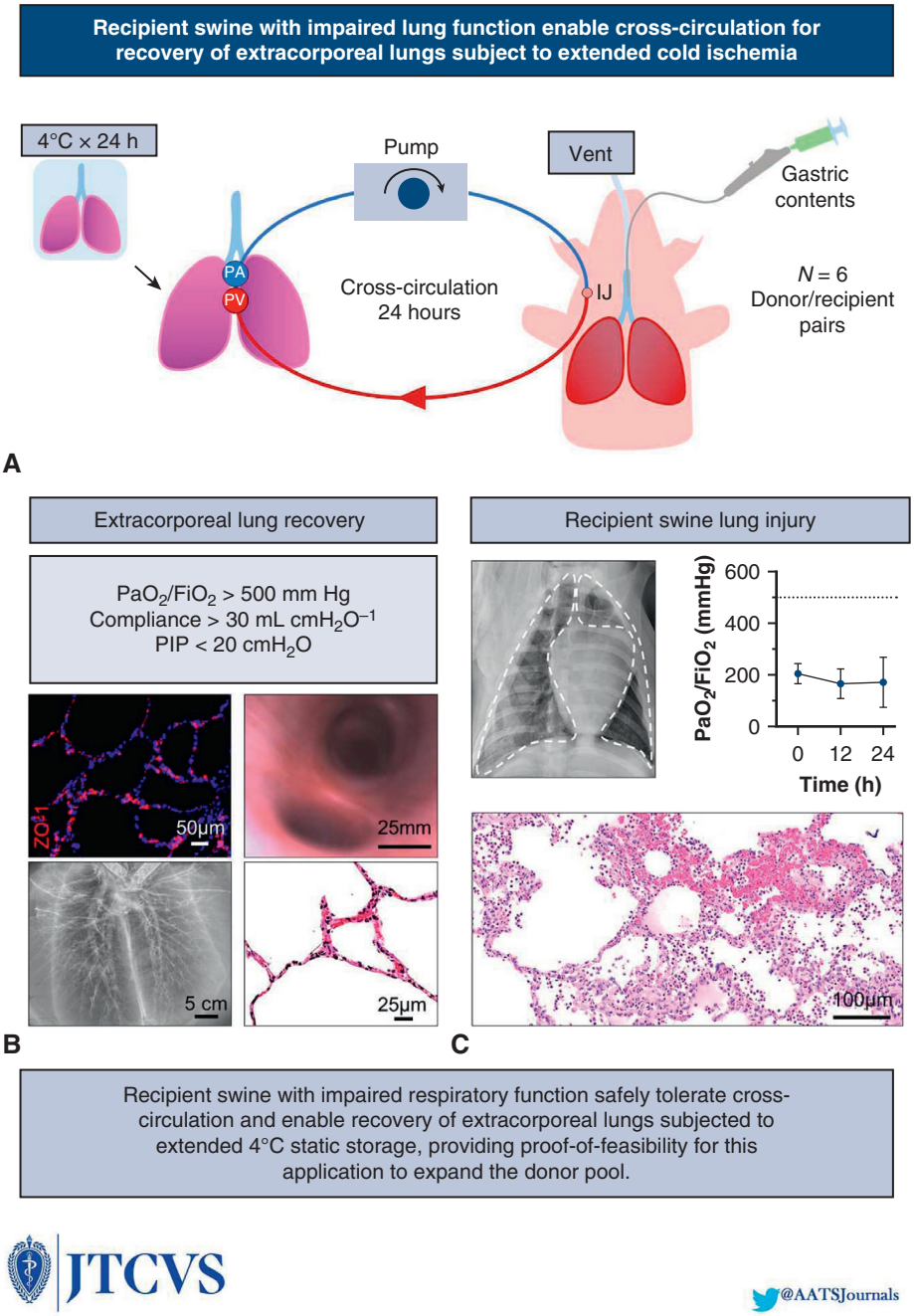


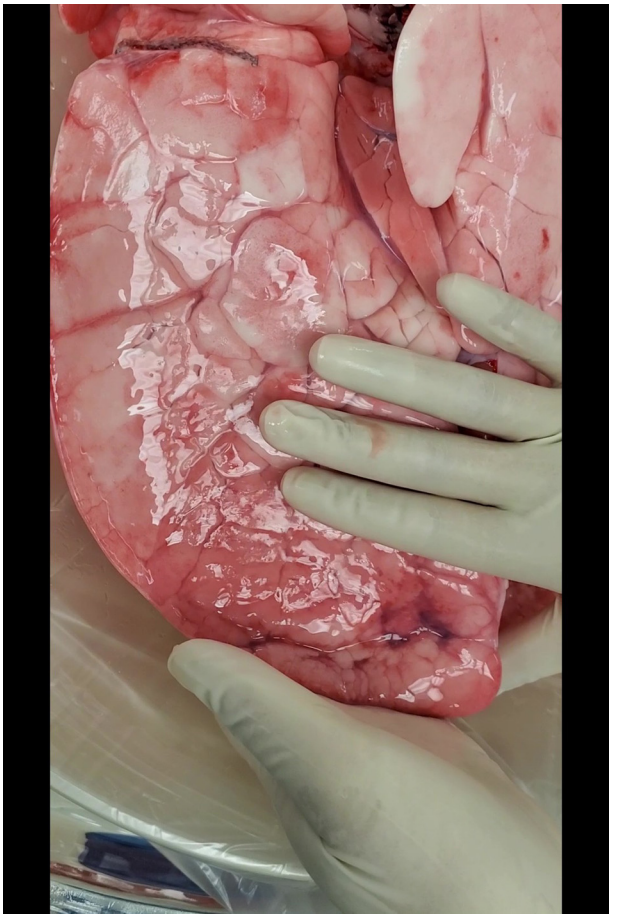
FIGURE 7. Recovery of donor extracorporeal lungs using cross-circulation with injured recipient swine. A, Donor extracorporeal lungs were subject to 24 hours of cold storage and were placed on cross-circulation for 24 hours with recipient swine that underwent lung injury with gastric aspiration. B, Extracorporeal lungs demonstrated functional, multiscale, and cellular recovery despite. C, recipient lung injury. *Pao_2*, Arterial oxygen tension; *Fio_2*, inspired oxygen fraction; *PIP*, peak inspiratory pressure.

studies modeling other etiologies of recipient lung disease will further clarify the threshold of injury that could potentially preclude extracorporeal lung recovery.

CONCLUSIONS

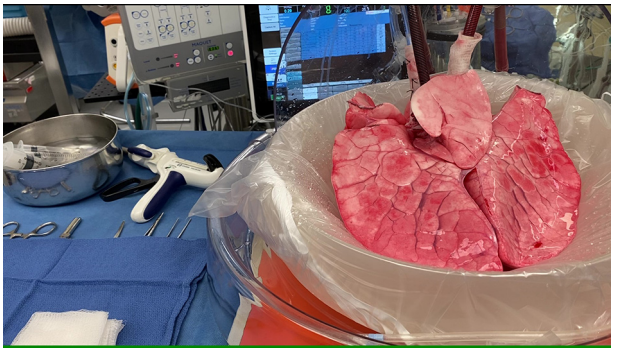
We demonstrate proof-of-feasibility that recipient swine with impaired respiratory function can (1) safely tolerate

(ZO-1) expression at 24 hours. Intact expression of K, connexin 43 (Cx 43) and L, CD31 at 24 hours of cross-circulation. M, Vascular viability indicated by acetylated LDL (acLDL) uptake in pulmonary artery after 24 hours of cross-circulation. *Box and whisker plots: lower and upper borders* represent lower and upper quartiles, *middle* represents median, *lower and upper whiskers* represent minimum and maximum values. *CD*, Cluster of differentiation; *Hpf*, high-powered field; *N*, elastase, neutrophil elastase; *OD*, optical density.



VIDEO 1. Manual recruitment of extracorporeal lungs on cross-circulation. Video available at: https://www.jtcvs.org/article/S0022-5223(23)00856-5/fulltext.

cross-circulation and (2) provide sufficient metabolic support to recover extracorporeal lungs damaged by 24 hours of 4 °C cold ischemia. We anticipate that this feasibility will extend to recipients with isolated chronic lung disease, who may be ideal candidates for this application of cross-circulation. We have robustly characterized the course of extracorporeal lung recovery after extended CIT from a global physiologic scale to a cellular level, which is a unique capability of this platform due to its durable, homeostatic milieu. We envision that this application of cross-circulation may increase allocation of lungs otherwise deemed unacceptable, leading to increased utilization of donor lungs.



VIDEO 2. Normothermic perfusion and ventilation of extracorporeal lungs on cross-circulation. Video available at: https://www.jtcvs.org/article/S0022-5223(23)00856-5/fulltext.

Conflict of Interest Statement

B.A.G. is a consultant for Xylyx Bio, Inc, which has commercial and licensing interests in cross-circulation. M.B. and G.V-N. own stock in Xylyx Bio, Inc. B.A.G., K.F., M.B., and G.V-N. are coinventors on a patent application (WO2018013849A1) for cross-circulation as a platform for extracorporeal organ recovery, regeneration, and maintenance. All other authors reported no conflicts of interest.

The *Journal* policy requires editors and reviewers to disclose conflicts of interest and to decline handling or reviewing manuscripts for which they may have a conflict of interest. The editors and reviewers of this article have no conflicts of interest.

References

1. Smith S, Trivedi JR, Fox M, Van Berkel VH. Donor lung utilization for transplantation in the United States. *J Heart Lung Transplant*. 2020;39:S374.
2. Singer JP, Chen J, Blanc PD, Leard LE, Kukreja J, Chen H. A thematic analysis of quality of life in lung transplant: the existing evidence and implications for future directions. *Am J Transplant*. 2013;13:839-50.
3. Cypel M, Yeung JC, Liu M, Anraku M, Chen F, Karolak W, et al. Normothermic ex vivo lung perfusion in clinical lung transplantation. *N Engl J Med*. 2011;364:1431-40.
4. Wallinder A, Ricksten SE, Hansson C, Riise GC, Silverborn M, Liden H, et al. Transplantation of initially rejected donor lungs after ex vivo lung perfusion. *J Thorac Cardiovasc Surg*. 2012;144:1222-8.
5. Raredon MSB, Rocco KA, Gheorghe CP, Sivarapatna A, Ghaedi M, Balestrini JL, et al. Biomimetic culture reactor for whole-lung engineering. *Biores Open Access*. 2016;5:72-83.
6. Lau AN, Goodwin M, Kim CF, Weiss DJ. Stem cells and regenerative medicine in lung biology and diseases. *Mol Ther*. 2012;20:1116-30.
7. Hozain AE, Tipograf Y, Pinezich MR, Cunningham KM, Donocoff R, Queen D, et al. Multiday maintenance of extracorporeal lungs using cross-circulation with conscious swine. *J Thorac Cardiovasc Surg*. 2020;159:1640-1.e18.
8. Hozain AE, O'Neill JD, Pinezich MR, Tipograf Y, Donocoff R, Cunningham KM, et al. Xenogeneic cross-circulation for extracorporeal recovery of injured human lungs. *Nat Med*. 2020;26:1102-13.
9. Kelly Wu W, Guenthart BA, O'Neill JD, Hozain AE, Tipograf Y, Ukita R, et al. Technique for xenogeneic cross-circulation to support human donor lungs ex vivo. *J Heart Lung Transplant*. 2023;42:335-44.
10. O'Neill JD, Guenthart BA, Kim J, Chicotka S, Queen D, Fung K, et al. Cross-circulation for extracorporeal support and recovery of the lung. *Nat Biomed Eng*. 2017;1:0037. https://doi.org/10.1038/s41551-017-0037
11. Guenthart BA, O'Neill JD, Kim J, Queen D, Chicotka S, Fung K, et al. Regeneration of severely damaged lungs using an interventional cross-circulation platform. *Nat Commun*. 2019;10:1985.
12. Pinezich M, Vunjak-Novakovic G. Bioengineering approaches to organ preservation ex vivo. *Exp Biol Med (Maywood)*. 2019;244:630-45.
13. Tipograf Y, Salna M, Minko E, Grogan EL, Agerstrand C, Sonett J, et al. Outcomes of extracorporeal membrane oxygenation as a bridge to lung transplantation. *Ann Thorac Surg*. 2019;107:1456-63.
14. Loer SA, Scheeren TW, Tarnow J. How much oxygen does the human lung consume? *Anesthesiology*. 1997;86:532-7.
15. Thabut G, Mal H, Cerrina J, Dartevelle P, Dromer C, Velly JF, et al. Graft ischemic time and outcome of lung transplantation: a multicenter analysis. *Am J Respir Crit Care Med*. 2005;171:786-91.

16. Okamoto T, Wheeler D, Farver CF, McCurry KR. Transplant suitability of rejected human donor lungs with prolonged cold ischemia time in low-flow acellular and high-flow cellular ex vivo lung perfusion systems. *Transplantation*. 2019;103:1799-808.
17. Kaslow SR, Reimer JA, Pinezich MR, Hudock MR, Chen P, Morris MG, et al. A clinically relevant model of acute respiratory distress syndrome in human-size swine. *Dis Model Mech*. 2022;15:dmm049603.
18. Login FH, Palmfeldt J, Cheah JS, Yamada S, Nejsum LN. Aquaporin-5 regulation of cell–cell adhesion proteins: an elusive "tail" story. *Am J Physiol Cell Physiol*. 2021;320:C282-92.
19. Hoetzenecker K, Schwarz S, Keshavjee S, Cypel M. Lung transplantation for acute respiratory distress syndrome. *J Thorac Cardiovasc Surg*. 2023;165:1596-601.
20. Leard LE, Holm AM, Valapour M, Glanville AR, Attawar S, Aversa M, et al. Consensus document for the selection of lung transplant candidates: an update from the International Society for Heart and Lung Transplantation. *J Heart Lung Transplant*. 2021;40:1349-79.
21. Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, et al. Incidence and outcomes of acute lung injury. *N Engl J Med*. 2005;353:1685-93.
22. Johnson ER, Matthay MA. Acute lung injury: epidemiology, pathogenesis, and treatment. *J Aerosol Med Pulm Drug Deliv*. 2010;23:243-52.
23. Ware LB, Matthay MA. Alveolar fluid clearance is impaired in the majority of patients with acute lung injury and the acute respiratory distress syndrome. *Am J Respir Crit Care Med*. 2001;163:1376-83.
24. Martin TR, Hagimoto N, Nakamura M, Matute-Bello G. Apoptosis and epithelial injury in the lungs. *Proc Am Thorac Soc*. 2005;2:214-20.
25. Racanelli AC, Kikkers SA, Choi AMK, Cloonan SM. Autophagy and inflammation in chronic respiratory disease. *Autophagy*. 2018;14:221-32.
26. Munshi L, Keshavjee S, Cypel M. Donor management and lung preservation for lung transplantation. *Lancet Respir Med*. 2013;1:318-28.
27. Ali A, Wang A, Ribeiro RVP, Beroncal EL, Baciu C, Galasso M, et al. Static lung storage at 10 °C maintains mitochondrial health and preserves donor organ function. *Sci Transl Med*. 2021;13:eabf7601.
28. Ali A, Hoetzenecker K, Luis Campo-Cañaveral de la Cruz J, Schwarz S, Barturen MG, Tomlinson G, et al. Extension of cold static donor lung preservation at 10 °C. *NEJM Evidence*. 2023;2(6). https://doi.org/10.1056/EVIDoa2300008
29. Gohrbandt B, Fischer S, Warnecke G, Avsar M, Sommer SP, Haverich A, et al. Glycine intravenous donor preconditioning is superior to glycine supplementation to low-potassium dextran flush preservation and improves graft function in a large animal lung transplantation model after 24 hours of cold ischemia. *J Thorac Cardiovasc Surg*. 2006;131:724-9.
30. Warnecke G, Sommer SP, Gohrbandt B, Fischer S, Hohlfeld JM, Niedermeyer J, et al. Warm or cold ischemia in animal models of lung ischemia–reperfusion injury: is there a difference? *Thorac Cardiovasc Surg*. 2004;52:174-9.
31. Sadaria MR, Smith PD, Fullerton DA, Justison GA, Lee JH, Puskas F, et al. Cytokine expression profile in human lungs undergoing normothermic ex-vivo lung perfusion. *Ann Thorac Surg*. 2011;92:478-84.
32. Leligdowicz A, Ross JT, Nesseler N, Matthay MA. The endogenous capacity to produce proinflammatory mediators by the ex vivo human perfused lung. *Intensive Care Med Exp*. 2020;8:56.
33. de Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia–reperfusion-induced lung injury. *Am J Respir Crit Care Med*. 2003;167:490-511.
34. Ukita R, Stokes JW, Wu WK, Talackine J, Cardwell N, Patel Y, et al. A large animal model for pulmonary hypertension and right ventricular failure: left pulmonary artery ligation and progressive main pulmonary artery banding in sheep. *J Vis Exp* 2021 July 15;(173):10.3791/62694.
35. Pohlmann JR, Akay B, Camboni D, Koch KL, Mervak BM, Cook KE. A low mortality model of chronic pulmonary hypertension in sheep. *J Surg Res*. 2012;175:44-8.

Key Words: cross-circulation, lung transplantation, normothermic organ perfusion, tissue engineering, acute lung injury, ex vivo lung perfusion, swine model

APPENDIX E1. SUPPLEMENTARY DISCUSSION

Comparison With Conventional Ex Vivo Lung Perfusion (EVLP)

Efforts to expand the donor pool have included the use of conventional EVLP, which has demonstrated an ability to recover marginal quality lungs through recirculation of an acellular or cellular perfusate. However, the duration of homeostatic support provided by EVLP is limited up to 12 hours and is insufficient for recovery of severely injured donor lungs and cellular and histological regeneration.[E1-E4] Notably, EVLP lacks the homeostatic regulation provided by the hepatic, pancreatic, renal, and neurohormonal systems that allow for long-term support and clearance of metabolites. Cross-circulation is a new approach first described by our group, which allows for circulation of whole blood between the extracorporeal lung and a live recipient providing multisystem physiologic regulation.[E5] Previous work from our group has demonstrated that lactate concentration in the perfusate quickly rises to 8.20 mM within 2 hours of EVLP, further increasing to 12.38 mM after 4 hours of EVLP but rapidly decreases to 2.98 mM within only 4 hours after transitioning to cross-circulation support.[E5] Attempts to modify EVLP with dialysis membranes have demonstrated limited improvements to extracorporeal lung function, which underscores the importance of the multisystem cross-talk that is provided by a whole recipient.[E6]

In addition, the long-term support provided by cross-circulation allows for a more robust multiscale evaluation of extracorporeal lung recovery, which includes not only gas exchange capability but also cellular viability, immunostaining analysis, and histopathologic evaluation. Previous work from our group has described the relative deficiencies with EVLP models in the scope of analysis during the course of extracorporeal lung perfusion.[E7] Specifically, EVLP platforms have been limited in the ability to report on cellular viability, functional assessments such as broncho- and vasoresponsiveness, microscopic analyses of histologic recovery, and thorough inflammatory profile trends.[E1,E2,E4,E8]

We believe that despite its limitations, EVLP remains an important clinical tool and cross-circulation represents a potential adjunct rather than replacement for conventional EVLP. We acknowledge that cross-circulation may be resource-intensive and also introduce the potential for infectious issues in the donor lung. Although this study did not examine the role of antibiotics, we believe that continued redosing of antibiotics would be wise in an actual clinical setting to protect not only the extracorporeal lungs but also the recipient from infectious complications. We envision that marginal quality lungs requiring only short periods of normothermic perfusion may be recovered using EVLP, whereas cross-circulation provides an opportunity to recover more severely damaged lungs, particularly those requiring a longer period of physiologic support for cellular regeneration or extensive therapeutic and bioengineering interventions.

Left Atrial Reconstruction Technique

Donor lungs are procured in the standard fashion as described in detailed procedure methods in Appendix E2. The left atrium is removed leaving a left atrial cuff, as depicted in Figure E1, *A*. Reconstruction of the left atrium circumvents the challenge of pulmonary venous outflow from multiple pulmonary veins. Our cross-circulation technique uses a closed left atrium, similar to the XVIVO EVLP platform, in which the left atrium is reconstructed and cannulated (Figure E1, *B*). Our group implements a vascular biobridge using donor aortic arch that is anastomosed to the left atrial cuff, which provides an endothelialized nonthrombogenic conduit for pulmonary venous drainage into the extracorporeal circuit. This theoretically provides an antithrombotic benefit for prevention of thrombotic complications in both the extracorporeal lung and the recipient swine. In addition, the elasticity of the vascular biobridge allows for increased compliance to ameliorate sudden pressure changes and facilitates laminar flow.[E5] The vascular biobridge and pulmonary artery are subsequently cannulated, and lungs de-aired prior to reperfusion with cross-circulation (Video 2).

APPENDIX E2. DETAILED PROCEDURE METHODS

Recipient Swine Sedation

Recipient swine (n = 6) underwent induction with intramuscular Telazol (5 mg kg^{-1}; Zoetis) and intravenous dexmedetomidine (0.005 mg kg^{-1}; Zoetis). General anesthesia was performed with continuous infusions of fentanyl citrate (0.01 mg kg^{-1}; Hikma Pharmaceuticals), midazolam (0.2 mg kg^{-1} h^{-1}; Avet Pharmaceuticals), and propofol (3-5 mg kg^{-1} h^{-1}; Pfizer). A femoral arterial line (Arrow International) was placed for continuous blood pressure monitoring and blood sampling. A femoral central venous catheter (Arrow International) was placed for continuous drug infusions.

Validation of Recipient Swine Lung Injury

Recipient swine vitals and ventilator settings were recorded at baseline and continuously after lung injury. Bronchoscopy and blood sampling was performed every 6 hours for assessment of inflammatory markers and hemolytic markers. Inflammatory markers (interferon-γ, interleukin [IL]-1α, IL-1β, IL-1Ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, tumor necrosis factor-α) were assessed by multiplex array (Eve Technologies). Hemolytic markers and activation markers (D-dimer, fibrinogen, plasma-free hemoglobin, P-selectin, M30) were analyzed using commercially available enzyme-linked immunosorbent

assays (Table E6). Blood samples were delivered to the Institute of Comparative Medicine Diagnostic Laboratory at Columbia University for complete blood count, basic metabolic panel, liver function tests, lactate dehydrogenase, and coagulation panels. Chest radiographs were performed at baseline, 2 hours after injury, and at experimental endpoint. Gross images of recipient lungs were obtained at the experimental end point. Lung tissue samples were obtained at the end point using a surgical stapler with medium/thick reloads for histopathologic analysis and immunohistochemical staining.

Donor Lung Procurement

Donor swine underwent induction and general anesthesia in a similar fashion as recipient swine. Animals were prepped and draped in sterile fashion and cefazolin (30 mg kg^{-1}; WG Critical Care) was administered before skin incision and median sternotomy. Then, 30,000 U of heparin was administered before cannulation of the main pulmonary artery. After exsanguination and presence of a non-perfusing heart rhythm, an antegrade flush with cold low-potassium dextran solution (Perfadex; XVIVO) containing alprostadil (25 mg kg^{-1}; Prostin VR Pediatric, Pfizer) was performed via the main pulmonary artery and the superior vena cava was ligated, and left atrial appendage and inferior vena cava were incised. Topical cooling was performed by packing the chest with sterile ice. Lungs were inflated, the trachea was stapled, and lungs were explanted and placed on ice. The heart was removed, leaving behind a left atrial cuff, and a retrograde flush with cold Perfadex was performed via the pulmonary veins. The aortic arch was dissected in preparation for a biobridge as previously described and the left subclavian and brachiocephalic branches were stapled.[E5,E7,E9,E10]

Lung Cannulation

The aortic arch was sutured to the left atrial cuff as a vascular biobridge, using a running 5-0 polypropylene suture (Figure E1, *A*). A 20- to 32-F cannula was secured in the main pulmonary artery and a 36-F cannula was secured in the aortic biobridge using 0-silk ties (Figure E1, *B*). An 8 mm endotracheal tube was secured in the trachea with 0-silk ties. Cold normal saline was used to flush and de-air the lungs. Once flushed, lungs were placed in a sterile double-lined basin containing warm normal saline.

Cross-Circulation

Circuit elements included a pump console (Jostra HL-20; Maquet), disposable pump (Rotaflow centrifugal pump; Maquet), and continuous pressure and flow monitor (VIPER software, G2 v1.26.4; Spectrum Medical) (Figure E1, *E*). Flow rates were maintained within 5%-10% of estimated cardiac output, with pulmonary artery pressures maintained <15 mm Hg and pulmonary vein pressures between 3 and 5 mm Hg. The recipient swine was maintained on a continuous heparin infusion with initial rate of 25 U kg^{-1} h^{-1}, and activated clotting time was monitored hourly using a whole-blood microcoagulation system (Hemochron; Accriva Diagnostics) with target range of 250 to 350 seconds.

Extracorporeal Lung Monitoring and Functional Analysis

Blood samples were obtained from the pulmonary artery and pulmonary vein cannulas every 6 hours and blood gas analysis was performed in the same manner as for the recipient blood samples. Dynamic compliance (C_{dyn} = tidal volume/[peak inspiratory pressure—positive end-expiratory pressure]) was calculated every 6 hours. Photographic (iPhone 13; Apple) and thermal infrared images (T430sx; FLIR) were obtained. Serial radiographs of the extracorporeal lung were obtained using a portable unit (PXP-16HF; United Radiology Systems). Lung weight was recorded every 6 hours using a scale (Denver Instrument Company).

Bronchoalveolar Lavage (BAL) Analysis

Bronchoscopic evaluation of the airways in both recipient and extracorporeal lungs was performed every 6 hours. A flexible 3.8-mm bronchoscope (aScope 4; Ambu) was inserted into the subsegmental bronchi of the left and right lower lobes. Sterile normal saline (5 mL) was injected and aspirated into a sterile specimen container (Busse). pH of BAL fluid samples was measured with pH strips (Ricca Chemical). BAL fluid sample smears were stained using a commercially available Kwik-Diff kit (Thermo Fisher Scientific) and imaged with a slide scanner (BX61VS; Olympus). BAL samples were centrifuged at 3500 rpm for 10 minutes at 4 °C, and supernatants were stored at −80 °C until further processing. BAL fluid protein concentrations were quantified by a commercially available protein assay (Pierce BCA Protein Assay; Thermo Fisher Scientific).

Molecular Pathology and Cytokine Analysis

Inflammatory, hemolytic, and activation markers in extracorporeal lungs were assessed in the same manner as for recipient swine.

Acetylated Low-Density Lipoprotein (LDL) Uptake Assay

To assess viability of the pulmonary endothelium at the experimental endpoint, biopsies of the left and right pulmonary arteries were obtained and placed in a 96-well plate (BD Falcon). Acetylated LDL, Alexa Fluor 594 conjugate (Thermo Fisher Scientific) was diluted 1:200 in DMEM/F12K cell culture media (Corning). Then, 150 μL of media containing acetylated LDL was added to the wells

containing vascular biopsies. The plate was then covered and incubated at 37 °C with gentle agitation for 4 hours. Samples were washed 5 times with phosphate-buffered saline and fixed in cold phosphate-buffered 4% paraformaldehyde for 24 hours. Samples were embedded in paraffin and sectioned at 5-μm thickness. Following deparaffinization and staining with DAPI, sections were examined using a fluorescent microscope (DMi8; Leica).

Vasoresponsiveness

After 24 hours of cross-circulation, the extracorporeal lung was decoupled from cross-circulation and placed on isolated EVLP. Epinephrine (4 mg; Vedco) was administered via the pulmonary artery cannula of the extracorporeal lung and pulmonary artery and pulmonary vein pressures were continuously recorded to assess vasoresponsiveness to adrenergic stimulation.

Bronchoresponsiveness

After 24 hours of cross-circulation, nebulized methacholine chloride (100 mg; Methapharm) was intratracheally administered. Peak inspiratory pressures were continuously monitored to assess airway smooth muscle tone.

Metabolic Activity Assay

To assess metabolic activity in extracorporeal lung tissue, samples were collected every 12 hours during cross-circulation. Lung tissue was finely minced and placed in a 96-well plate. Alamar Blue reagent (Thermo Fisher Scientific) was diluted 1:10 in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum. Then, 150 μL of alamarBlue reagent was added to wells containing lung tissue and empty wells (negative control). The plate was incubated at 37 °C protected from light for 2 hours with gentle agitation. Absorbance was measured at 570 nm and normalized to 600 nm.

Myeloperoxidase Activity Assay

Lung tissue samples were collected every 12 hours during cross circulation and snap frozen in liquid nitrogen and stored at −80 °C until further processing. Myeloperoxidase activity was quantified using a commercially available assay (Abcam).

Histopathologic Analysis

Donor lung samples were obtained in situ, after 24 hours of cold ischemia, and every 12 hours on cross-circulation using a surgical stapler with medium/thick reloads. Tissue specimens were fixed in cold phosphate-buffered 4% paraformaldehyde for 24 to 48 hours, embedded in paraffin, and sectioned at 5-μm thickness. Tissue sections were stained with hematoxylin and eosin, Alcian blue, trichrome, and pentachrome by the histology services of the Department of Molecular Pathology at Columbia University. Histologic evaluation of extracorporeal lungs after 24 hours of cold ischemia and before initiation of cross-circulation is summarized in Figure E3.

Immunohistochemical Staining

Tissue sections were deparaffinized and antigen retrieval was performed with boiling citrate buffer (pH 6.0). Sections were blocked with 10% donkey serum in phosphate-buffered saline for 2 hours at room temperature. Antibodies (diluted 1:100 in phosphate-buffered saline) or phosphate-buffered saline alone (negative control) were applied to tissue sections and incubated for 12 hours at 4 °C. Secondary antibodies were diluted 1:200 and applied to sections followed by incubation at room temperature for 2 hours. Following DAPI staining, sections were mounted with Vectashield mounting medium (Vector Laboratories) and imaged using a fluorescent microscope (DMi8; Leica). Terminal deoxynucleotidyl transferase dUTP nick end labeling assay and additional stains for neutrophil elastase and CD163 were performed by the histology services of the Department of Molecular Pathology at Columbia University. A list of primary and secondary antibodies used is shown in Table E5.

Blinded Pathologic Review

An experienced pulmonary pathologist blinded to study timepoints performed histologic review of tissue samples to generate a lung injury score according to previously described methods.[E5,E7,E9,E10] Scoring was based on number of apoptotic cells per high-power field (hpf; 40×) (0 → <2, 1 → 3-5, 2 → 6-10, 3 → >10), number of neutrophils per hpf (0 → <5, 1 → 6-10, 2 → 11-20, 3 → >20), alveolar edema (0 → <5% of all alveoli contained edema fluid, 1 → 6T-25%, 2 → 26T-50%, 3 → >50%), interstitial infiltrate (0 → none per hpf, 1 → <50 per hpf, 2 → 50-100 per hpf, 3 → 100 per hpf), and interstitial edema (0 → none, 1 → 1x width vessel media, 2 → ≥2× width vessel media).

Statistical Analysis and Animal Use Justification

The number of animals used in this study was calculated based on the resource equation approach as described by Arifin and colleagues.[E11] The minimum number of animals was calculated using the following formula: minimum $N = 10/(r-1) + 1$, where $r = 5$ and represents the number of repeated measurements throughout 24 hours of cross-circulation. We utilized an additional 4 animals (2 donors and 2 recipients) to obtain a final N of 6, in order to further support proof of feasibility. One-way analysis of variance and paired Student t tests were performed using statistical analysis software (Prism Version 9, GraphPad).

E-References

E1. Nakajima D, Liu M, Ohsumi A, Kalaf R, Iskender I, Hsin M, et al. Lung lavage and surfactant replacement during ex vivo lung perfusion for treatment of gastric acid aspiration–induced donor lung injury. *J Heart Lung Transplant*. 2017;36:577-85.

E2. Inci I, Hillinger S, Arni S, Kaplan T, Inci D, Weder W. Reconditioning of an injured lung graft with intrabronchial surfactant instillation in an ex vivo lung perfusion system followed by transplantation. *J Surg Res*. 2013;184:1143-9.

E3. Meers CM, Wauters S, Verbeken E, Scheers H, Vanaudenaerde B, Verleden GM, et al. Preemptive therapy with steroids but not macrolides improves gas exchange in caustic-injured donor lungs. *J Surg Res*. 2011;170:e141-8.

E4. Khalifé-Hocquemiller T, Sage E, Dorfmuller P, Eddahibi S, Fadel E. 394 Ex vivo perfusion worsened lung injuries induced by gastric acid aspiration in pigs. *J Heart Lung Transplant*. 2011;30:S135-6.

E5. O'Neill JD, Guenthart BA, Kim J, Chicotka S, Queen D, Fung K, et al. Cross-circulation for extracorporeal support and recovery of the lung. *Nat Biomed Eng*. 2017;1:0037.

E6. Buchko MT, Himmat S, Stewart CJ, Hatami S, Dromparis P, Adam BA, et al. Continuous hemodialysis does not improve graft function during ex vivo lung perfusion over 24 hours. *Transplant Proc*. 2019;51:2022-8.

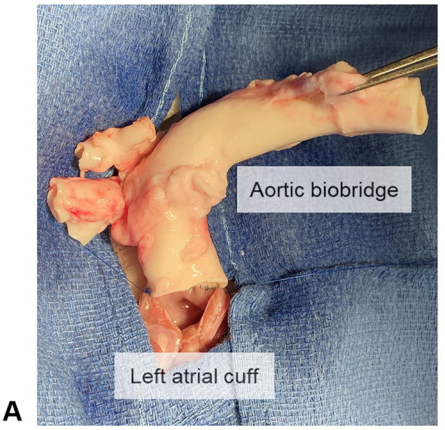
E7. Guenthart BA, O'Neill JD, Kim J, Queen D, Chicotka S, Fung K, et al. Regeneration of severely damaged lungs using an interventional cross-circulation platform. *Nat Commun*. 2019;10:1985.

E8. Meers CM, Tsagkaropoulos S, Wauters S, Verbeken E, Vanaudenaerde B, Scheers H, et al. A model of ex vivo perfusion of porcine donor lungs injured by gastric aspiration: a step towards pretransplant reconditioning. *J Surg Res*. 2011;170:e159-67.

E9. Hozain AE, Tipograf Y, Pinezich MR, Cunningham KM, Donocoff R, Queen D, et al. Multiday maintenance of extracorporeal lungs using cross-circulation with conscious swine. *J Thorac Cardiovasc Surg*. 2020;159:1640-53.e18.

E10. Hozain AE, O'Neill JD, Pinezich MR, Tipograf Y, Donocoff R, Cunningham KM, et al. Xenogeneic cross-circulation for extracorporeal recovery of injured human lungs. *Nat Med*. 2020;26:1102-13.

E11. Arifin WN, Zahiruddin WM. Sample size calculation in animal studies using resource equation approach. *Malaysian J Med Sci*. 2017;24:101-5.



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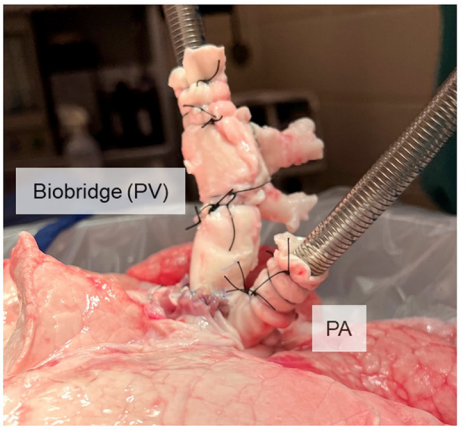

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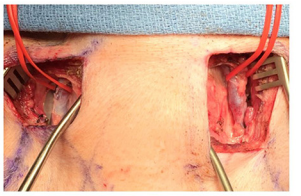

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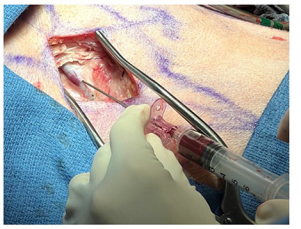

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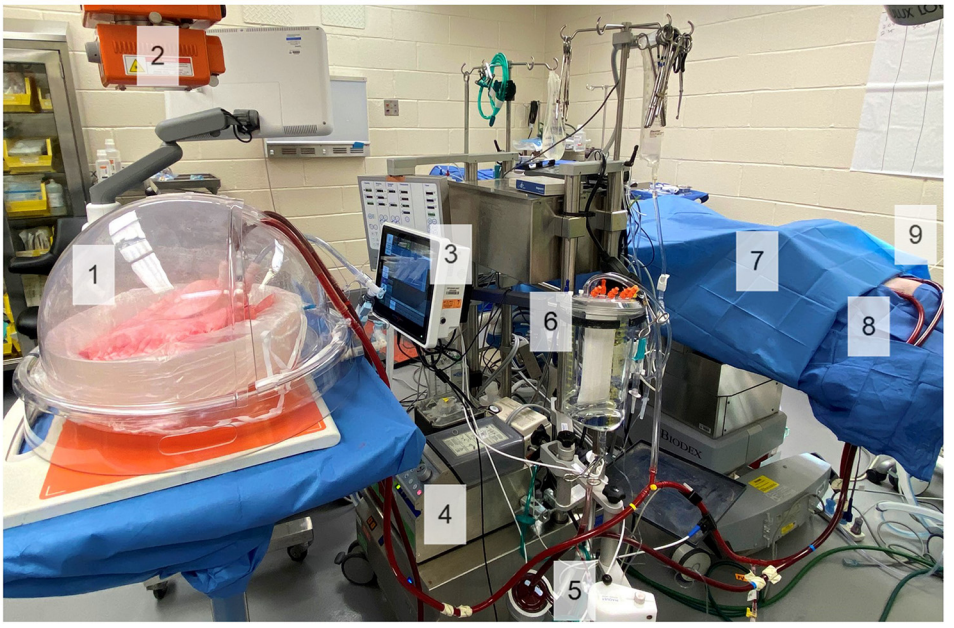

E

1 Humidified organ chamber
2 X-ray
3 Perfusion data acquisition system
4 Pump controls
5 Pump
6 Reservoir
7 Anesthetized recipient
8 Recipient inflow cannula
9 Recipient outflow cannula

FIGURE E1. Operative technique and setup. A, Anastomosis of aortic biobridge to left atrial cuff. B, Cannulation of lungs via pulmonary artery (*PA*) inflow and biobridge for pulmonary vein (PV) drainage. C, Bilateral cutdown exposing internal jugular veins of recipient swine and D, cannulation of internal jugular vein via Seldinger technique. E, Operating room setup during cross-circulation.

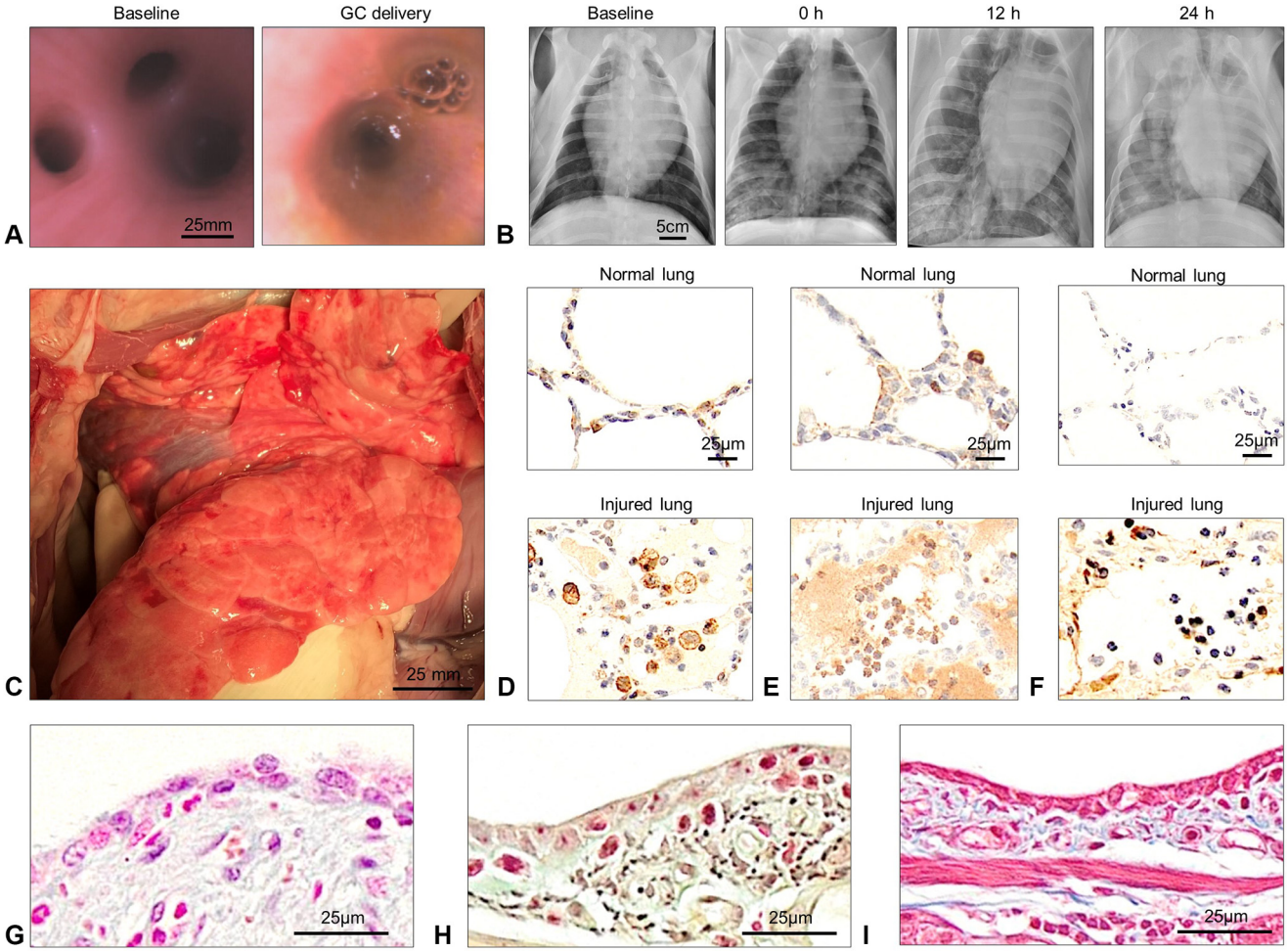

FIGURE E2. Recipient lung injury. A, Bronchoscopy at baseline and during gastric content (*GC*) delivery. B, Radiograph of the chest at baseline before injury and throughout cross-circulation. C, Gross appearance of recipient lungs after 24 hours of cross-circulation. Immunostaining of D, CD163 + macrophages and E, N. elastase, and F, TUNEL staining. G, Alcian blue staining demonstrating loss of mucus-containing goblet cells. H, Pentachrome and I, trichrome staining demonstrating loss of ciliated columnar epithelium.

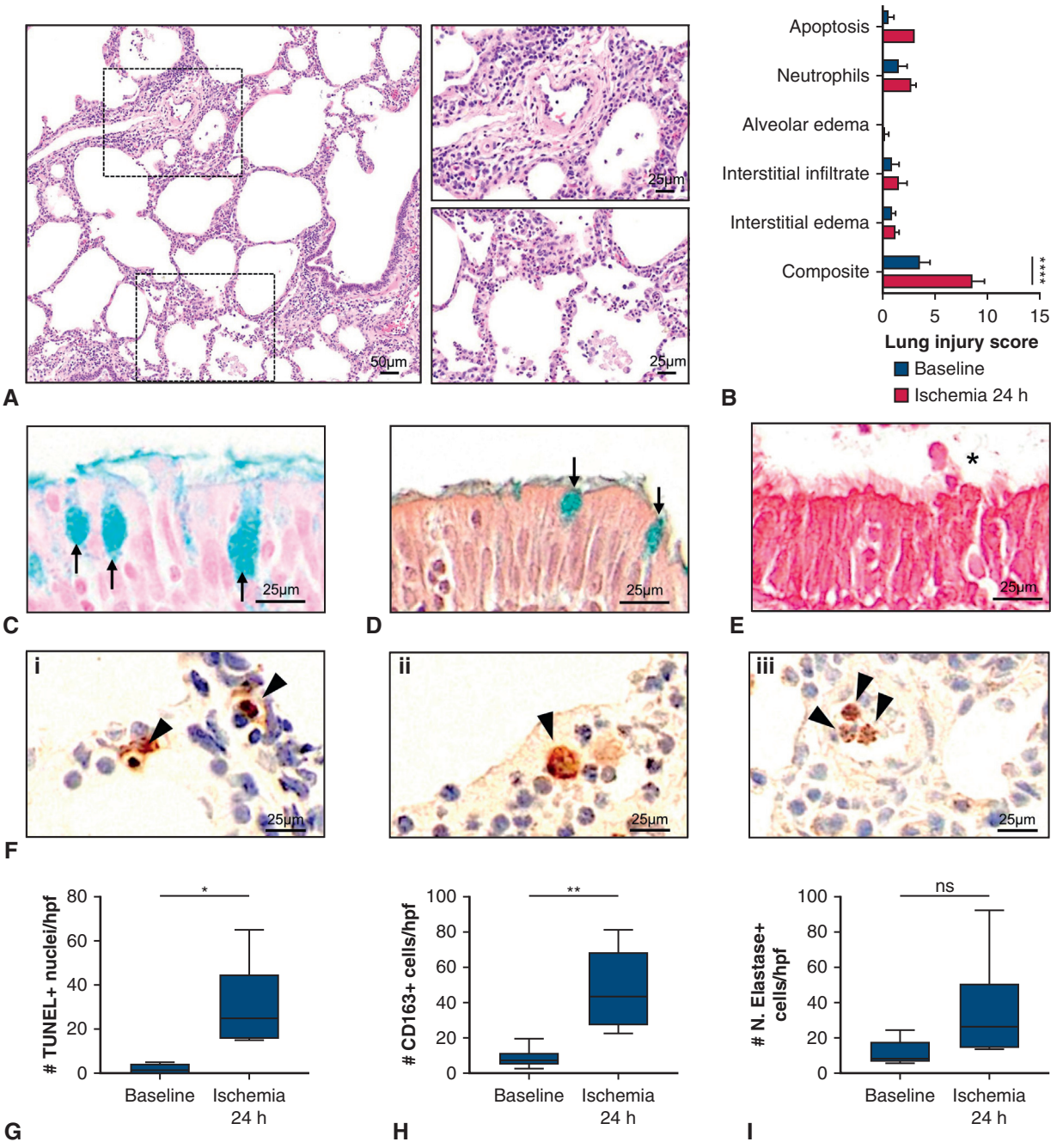

FIGURE E3. Histologic evaluation of extracorporeal lung after 24-hour cold ischemia injury. A, Hematoxylin and eosin staining of extracorporeal lungs after 24 hours cold ischemia before reperfusion. *Insets* demonstrate interstitial and airway infiltrates. B, Lung injury score obtained after 24 hours of cold ischemia. C, Alcian blue (*arrows* represent mucus-containing goblet cells) and D, pentachrome staining demonstrating intact mucus-containing goblet cells (represented by *arrows*) and E, preserved ciliated epithelium with neutrophil extravasation into airway space (represented by *asterisk*). F, TUNEL + cells (positive stain repressed by *arrowheads*) (i) and immunostaining of CD163 + macrophages (positive stain represented by *arrowheads*) (ii) and neutrophils (positive stain represented by *arrowheads*) (iii). Quantification of G, TUNEL + cells, H, CD163 + macrophages, and I, neutrophils after 24 hours of cold ischemia. *TUNEL*, Terminal deoxynucleotidyl transferase dUTP nick end labeling; *CD*, cluster of differentiation.

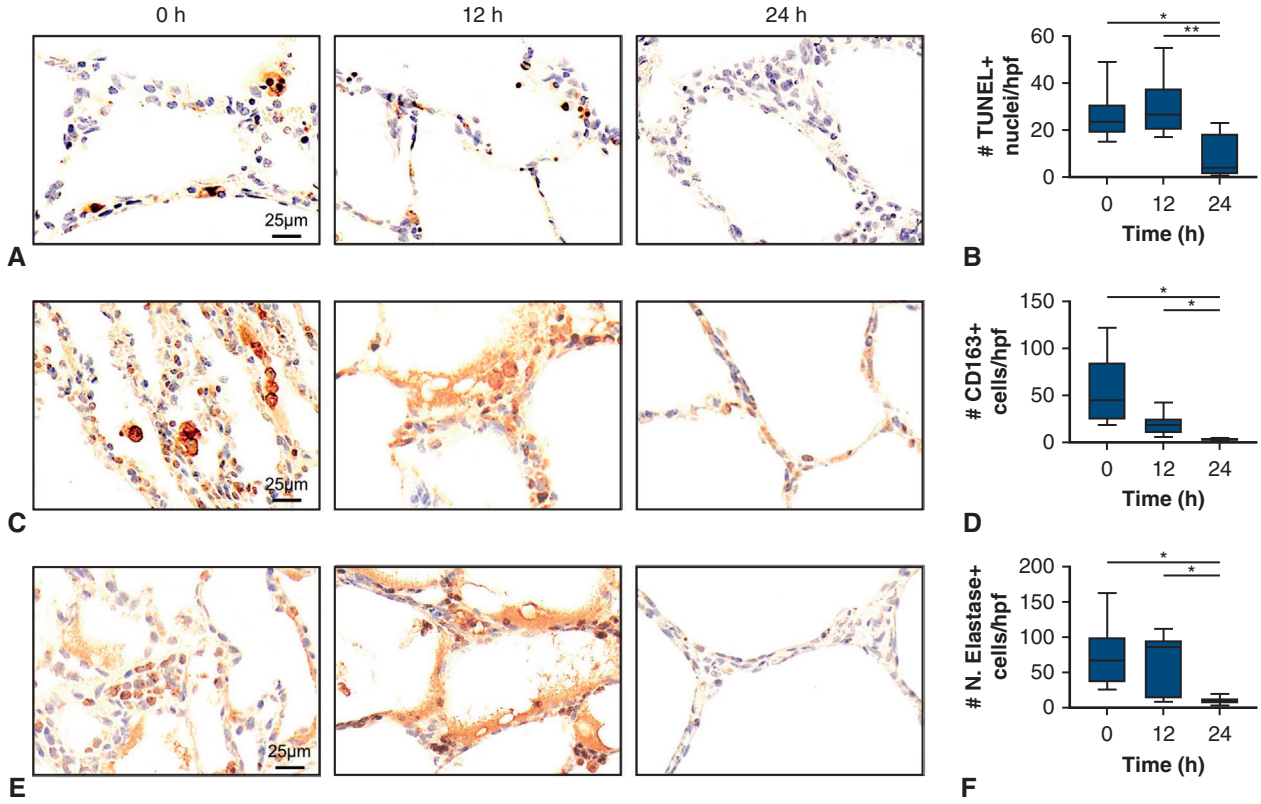

FIGURE E4. TUNEL, CD163, and N. Elastase stains in extracorporeal lung throughout cross-circulation. A, TUNEL staining throughout 24 hours of cross-circulation and B, quantification of TUNEL + cell counts (*$P \leq .05$, **$P \leq .01$). C, CD163 staining throughout 24 hours of cross-circulation and D, quantification of CD163 + cells (*$P \leq .05$). E, N. elastase immunostaining throughout cross-circulation and F, quantification of neutrophils (*$P \leq .05$). *TUNEL*, Terminal deoxynucleotidyl transferase dUTP nick end labeling; *CD*, cluster of differentiation; *N*, elastase.

TABLE E1. Recipient swine gastric content re-doses

Recipient animal no.	Gastric content re-doses
1	3
2	2
3	2
4	2
5	7
6	5

Shown is a summary of number or gastric content doses for each recipient animal.

TABLE E2. Recipient cytokines in BAL fluid

Inflammatory cytokines, pg/mL	Time, h			
	Baseline	0	12	24
IL-1α	16.1 ± 13.1	677.1 ± 407.4	589.9 ± 318.5	530.5 ± 136.0
IL-1Ra	67.9 ± 51.2	5412.2 ± 765.1	10,920.0 ± 5898.1	10,324.2 ± 5691.6
IL-1β	76.4 ± 64.9	23,262.2 ± 13,017.3	19,982.5 ± 8423.2	25,572.8 ± 9996.3
IL-4	10.8 ± 1.6	30.9 ± 27.6	40.6 ± 34.6	25.9 ± 22.2
IL-6	1.6 ± 1.0	1189.3 ± 419.8	1538.8 ± 863.1	898.2 ± 473.2
IL-8	208.0 ± 223.9	14,155.3 ± 8226.2	20,759.2 ± 3049.7	22,244.7 ± 1296.9
IL-10	13.8 ± 7.2	55.9 ± 7.5	93.2 ± 30.5	241.7 ± 185.7
IL-12	13.5 ± 8.9	122.7 ± 72.6	61.9 ± 14.7	76.7 ± 49.1
IL-18	138.4 ± 62.3	634.7 ± 528.6	561.3 ± 83.4	663.4 ± 351.4
M30	144.6 ± 27.5	127.9 ± 8.7	121.6 ± 1.8	127.7 ± 9.5

Quantification of inflammatory cytokines in recipient BAL fluid at baseline prior to injury and throughout cross-circulation (n = 6). All values represent mean ± standard deviation. *IL*, Interleukin.

TABLE E3. Recipient serum cytokines

Inflammatory cytokines, pg/mL	Time (h)		
	0	12	24
IFN-γ	387.6 ± 175.9	168.6 ± 19.1	394.9 ± 310.8
IL-1α	18.0 ± 11.6	18.9 ± 14.2	16.9 ± 11.5
IL-1Ra	4854.9 ± 3617.2	5405.5 ± 2963.4	1619.8 ± 816.2
IL-1β	242.3 ± 111.3	256.1 ± 145.7	233.2 ± 123.4
IL-2	149.5 ± 70.6	166.1 ± 72.7	133.0 ± 50.3
IL-4	440.6 ± 262.3	615.5 ± 481.3	602.1 ± 467.4
IL-6	261.7 ± 135.6	121.0 ± 38.6	113.9 ± 41.2
IL-10	734.3 ± 186.5	560.1 ± 243.3	477.9 ± 252.7
IL-12	1153.9 ± 355.2	767.6 ± 298.3	377.1 ± 135.1
IL-18	920.5 ± 400.5	616.8 ± 417.9	511.7 ± 304.9
TNF-α	112.9 ± 42.5	58.7 ± 33.2	38.7 ± 30.6
P-selectin	15.0 ± 3.2	15.6 ± 3.2	15.8 ± 3.4
M30	296.8 ± 76.6	133.5 ± 36.7	115.3 ± 23.2

Quantification of inflammatory cytokines in recipient serum throughout 24 hours of cross-circulation (n = 6). All values represent mean ± standard deviation. *IFN*, Interferon; *IL*, interleukin; *TNF*, tumor necrosis factor-α.

TABLE E4. Cytokines in extracorporeal lung BAL fluid

Inflammatory cytokines, pg/mL	Time (h)		
	0	12	24
IL-1α	37.9 ± 30.3	42.2 ± 19.1	59.5 ± 58.4
IL-1Ra	1634.4 ± 1066.4	1048.7 ± 1039.7	385.1 ± 99.9
IL-1β	846.7 ± 787.5	1337.3 ± 1224.1	1110.9 ± 1092.4
IL-4	11.2 ± 3.6	10.0 ± 5.4	15.5 ± 11.6
IL-6	35.3 ± 30.0	1030.7 ± 366.6	961.8 ± 419.8
IL-8	3212.5 ± 3333.8	2595.3 ± 939.6	6647.8 ± 5323.3
IL-10	14.6 ± 0.5	6.6 ± 1.8	14.3 ± 11.0
IL-12	219.2 ± 199.0	210.7 ± 239.6	89.9 ± 59.9
IL-18	1424.3 ± 1413.9	623.3 ± 191.7	146.4 ± 90.2
M30	145.5 ± 23.8	124.3 ± 5.4	128.0 ± 6.0

Quantification of inflammatory cytokines in BAL fluid of extracorporeal lungs throughout 24 hours of cross-circulation (n = 6). All values represent mean ± standard deviation.
IL, Interleukin.

TABLE E5. Primary and secondary antibodies

Antibodies	Application	Catalog number	Dilution
Primary			
Rabbit anti-caspase 3	IHC	ab13847	1:400
Rabbit anti-CD31	IHC	ab28364	1:100
Rabbit anti-CD163	IHC	ab28364	1:800
Rabbit anti-connexin 43	IHC	ab11370	1:100
Rabbit anti-neutrophil elastase	IHC	ab68672	1:250
Rabbit anti-ZO-1	IHC	ab216880	1:100
Rabbit anti-ZO-3	IHC	ab191143	1:100
Rabbit anti-aquaporin 5	IHC	ab78486	1:100
Secondary			
Goat anti-rabbit IgG (594)	IHC	ab150080	1:200
Biotinylated goat anti-rabbit IgG	IHC	BA-1000	1:200

IHC, Immunohistochemistry; *CD*, cluster of differentiation; *ZO-1*, zonula occludens-1; *ZO-3*, zonula occludens-3; *IgG*, immunoglobulin G.

TABLE E6. ELISA kit product information

Marker	Application	Catalog number
D-dimer	Blood	LS-F56441
Fibrinogen	Blood	MBS9308812
M30	BAL/blood	MBS751241
Plasma free hemoglobin	Blood	MBS267146
P-selectin	Blood	MBS030206

BAL, Bronchoalveolar lavage.