Interactions of Cardiopulmonary Bypass and Erythrocyte Transfusion in the Pathogenesis of Pulmonary Dysfunction in Swine

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ABSTRACT

Background: Allogeneic erythrocyte transfusion in cardiac surgical patients is associated with a fourfold increase in pulmonary complications. Our understanding of the processes underlying these observations is poor and there is no experimental model of transfusion-related acute lung injury that shows homology to cardiac surgical patients. Our objective was to develop a novel swine recovery model to determine how two clinical risk factors, allogenic erythrocyte transfusion and cardiopulmonary bypass, interact in the genesis of postcardiac surgery acute lung injury.

Methods: Thirty-six pigs were infused with allogeneic 14- or 42-day-old erythrocytes or they underwent cardiopulmonary bypass with or without transfusion of 42-day erythrocyte. Controls received saline. All pigs were recovered and assessed for pulmonary dysfunction, inflammation, and endothelial activation at 24 h.

Results: Transfusion of stored allogeneic erythrocytes in pigs compared with sham caused pulmonary dysfunction characterized by reduced lung compliance (mean difference −3.36 [95% CI, −5.31 to −1.42] ml/cm H_2O), an increase in protein levels in bronchoalveolar lavage fluid, histological lung injury inflammation, and endothelial activation. Transfusion of blood stored for up to 42 days resulted in greater protein levels in bronchoalveolar lavage fluid, macrophage infiltration, platelet activation, and depletion of T-lymphocytes in recipient lungs *versus* 14-day-old blood. Transfusion interacted with cardiopulmonary bypass to increase lung injury in the absence of platelet activation.

Conclusions: In this novel large animal model of allogeneic erythrocyte transfusion, pulmonary dysfunction occurs in the absence of any priming event, is increased when combined with other inflammatory stimuli, and is mediated by monocyte activation and T-lymphocyte depletion.

What We Already Know about This Topic

- Transfusion of allogeneic erythrocytes increases the risk of pulmonary morbidity postcardiac surgery.
- Using a novel *in vivo* porcine model of pulmonary dysfunction, this study determined whether (1) the transfusion of older erythrocytes would cause greater pulmonary dysfunction compared with younger erythrocytes; and (2) erythrocyte transfusion would interact with cardiopulmonary bypass to increase the severity of pulmonary dysfunction.

What This Article Tells Us That Is New

- Allogeneic erythrocyte transfusion of older erythrocytes causes pulmonary dysfunction, which is characterized by marked neutrophil/macrophage infiltration. Moreover, transfusion interacted with cardiopulmonary bypass to increase lung injury.

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PULMONARY morbidity is an important contributor to mortality and resource use after cardiac surgery with cardiopulmonary bypass (CPB).[1,2] Transfusion of allogeneic erythrocytes increases the risk of pulmonary morbidity post-cardiac surgery by as much as fourfold.[3,4] This effect may be influenced by the duration of erythrocyte storage before transfusion. Koch *et al.*[4] demonstrated an increased risk of respiratory insufficiency and the need for prolonged ventilation in patients receiving erythrocytes stored for greater than 14 days *versus* those receiving blood stored for less than 14 days. Transfusion-related acute lung injury (TRALI), defined by a consensus definition[5] of hypoxaemia ($Pa_{O_2}/F_{I_{O_2}}$ <300 mmHg), bilateral infiltrates on chest radiograph, pulmonary artery occlusion pressure of less than 18 mmHg, and the acute onset of features within 6 h after a transfusion is rare, occurring in 2.4% of cardiac surgical patients.[6] However, transfusion-associated circulatory overload and transfusion-associated dyspnoea are other syndromes of transfusion-related morbidity, which overlap with the features of TRALI. These syndromes are poorly defined and consistently under-reported. Consequently, the true incidence of transfusion-associated pulmonary morbidity is unclear.[7] Moreover, the associations between pulmonary complications and erythrocyte transfusion are derived from observational studies, so causality has therefore, not been established, and the underlying pathophysiological mechanisms are poorly understood. The latter can be attributed to the lack of experimental models with homology to TRALI, as observed in a clinical setting. Current TRALI models are also limited by cross-species or *ex-vivo* design, the use of priming events such as lipopolysaccharide that have limited homology to clinical events, and methods of erythrocyte storage dissimilar to those used for human erythrocytes.[8–12] At present, there is no large animal model where transfusion of an allogeneic cellular blood component causes acute lung injury. The objectives of this study were therefore: (1) to determine whether transfusion of allogeneic erythrocyte causes pulmonary dysfunction in swine, (2) to assess whether erythrocyte storage duration affects severity of pulmonary dysfunction, and (3) to evaluate the interaction of erythrocyte transfusion with CPB in the genesis of postcardiac surgery pulmonary dysfunction. We hypothesized that the transfusion of older erythrocytes would cause greater pulmonary dysfunction compared with younger erythrocytes and that erythrocyte transfusion would interact with CPB to increase the severity of pulmonary dysfunction.

Materials and Methods

Animals received care in accordance with and under license of the Animals (Scientific Procedures) Act 1986 (London, United Kingdom) and conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (Publication No. 85-23, revised 1996). The study received local institutional review board (University of Bristol, Bristol, United Kingdom) approval.

Intervention

Thirty-six pigs were quasirandomized to the following groups:

1. Experiment 1: (1) **Sham (n = 7)**, neck dissection plus 500 ml crystalloid infusion; (2) **D14 Tx (n = 6)**, neck dissection plus 500 ml (2 units) of 14-day-old stored porcine erythrocyte units; (3) **D42 Tx (n = 7)**, neck dissection plus 500 ml (2 units) of 42-day-old stored porcine erythrocyte units.
2. Experiment 2: (1) **Sham**, neck dissection plus 500 ml crystalloid infusion; (2) **CPB (n = 7)**, 2.5 h of CPB plus 500 ml of crystalloid infusion; (3) **CPB + D42 Tx (n = 9)**, 2.5 h of CPB plus 500 ml (2 units) of 42-day-old stored porcine erythrocyte units.

Porcine Erythrocyte Preparation

Fifteen adult female Large-White-Landrace crossbred pigs (80–100 kg) were used to obtain packed erythrocytes. Blood from the external jugular vein was collected in citrate-phosphate-dextrose, leucodepleted, buffy coat removed, and stored in sucrose-adenosine-glucose-mannitol using the Leukotrap WB system (Pall Medical, Portsmouth, United Kingdom) at 4°C for either 14, or 42 days, according to the manufacturer's instructions and National Health Service Blood and Transplant Standards for Human Blood. In transfused pigs, donor and recipient blood was cross-matched using a visual agglutination test. Transfused pigs received packed erythrocytes from a single porcine donor.

In Vivo *Erythrocyte Transfusion Models*

Thirty-six adult female farm-bred Large-White-Landrace crossbred pigs (50–70 kg) were weighed and anaesthetized with ketamine (100 mg/kg) and halothane (1.5–2.0%) with nitrous oxide 50% in oxygen. Animals were intubated and positive pressure ventilation commenced in a circle circuit using a Penlon Nuffield 200 (Abingdon, Oxford, United Kingdom) initially to achieve peak inspiratory pressures of 30 cm H_2O in a 1:4 (inspiratory:expiratory) ratio, with modifications to maintain Pa_{CO_2} between target values of 35 and 45 mmHg. Venous access and measurement of central venous pressure was achieved through direct puncture of the left external jugular vein. Arterial blood pressure was continuously monitored *via* a 20-gauge Vygon catheter (Vygon Ltd., Swindon, United Kingdom) placed in the left common carotid artery. Core body temperature was assessed using a rectal temperature probe. Central venous pressure (8–12 mmHg), hydration, and sodium load (500 ml/h, 0.9% normal saline) were strictly controlled. Postintervention, all animals were recovered, and reanesthetized and reevaluated after 24 h. A schematic of our experimental design is provided in Supplemental Digital Content 1, figure 1, http://links.lww.com/ALN/A929, which is a figure of our experimental design.

CPB was performed, as described previously.[13,14] Total CPB time was 2.5 h. Anesthesia, surgical incisions, and

monitoring were performed as for transfusion experiments. After 30 min of CPB or sham procedure, 500 ml of crystalloid or allogeneic erythrocytes were transfused over 2 h during the remainder of the intervention period.

Outcomes

Storage-related Changes in Stored Porcine Erythrocyte Units. Five milliliter aliquots were removed from representative bags for evaluation of storage-related changes. Biochemical changes in the supernatant, plasma hemoglobin using spectromorphometry, and erythrocyte adenosine triphosphate concentrations using high-performance liquid chromatography, were assessed at weekly intervals, as previously described.[13,15] The hemolysis index was defined as plasma (hemoglobin × hematocrit)/donor unit hemoglobin. Scanning electron microscopy was performed of stored porcine erythrocytes at days 0, 14, and 42 of storage.

Assessment of Pulmonary Dysfunction. Pulmonary dysfunction was determined according to functional, histological, and biochemical parameters of acute lung injury, as recommended by the American Thoracic Society.[16] Lung compliance, Pa_{O_2}/F_{IO_2} ratio, airways resistance, and work of breathing were measured *in-vivo* at baseline, 1.5 and 24 h postintervention, using the SERVO-i Universal Ventilator (Maquet GmbH, Rastatt, Germany),which used volume-controlled ventilation with a tidal volume of 10 ml/kg, F_{IO_2} of 0.5, respiratory rate of 12 breaths/min, and peak end-expiratory pressure of 5 cm H_2O. Total protein in bronchoalveolar lavage samples was measured using a Bradford Protein Assay (Quick Start Bradford Protein Assay, Hercules, CA) at 24 h postintervention by an investigator blinded to intervention allocation. The lower lobe of the left lung was harvested 24 h postintervention, immediately fixed in 10% formalin, and six lung sections taken sequentially across the resected lung were stained with hematoxylin and eosin. A histology scoring system for lung injury was used by investigators blinded to intervention allocation, as previously described.[16] Briefly, the following parameters were scored on a scale of 0–2: (1) neutrophils in the alveolar space, (2) neutrophils in the interstitial space, (3) hyaline membranes, (4) proteinaceous debris filling the airspaces, and (5) alveolar septal thickening. The sum of each of the five variables (each with a different weighting) was normalized to the number of fields evaluated. The resulting score is a continuous value between 0 and 1 (inclusive). The mean lung injury score was then obtained for each group.

Pulmonary Inflammatory and Platelet Cell Infiltration and Activation, and Endothelial Activation. Confocal immunofluorescence was performed on 5-μm sections of frozen lung tissue (six sections per pig) from four pigs per group for macrophages (MAC-387; Abcam, Cambridge, United Kingdom), T-lymphocytes (anti cd-3; clone FY1H2 [IgG1], kind gift from Professor Michael Bailey, Ph.D., Bristol Veterinary School, University of Bristol, Bristol, United Kingdom), total platelets (anti-CD41, AbD Serotec, Kidlington, United Kingdom), and activated platelets (antiplatelet-activating complex-1; BD Bioscience, Oxford, United Kingdom). Neutrophils were quantified on hematoxylin and eosin sections.

Tumor necrosis factor-α (R&D Systems, Abingdon, United Kingdom) was measured in homogenized lungs obtained 24 h postintervention using solid phase enzyme-linked immunosorbent assay according to manufacturer's protocol, and normalized to lung protein concentrations.

Western blotting was used to assess pulmonary endothelial activation and inflammation in lung homogenates. Polyclonal antibodies to E-selectin (R&D Systems) P-selectin (SantaCruz Biotechnology, Dallas, TX), endothelin-1 (Acris Antibodies, Herford, Germany), and Vascular Endothelial-cadherin (SantaCruz Biotechnology) were used. Blots were quantified by densitometry normalized to β-Actin (SantaCruz Biotechnology), as previously described.[13]

Statistical Analysis

The current study represents an analysis of pulmonary dysfunction from a series of experiments powered to detect differences in creatinine clearance as a primary endpoint.[13,14,17] The study outcomes to assess pulmonary dysfunction were prespecified secondary outcomes in these experiments, however, no power calculation was performed *a priori*, and our analysis should be considered exploratory.

Comparisons between groups were performed using one-way and repeated measures ANOVA with the Bonferroni correction. General linear model ANOVA, evaluating time, group, and the interaction of time and group, was used for repeated measures with adjustment for baseline values. Data were reported throughout as mean (±SEM) for normally distributed or as geometric mean (±95% CIs) for nonnormally distributed data. Treatment differences were reported as mean difference (95% CIs) or as the ratio of geometric means (95% CIs). Statistical significance was defined as a P value less than 0.05, using two-tailed tests. All analyses were conducted using SPSS 18.0 (SPSS Inc., Chicago, IL).

Results

Thirty-two of 36 animals completed the experimental protocol to recovery and reassessment. Baseline characteristics, including lung function, lung tidal volumes, gas exchange, and inflammatory markers were similar between groups (see appendices 1 and 2). CPB pigs required a greater volume of intravenous fluids due to the pump priming volume. Four experiments (2 pigs in D14 Tx group and 2 pigs in CPB + Tx group) were terminated prematurely due to cardiovascular instability or refractory hypoxemia. These pigs were excluded from our analyses. To assess whether this may represent a source of bias, our analysis was repeated incorporating these animals up until the time of death. However, this did not alter our findings (see Supplemental Digital Content 1, tables 1–4, http://

links.lww.com/ALN/A929, which are tables demonstrating the sensitivity analyses for experiment 1 and 2).

Stored Porcine Erythrocytes Develop a "Storage Lesion"

To determine homology between porcine and human erythrocyte units, biochemical changes of porcine erythrocytes were analyzed and compared with human data (fig. 1A). Hematocrit concentrations remained constant during storage, although the mean hematocrit was lower in porcine units compared with human units, a reflection of the lower hematocrit in porcine venous blood. The day-14 porcine erythrocyte units developed a storage lesion, which showed considerable homology to that observed in 42-day-old human erythrocyte units, as shown by an increase in potassium and oxygen levels and a decrease in sodium and erythrocyte adenosine triphosphate concentrations. However, acidic pH in sucrose-adenosine-glucose-mannitol stored porcine units appeared to inhibit glycolysis; glucose was not utilized and adenosine triphosphate levels became depleted more rapidly than those observed in human units. Concentrations of 2,3-diphosphoglycerate in porcine erythrocytes decreased over storage time in a similar manner to human erythrocytes. The hemolysis index was higher in porcine units at day 42, but the mean hemolysis index (mean 0.87 [±0.59]) remained less than 1, a quality control standard for human erythrocyte storage. Porcine erythrocytes underwent morphological change over storage time, characterized by the loss of biconcavity and development of echinocytosis, as observed on scanning electron microscopy (fig. 1B).

Transfusion of 14-day and 42-day Porcine Erythrocytes Causes Pulmonary Dysfunction in Swine

Pulmonary Dysfunction. To determine whether stored erythrocytes caused pulmonary dysfunction, adult pigs received an allogeneic transfusion with 14- or 42-day-old erythrocytes and lung function was assessed *in vivo* at 1.5 and 24 h posttransfusion. Despite a sustained rise in hematocrit, there was no difference between the groups in $Pa_{O_2}/F_{I_{O_2}}$ ratio after erythrocyte transfusion (fig. 2, A and B). There was no difference in other measures of pulmonary function including tidal volumes, Pa_{CO_2}, or central venous pressures between groups or over time (fig. 2, C–E). D14 and D42 erythrocyte transfusion caused significant reductions in lung compliance at both 1.5 and 24 h compared with sham (fig. 3A). Both D14 and D42 Tx pigs demonstrated histological evidence of pulmonary injury characterized by neutrophils in the interstitium and alveolar space, hyaline membrane formation, alveolar wall thickening, proteinaceous debris in the alveolar space, and significantly elevated lung injury scores (fig. 3, B and C). Erythrocyte transfusion increased protein concentrations in bronchoalveolar lavage fluid compared with sham, although, this was significantly less in pigs receiving D14 *versus* D42 transfusions (fig. 3D).

Inflammation and Endothelial Activation. Both D14 and D42 erythrocytes caused significant pulmonary neutrophil infiltration compared with sham ($P < 0.001$; fig. 4). Erythrocyte transfusion induced a pulmonary macrophage infiltrate, T-lymphocyte depletion, platelet sequestration (CD41), and activation (platelet activating complex-1), which was significantly greater in pigs receiving D42 *versus* D14 erythrocytes (fig. 4, A and B). The transfusion of 42-day-old erythrocytes was also associated with greater tumor necrosis factor-α concentrations in porcine lung tissue (fig. 4C).

To determine the effect of erythrocyte transfusion on pulmonary endothelial activation, lung homogenates from sham and transfused pigs underwent Western blot analysis and were probed with antibodies to markers of endothelial activation. Both D14 and D42 erythrocytes caused pulmonary endothelial activation evinced by similar up-regulation of E-selectin and P-selectin in lung homogenates, and resulted in down-regulation of VE-cadherin, an endothelial cell gap junction protein, indicative of increased endothelial permeability (fig. 4, D and E). Up-regulation of endothelin-1, a marker of lung inflammation and oxidative stress, was much greater in pigs receiving D42 erythrocytes (fig. 4, D and E).

Erythrocyte Transfusion Worsens Pulmonary Dysfunction in the Presence of CPB

CPB resulted in hemodilutional anemia (fig. 5), a significant reduction in lung compliance and arterial oxygen tension at 1.5 h, which improved at 24 h (fig. 6), a nonsignificant rise in histological lung injury scores (fig. 6C) and a significant increase in protein concentration in bronchoalveolar lavage fluid at 24 h, compared with sham (fig. 6D). CPB also caused a pulmonary inflammatory infiltrate characterized by an increase in neutrophils, macrophages, and T-lymphocytes (fig. 6E and appendix 3A), which was associated with an increase in tumor necrosis factor-α concentrations in porcine lung tissue (appendix 3B). CPB resulted in a reduction in total platelets, as indicated by CD41 staining but caused an increase in the number of activated platelets in lung tissue (fig. 6E). CPB also increased P-selectin and endothelin-1, and attenuated VE-cadherin expression, but had no significant effect on E-selectin expression (fig. 6F and appendix 3C).

Transfusion of D42 erythrocytes to pigs undergoing CPB reversed hemodilutional anemia (fig. 5A), but increased severity of pulmonary dysfunction by reducing lung compliance at 24 h, despite augmenting arterial oxygen tensions and increasing lung injury scores, bronchoalveolar lavage protein concentration, pulmonary neutrophil and macrophage infiltration, and pulmonary endothelial activation (E-selectin expression) compared with CPB alone (fig. 6 and appendix 3). In contrast, erythrocyte transfusion + CPB had no effect on pulmonary T-lymphocytes, tumor necrosis factor-α concentrations, platelet activation, and P-selectin and endothelin-1 expression (fig. 6 and appendix 3).

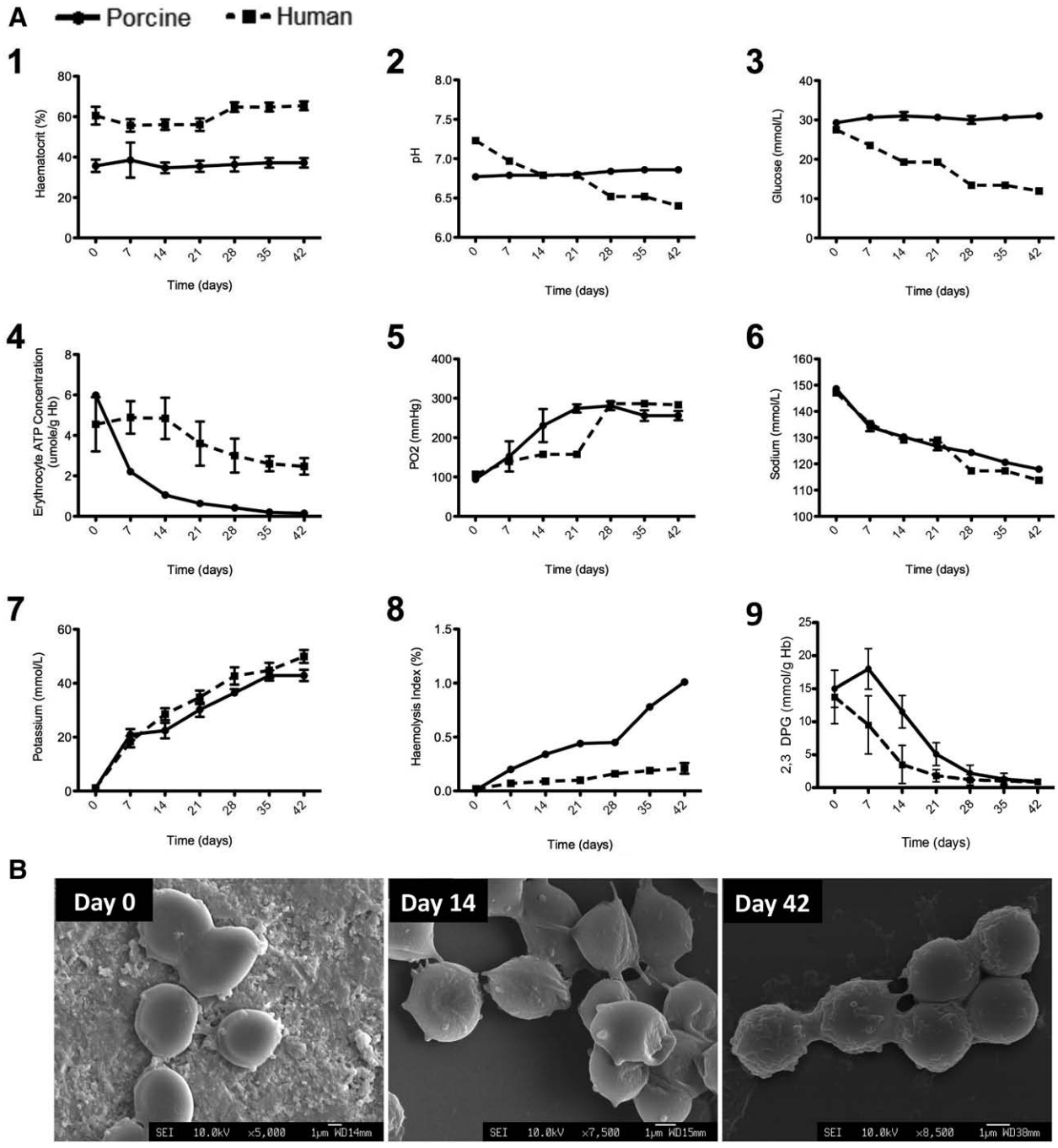

Fig. 1. Storage of porcine and human red cells causes erythrocyte degradation. (*A*) Weekly biochemical analyses of supernatant in porcine and human erythrocyte units. (*1*) Hematocrit levels are steady over time in both stored porcine and human erythrocyte units. (*2*) Acidic-starting pH in porcine units inhibits anaerobic glycolysis and therefore, (*3*) glucose is not utilized and (*4*) adenosine triphospate (determined by using high-performance liquid chromatography) is rapidly depleted. (*5*) Oxygen tension, (*6*) sodium, and (*7*) potassium concentrations in erythrocyte unit supernatant changes are similar to human erythrocyte units. (*8*) Hemolysis index, derived from plasma hemoglobin measurements, rose steadily during storage but remained less than 1%. *Solid lines* represent porcine erythrocytes and *dashed lines* represent human erythrocytes. (*9*) 2,3-Diphosphoglycerate (2,3-DPG) concentrations decreased over storage time in both porcine and human erythrocyte units. (*B*) Representative scanning electron microscopy images of stored porcine erythrocytes at days 0, 14, and 42 demonstrates changes in erythrocyte morphology and eichinocytosis characteristic of storage-related changes and adenosine triphosphate depletion. Data represents mean (±SEM). ATP = adenosine triphosphate; P_{O_2} = oxygen tension.

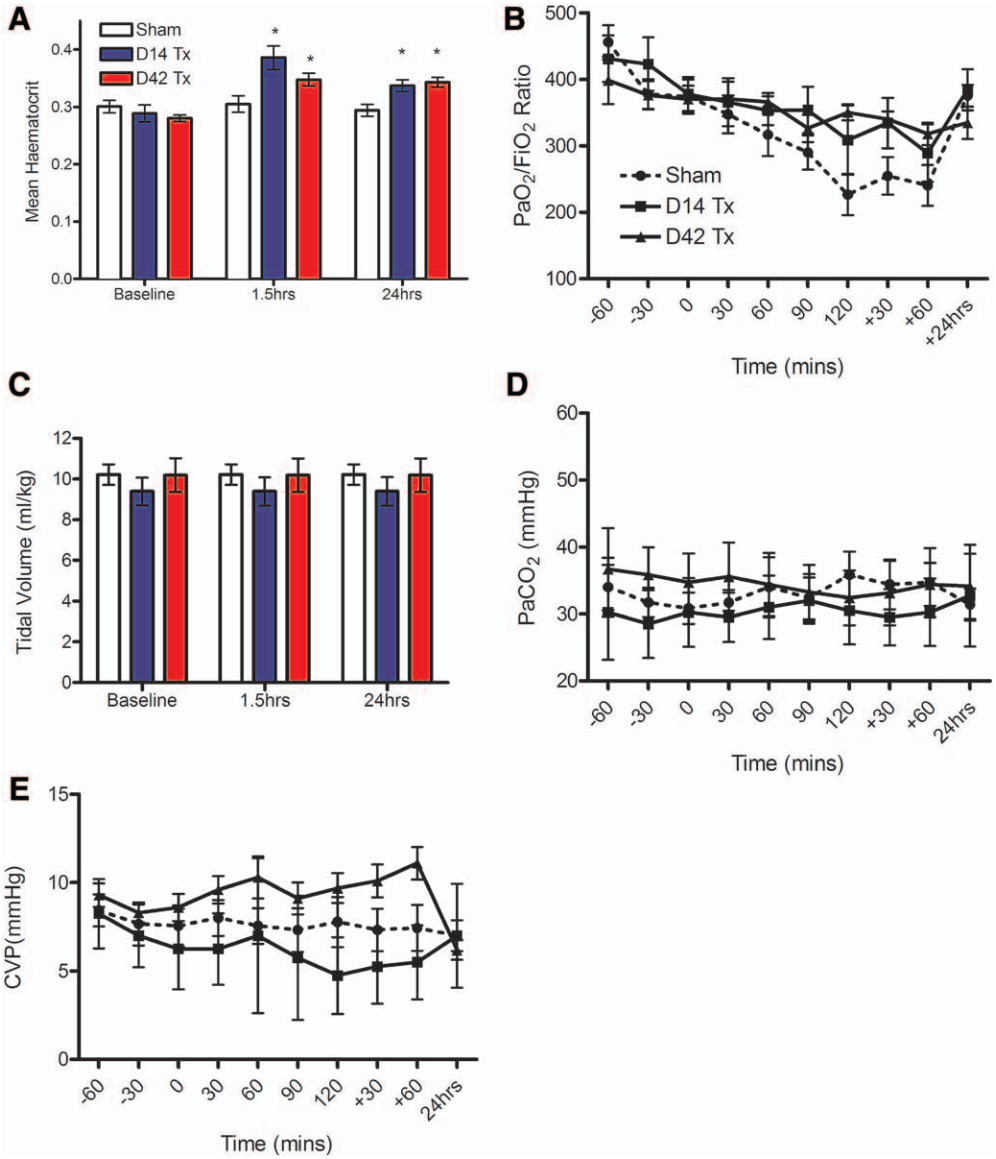

Fig. 2. Hematocrit, lung function, and physiological variables for sham, 14-day erythrocyte transfusion, and 42-day erythrocyte transfusion pigs. (*A*) Mean hematocrit, (*B*) Pao_2/Fio_2 ratios, (*C*) tidal volumes per kilogram of body weight, (*D*) arterial carbon dioxide tension, and (*E*) central venous pressure. Data represents mean (±SEM). * $P < 0.05$ *versus* sham. CVP = central venous pressure; Fio_2 = fraction of inspired oxygen; $Paco_2$ = arterial carbon dioxide tension; Pao_2 = arterial oxygen tension; Tx = transfusion.

Discussion

Main Findings

We describe a novel *in-vivo* porcine model of pulmonary dysfunction, using a protocol for the preparation of allogeneic porcine erythrocyte units identical to that used by the United Kingdom National Health Service Blood and Transplant. Our main findings are: (1) allogeneic erythrocyte transfusion causes pulmonary dysfunction in the absence of any clear priming event; (2) pulmonary dysfunction is characterized by pulmonary neutrophil infiltration and endothelial cell activation; (3) older blood is associated with increased macrophage infiltration, T-lymphocyte depletion, and platelet activation compared with younger blood; (4) stored erythrocyte transfusion in the presence of CPB exacerbates pulmonary dysfunction characterized by marked neutrophil and macrophage infiltration, endothelial activation but not platelet activation.

Strengths and Limitations

The current porcine model has significant advantages over other *in vivo* models because: (1) transfusion of cross-matched allogeneic erythrocytes in swine mimics clinical transfusion as compared with models that have used either syngeneic blood or xenotransfusion[8,10,11]; (2) stored, leukodepleted erythrocytes were used to induce pulmonary dysfunction, which again mimics clinical transfusion as compared with the use of plasma or membrane-derived proinflammatory factors,

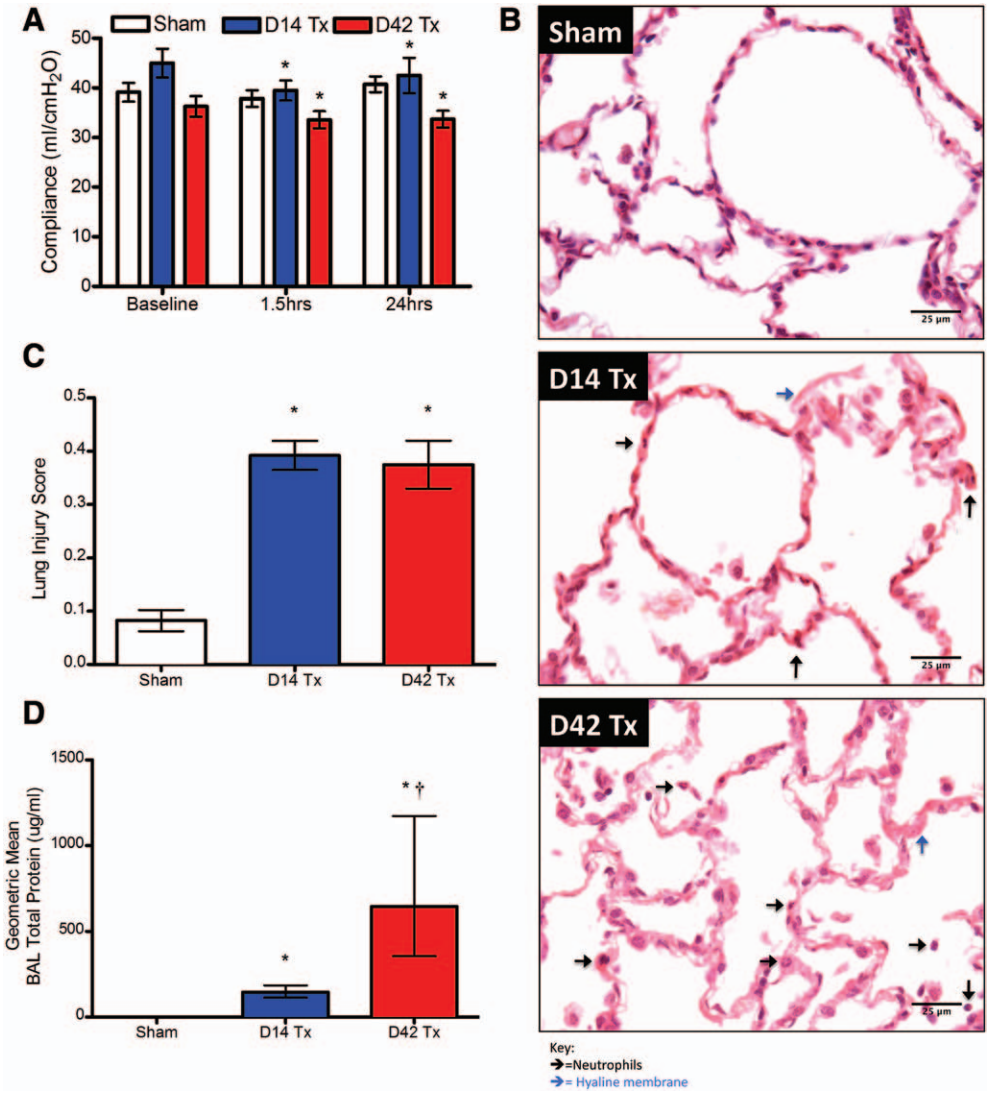

Fig. 3. Allogeneic erythrocyte transfusion causes pulmonary dysfunction in pigs. (*A*) Decreased lung compliance is observed in transfused pigs. (*B*) Representative hematoxylin and eosin-stained lung sections (×600) demonstrate normal lung morphology in sham pigs. Interstitial neutrophils (*black arrows*), hyaline membranes (*blue arrows*), and proteinaceous debris in the alveolar space are evident in pigs receiving 14-day-old erythrocytes. These changes are more marked with neutrophils in the alveolar space and alveolar wall thickening in pigs receiving 42-day old blood. (*C*) Lung injury scores were significantly elevated in both groups of pigs receiving erythrocyte transfusion. (*D*) Total protein in bronchoalveolar lavage fluid was significantly increased in transfused pigs and this was more marked in pigs receiving 42-day old blood. Data represents mean (±SEM) for normally distributed data or geometric mean (±95% CI) for nonnormally distributed data. * $P < 0.05$ *versus* sham. † $P < 0.05$ *versus* D14 Tx. BAL = bronchoalveolar lavage; Tx = transfusion.

nonphysiological doses of proinflammatory components of the storage supernatant, or antibodies[10,11,18]; (3) the combination of erythrocyte transfusion with a clinical priming event, CPB, models a common clinical scenario associated with pulmonary dysfunction unlike other models[10,11,18]; (4) the model allows us to link changes in biochemical and histological parameters to clinically relevant outcomes, such as changes in lung compliance, in a model homologous to human physiology. Swine are excellent models of respiratory disease as anatomy, biochemistry, physiology, size, and genetics resemble those of humans.[19] As a result, porcine lungs have been used to study many respiratory diseases and therapeutics, including surfactant function and therapy,[20] reperfusion injury,[21] pulmonary artery hypertension,[22] and the effects of mechanical ventilation.[17] Importantly, our results are comparable with a recent clinical study in which healthy volunteers when transfused with autologous blood developed subclinical pulmonary dysfunction associated with increases in markers of pulmonary inflammation.[23]

Our results must also be interpreted with appropriate consideration of the limitations of this preclinical model. Importantly, we did not detect hypoxia in this model, the principal feature of TRALI, and the changes in lung compliance,

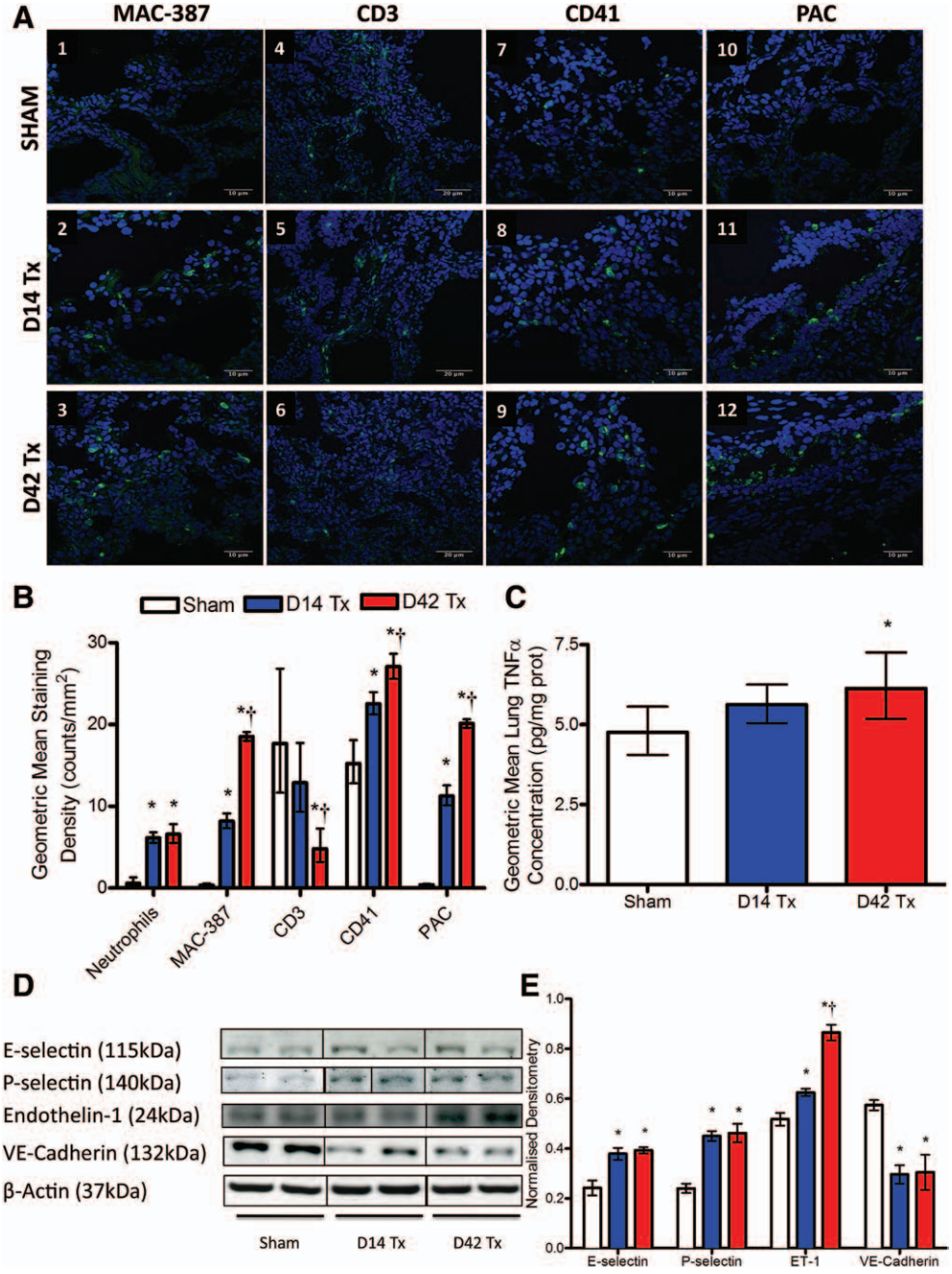

Fig. 4. Allogeneic erythrocyte transfusion causes neutrophil and macrophage infiltration, T-lymphocyte depletion, platelet activation, increased tumor necrosis factor-α concentration, and endothelial activation in porcine lung tissue at 24 h. (*A*) Immunofluorescent staining for (*1–3*) macrophages using the MAC-387 antibody, (*4–6*) T-lymphocytes using the CD-3 antibody, (*7–9*) constitutive platelets using the CD41 antibody, and (*10–12*) activated platelets using the platelet-activating complex (PAC; anti-gpIIb/IIIa, $\alpha_{IIb}\beta_3$ epitope) antibody is shown in *green* and 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei in *blue* in porcine lung tissue (×630, MAC-387, CD41, PAC; ×400, CD3) for sham, 14-day, and 42-day erythrocyte transfused pigs. (*B*) Quantification of inflammatory cell and platelet staining demonstrates that 42-day erythrocyte transfusion elicits a significant macrophage infiltrate, T-lymphocyte depletion, and platelet activation, which is attenuated by 14-day erythrocyte transfusion. Quantification of lung neutrophil infiltration based on H&E sections demonstrates significantly increased lung neutrophil counts in both 14- and 42-day transfused pigs. (*C*) Tumor necrosis factor-α concentration is increased in whole lung lysates of 42-day transfused pigs, which is determined using solid phase enzyme-linked immunosorbent assay. (*D* and *E*) Western blot analysis demonstrates that markers of endothelial activation (E-selectin, P-selectin, endothelin-1) are increased in whole lung lysates of pigs that have received erythrocyte transfusion. Erythrocyte transfusion also reduces expression of VE-Cadherin. Images from the same gel have been grouped, as indicated by *black dividing lines*. Data represents mean (±SEM) for normally distributed data or geometric mean (±95% CI) for nonnormally distributed data. * $P < 0.05$ *versus* sham. † $P < 0.05$ *versus* D14 Tx. ET-1 = endothelin-1; H&E = hematoxylin and eosin; kDA = kilodaltons; prot = protein; TNFα = tumor necrosis factor-α; Tx = transfusion.

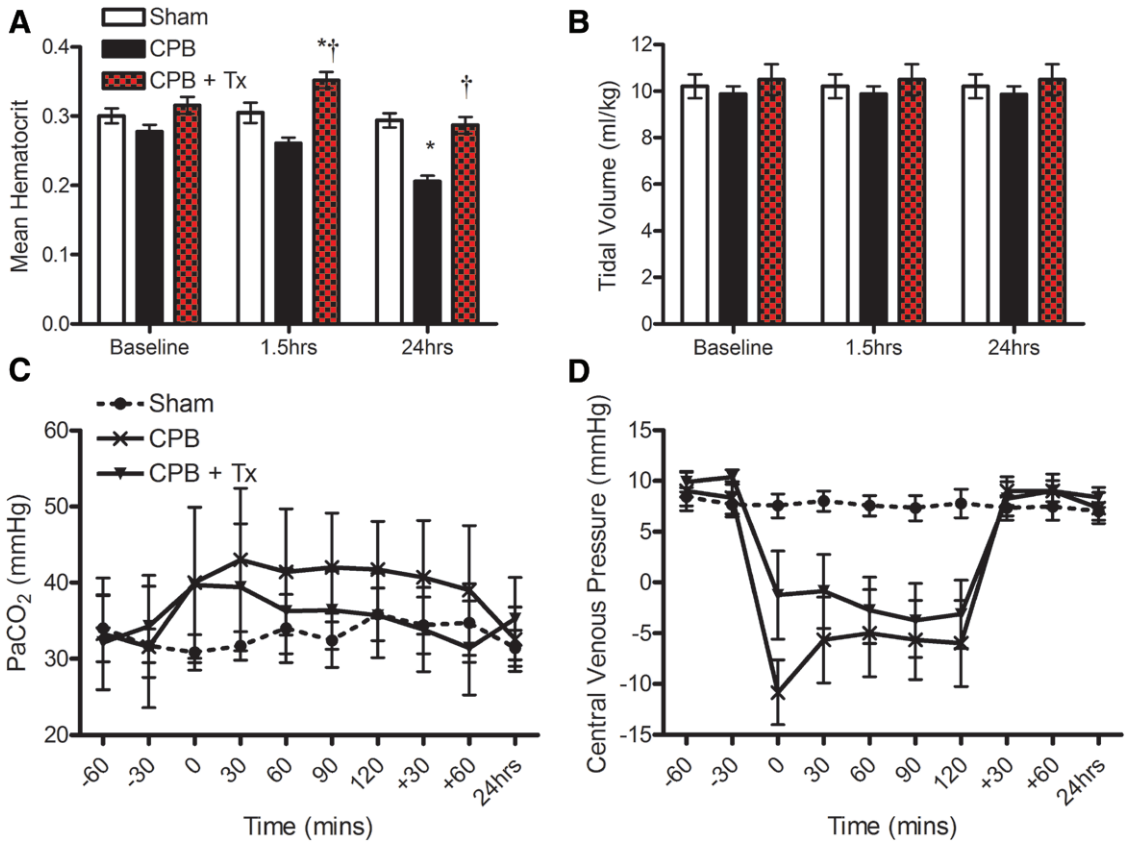

Fig. 5. Hematocrit, lung function and physiological variables for sham, cardiopulmonary bypass (CPB), and CPB + transfusion (Tx) pigs. (*A*) Hematocrit, (*B*) tidal volumes per kilogram of body weight, (*C*) arterial carbon dioxide tensions, and (*D*) central venous pressure. Data represents mean (±SEM). * $P < 0.05$ *versus* sham, † $P < 0.05$ *versus* CPB. $Paco_2$ = arterial carbon dioxide tension.

although statistically significant, are unlikely to equate to clinical TRALI. There are several possible reasons for this. First, we evaluated posttransfusion lung injury only at two time points, 1.5 and 24 h. By evaluating these relatively early time points, we may not have detected important late pathological changes, for example postcardiac surgery TRALI typically manifests later in the clinical setting, often 48–72 h postsurgery.[3] Second, stored erythrocytes are known to preserve gas exchange resulting in systemic normoxia, despite the increased oxygen affinity secondary to 2,3-diphosphoglycerate depletion.[24,25] However, transfusion of stored erythrocytes causes microvascular defects in the recipients, which leads to tissue hypoxia which is not evident systemically. This has been shown in experimental studies in baboons and hamsters[26,27] and is in agreement with the findings of the study by Weiskopf *et al.*,[23] where autologous erythrocyte transfusion resulted in subtle deficits in gas exchange (defined by a change in the alveolar to arterial difference in oxygen partial pressure) but no significant change in arterial oxygen tensions. Third, it is possible that the functional effects we observed might be accentuated by preexisting disease states where they become clinically significant, as has been reported in a clinical study.[28] These limitations notwithstanding, it must also be remembered that severe respiratory distress and hypoxia are not appropriate in a recovery model for reasons of refinement and three experiments in the current study were terminated prematurely for this reason. Severe hypoxia in these animals was most evident at approximately 6–8 h postintervention and was therefore, not reflected in our results.

Another limitation is that the porcine erythrocyte storage lesion in our study had important differences to the human storage lesion. Stored porcine erythrocytes do not utilize glucose during storage unlike stored human erythrocytes for two reasons; first porcine erythrocytes, unlike other mammalian erythrocytes, contain a hexokinase III, which is responsible for 98% of the total glucose phosphorylating activity.[29] The presence of this hexokinase reduces the erythrocyte cell membranes ability to transport glucose and therefore, porcine erythrocytes have a reduced capacity to metabolize glucose.[30] Second, the starting acidic pH inhibits relevant enzyme systems. As a result glycolysis does not occur and therefore, a reduction in pH and a rise in lactate are not observed. Erythrocyte adenosine triphosphate concentrations rapidly decline, as they are utilized as the only energy source, but not replenished *via* glycolysis. Despite this limitation, we have established a model of allogenic erythrocyte transfusion, although with an advanced storage lesion, and demonstrated that transfusion of these cells results in a sustained increase in hematocrit for up to 24 h.

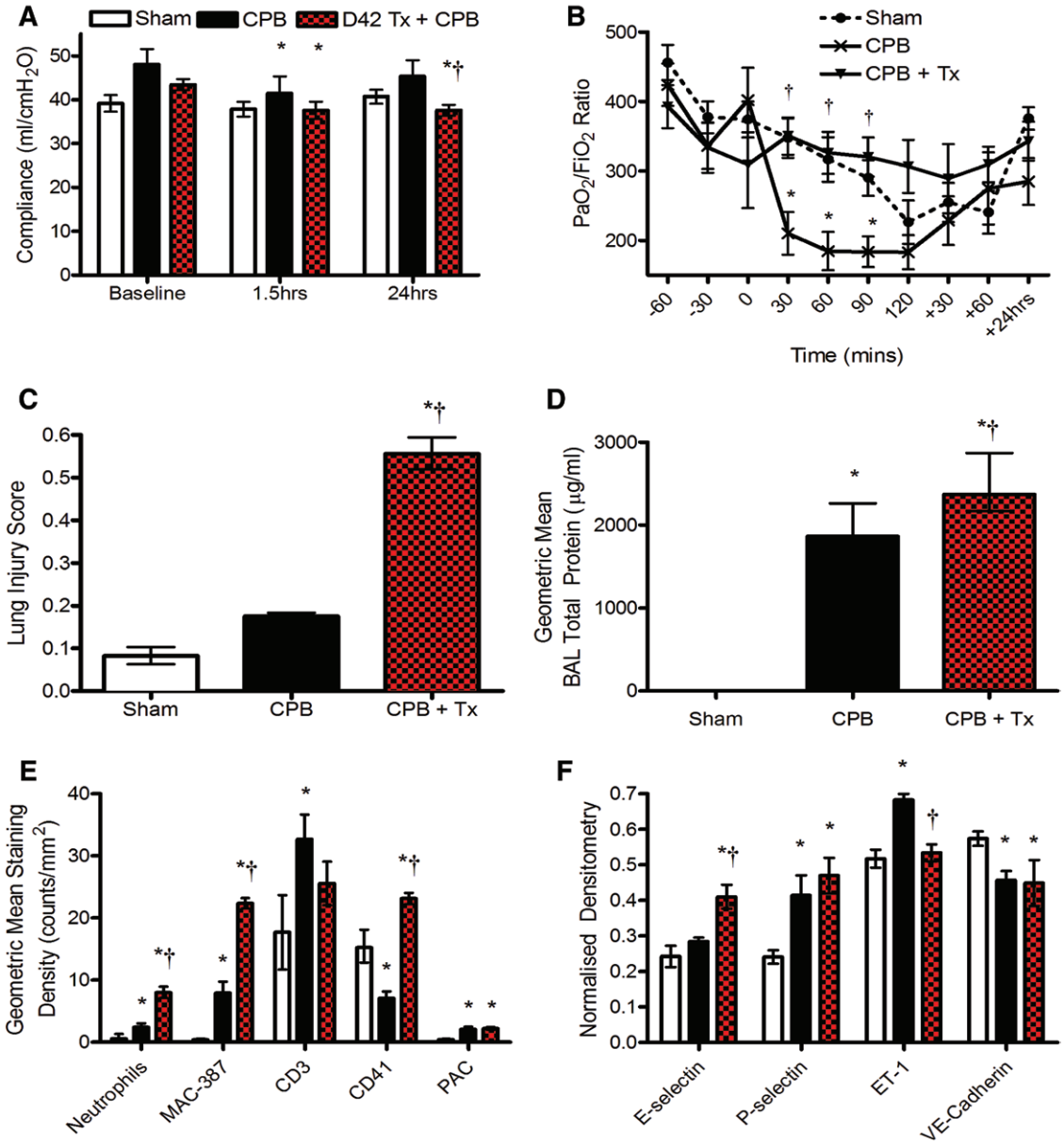

Fig. 6. Erythrocyte transfusion (Tx) in the presence of cardiopulmonary bypass (CPB) augments pulmonary dysfunction. (*A*) Lung compliance was significantly reduced at both time points in pigs receiving CPB with erythrocyte transfusion. (*B*) Arterial oxygen tensions were increased in pigs receiving CPB with erythrocyte transfusion. Time points –60 and –30 represent baseline measurements, 0–120 min represent intervention time point measurements, +30 and +60 represent 1.5 h postintervention measurements, and +24 h represent 24 h postintervention measurements. (*C*) CPB + erythrocyte transfusion increased lung injury scores. (*D*) CPB + erythrocyte transfusion increased total protein content in bronchoalveolar lavage fluid. (*E*) CPB + erythrocyte transfusion increased pulmonary neutrophil and macrophage infiltrate. CPB caused T-lymphocyte infiltration. Erythrocyte transfusion in the presence of CPB prevented T-lymphocyte infiltration. CPB + erythrocyte transfusion augmented pulmonary platelet infiltration but did not affect platelet activation. (*F*) Erythrocyte transfusion in the presence of CPB augmented E-selectin expression, but did not alter P-selectin and VE-cadherin expression compared with CPB alone. Erythrocyte transfusion also prevented CPB-induced endothelin-1 (ET-1) expression. Data represents mean (±SEM) for normally distributed data or geometric mean (±95% CI) for nonnormally distributed data. * $P < 0.05$ *versus* sham. † $P < 0.05$ *versus* CPB. BAL = bronchoalveolar lavage; F_{IO_2} = fraction of inspired oxygen; PAC = platelet-activating complex; Pa_{O_2} = arterial oxygen tension.

Finally, although the sham group presented with the same fluid load as the experimental groups, 500 ml of crystalloid would not have the same effect on intravascular volume. Although difference in the 14- and 42-day-old blood groups argue against a pure volume effect, this does represent a limitation in the study.

Translational Relevance

Our observations are consistent with previous studies showing that neutrophils and endothelial activation play an important role in the development of TRALI.[31] Our findings are consistent with a two-event hypothesis of TRALI,

in which CPB and transfusion interact to cause more severe TRALI than either risk factor in isolation. However, for the first time, we demonstrate that erythrocyte transfusion in the absence of any identified priming event can also cause pulmonary dysfunction in an *in-vivo* large animal model using an allogeneic cellular blood component. This finding is unlikely to have been confounded by: (1) a preexisting inflammatory state, as leukocyte counts, C-reactive protein levels, Pao_2/Fio_2 ratios, and core temperatures were within normal limits for swine and similar between groups at baseline; (2) circulatory overload, as central venous pressures and total volumes of intravenous fluids infused were comparable between groups; (3) immune cross-reactivity, as all blood was cross-matched. One consideration is that anesthesia and ventilation served as a priming event, however, we do not believe this to have confounded our results because ventilation was standardized across the groups and did not elicit any evidence of pulmonary injury or inflammation by the measures used.

Our study also suggests an important role for macrophage infiltration and T-lymphocyte depletion in the pathogenesis of erythrocyte transfusion-mediated pulmonary dysfunction. These findings are supported by recent observations in rodents.[32–34] Our results also suggest that postcardiac surgery pulmonary dysfunction associated with erythrocyte transfusion may occur despite significant attenuation of platelet activation, in this case as a result of CPB. This is in contrast to recent studies in mice, which demonstrates that depletion of platelets or antiplatelet therapy prevents antibody-mediated TRALI.[18] The pathogenesis of CPB-induced acute lung injury in this model differs from lipopolysaccharide-induced acute lung injury and highlights the importance of developing animal models with more clinically relevant insults to aide data interpretation.

In conclusion, we have developed a novel *in-vivo* porcine model of pulmonary dysfunction, using allogeneic porcine erythrocytes that has homology to cardiac surgical patients. These findings are consistent with observational studies in cardiac surgical patients showing strong associations between erythrocyte transfusion and organ injury.[3,4,6] Transfusion-mediated pulmonary dysfunction in this model occurs both in the absence, and the presence of CPB. This model represents an ideal platform for the evaluation of therapies that prevent pulmonary dysfunction before translation into the clinical setting.

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Appendix 1. Experiment 1: Baseline Characteristics

	Sham (n = 7)	D14 Tx (n = 4)	D42 Tx (n = 7)	*P* Value
Weight, kg*	58.5 (52.8–65.0)	59.5 (45.5–77.7)	59.9 (52.5–68.5)	0.95
Total IV fluids over 24 h, ml*	4,567.7 (4,403.5–4,739.2)	4,499.9 (4,499.9–4,499.9)	4,637.7 (4,421.8–4,863.0)	0.51
Hematocrit	0.32 (0.02)	0.29 (0.03)	0.29 (0.02)	0.06
Tidal volumes, ml/kg	10.21 (1.33)	9.39 (1.37)	10.19 (2.18)	0.48
Compliance, ml/cm H_2O	39.1 (5.01)	45.0 (5.77)	36.3 (5.50)	0.06
Inspiratory airways resistance, cm $H_2O \cdot l^{-1} \cdot s^{-1}$)	6.57 (2.07)	5.75 (2.50)	6.71 (1.70)	0.74
Work of breathing, J*	1.15 (1.02–1.31)	1.02 (0.89–1.18)	1.19 (1.07–1.31)	0.15
Pa_{O_2}, mmHg	221.00 (61.34)	242.25 (11.11)	202.14 (36.41)	0.39
$Pa_{O_2}/F_{I_{O_2}}$ ratio	442.00 (122.68)	484.5 (22.23)	404.29 (72.81)	0.39
Plasma leukocyte count, $\times 10^9$/l	18.96 (5.26)	16.85 (9.73)	17.94 (3.72)	0.92
Serum C-reactive protein, mg/l*	1.51 (0.55–4.15)	1.00 (1.00–1.00)	1.57 (0.52–4.78)	0.76
Rectal temperature, °C	37.8 (1.04)	37.6 (0.77)	37.9 (0.59)	0.87

Data expressed as mean (SD).

* Nonnormally distributed data expressed as geometric mean (± 95% CI).

$F_{I_{O_2}}$ = fraction of inspired oxygen; IV = intravenous; Pa_{O_2} = arterial oxygen tension; Tx = transfusion.

Appendix 2. Experiment 2: Baseline Characteristics

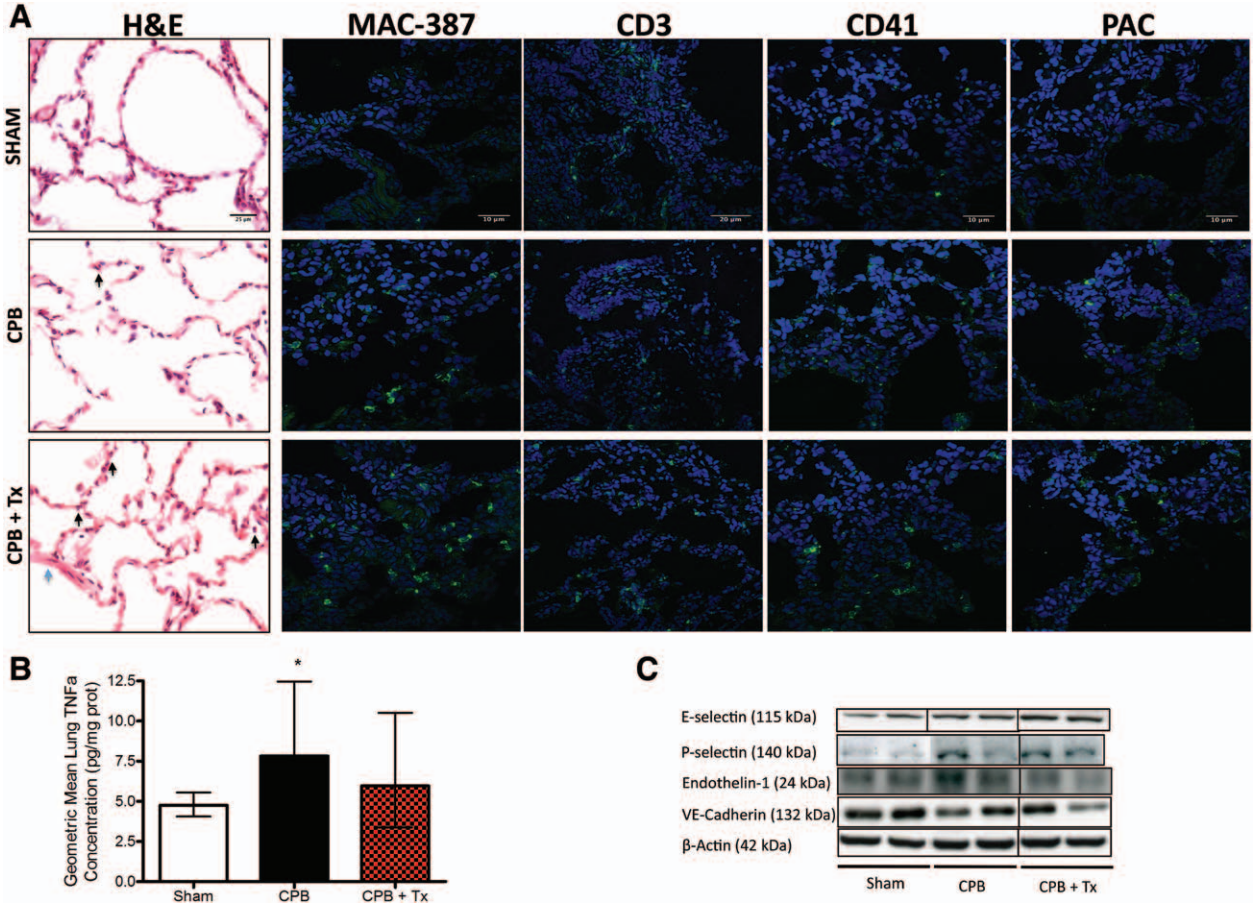
	Sham (n = 7)	CPB (n = 7)	CPB + D42 Tx (n = 7)	*P* Value
Weight, kg*	58.5 (52.8–65.0)	56.9 (53.5–60.5)	57.5 (52.6–62.8)	0.85
Total IV fluids over 24 h, ml*	4,567.7 (4,403.5–4,739.2)	5,614.4† (5,065.2–6,224.4)	5,236.0† (4,545.7–6,029.8)	<0.01
Hematocrit	0.32 (0.02)	0.29 (0.02)	0.31 (0.03)	0.08
Tidal volumes, ml/kg	10.21 (1.33)	9.86 (0.89)	10.49 (1.75)	0.32
Compliance, ml/cm H_2O	39.1 (5.01)	48.0 (9.52)	43.4 (3.25)	0.06
Inspiratory airways resistance, cm $H_2O \cdot l^{-1} \cdot s^{-1}$)	6.57 (2.07)	5.71 (0.76)	6.43 (1.72)	0.58
Work of breathing, J*	1.15 (1.02–1.31)	1.02 (0.93–1.11)	1.03 (0.92–1.14)	0.11
Pa_{O_2}, mmHg	221.00 (61.34)	209.86 (41.36)	221.14 (31.94)	0.87
$Pa_{O_2}/F_{I_{O_2}}$ ratio	442.00 (122.68)	419.71 (82.72)	442.29 (63.89)	0.87
Plasma leukocyte count, $\times 10^9$/l	18.96 (5.26)	14.56 (8.41)	15.16 (8.27)	0.50
Serum C-reactive protein, mg/l*	1.51 (0.55–4.15)	1.50 (0.56–4.04)	1.00 (1.00–1.00)	0.62
Rectal temperature, °C	37.8 (1.04)	37.9 (0.37)	37.9 (0.79)	0.91

Data expressed as mean (SD).

* Nonnormally distributed data expressed as geometric mean (± 95% CI). † $P < 0.05$ *vs.* sham.

CPB = cardiopulmonary bypass; $F_{I_{O_2}}$ = fraction of inspired oxygen; IV = intravenous; Pa_{O_2} = arterial oxygen tension; Tx = transfusion.

Appendix 3



Experiment 2: Pulmonary inflammation and endothelial activation. (*A*) Representative hematoxylin and eosin-stained lung sections (×600) demonstrate normal lung morphology in sham pigs. Interstitial neutrophils (*black arrows*) are evident in pigs receiving cardiopulmonary bypass (CPB) alone. Interstitial neutrophils (*black arrows*), hyaline membranes (*blue arrows*), and proteinaceous debris and neutrophils in the alveolar space in pigs receiving CPB + Tx. Immunofluorescent staining for macrophages using the MAC-387 antibody, T-lymphocytes using the CD-3 antibody, constitutive platelets using the CD41 antibody, and activated platelets using the platelet-activating complex (PAC; anti-gpIIb/IIIa, αIIbiβ3 epitope) antibody is shown in *green* and 4β,6-diamidino-2-phenylindole-stained nuclei in *blue* in porcine lung tissue (×630, MAC-387, CD41, PAC; ×400, CD3) for sham, CPB, and CPB + Tx pigs. (*B*) Tumor necrosis factor-α (TNF-α) concentration measured in whole lung lysates using solid phase enzyme-linked immunosorbent assay. (*C*) Western blot analysis for markers of endothelial activation (P-selectin, E-selectin, Endothelin-1, VE-Cadehrin) in whole lung lysates of pigs. Images from the same gel have been grouped indicated by *black dividing lines*. kDa = kilodaltons; Tx = transfusion.