Published in final edited form as:

Xenotransplantation. 2011 ; 18(2): 94–107. doi:10.1111/j.1399-3089.2011.00633.x.

Absence of Gal epitope prolongs survival of swine lungs in an ex vivo model of hyperacute rejection

Bao-Ngoc H. Nguyen¹, Agnes M. Azimzadeh¹, Carsten Schroeder^{1,2}, Thomas Buddensick¹, Tianshu Zhang¹, Amal Laaris¹, Megan Cochrane³, Henk-Jan Schuurman⁴, David H. Sachs³, James S. Allan³, and Richard N. Pierson III¹

¹Department of Surgery, University of Maryland and Baltimore VAMC, Baltimore, MD

²Department of Surgery, Case Western University, Cleveland, OH

³Transplantation Biology Research Center, Massachusetts General Hospital, Boston, MA

⁴Formerly at Immerge Biotherapeutics Inc, Cambridge, MA, USA

Abstract

Background—Galactosyl transferase gene knock-out (GalTKO) swine offer a unique tool to evaluate the role of the Gal antigen in xenogenic lung hyperacute rejection.

Methods—We perfused GalTKO miniature swine lungs with human blood. Results were compared with those from previous studies using wild-type and human decay-accelerating factor-transgenic (hDAF^{+/+}) pig lungs.

Results—GalTKO lungs survived 132 ± 52 min compared to 10 ± 9 min for wild-type lungs ($P = 0.001$) and 45 ± 60 min for hDAF^{+/+} lungs ($P = 0.18$). GalTKO lungs displayed stable physiologic flow and pulmonary vascular resistance (PVR) until shortly before graft demise, similar to autologous perfusion, and unlike wild-type or hDAF^{+/+} lungs. Early (15 and 60 min) complement (C3a) and platelet activation and intrapulmonary platelet deposition were significantly diminished in GalTKO lungs relative to wild-type or hDAF^{+/+} lungs. However, GalTKO lungs adsorbed cytotoxic anti-non-Gal antibody and elaborated high levels of thrombin; their demise was associated with increased PVR, capillary congestion, intravascular thrombi and strong CD41 deposition not seen at earlier time points.

Conclusions—In summary, GalTKO lungs are substantially protected from injury but, in addition to anti-non-Gal antibody and complement, platelet adhesion and non-physiologic intravascular coagulation contribute to Gal-independent lung injury mechanisms.

Keywords

alphaGal; antibodies; ex vivo lung perfusion; genetically engineered; hyperacute rejection; lung; swine; Xenotransplantation

© 2011 John Wiley & Sons A/S.

Address reprint request to A. M. Azimzadeh, Department of Surgery, University of Maryland, 10 S. Pine Street, MSTF Building, Room 434C, Baltimore, MD 21201, USA (aazimzadeh@smail.umaryland.edu).

Author contributions

B.-N.H. Nguyen, A.M. Azimzadeh, C. Schroeder, T. Zhang, T. Buddensick, A. Laaris, M. Cochrane, and J.S. Allan contributed to the conduct of ex vivo perfusion and/or in vitro experiments and data analysis/interpretation. B.-N.H. Nguyen, A.M. Azimzadeh, J.S. Allan and R.N. Pierson III contributed to drafting the article. H.-J. Schuurman, D.H. Sachs, J.S. Allan and R.N. Pierson III contributed to the experimental design, logistical support and revision of article.

Introduction

The shortage of human organ donors remains a major limitation to the field of transplantation [1], and might be solved using xenografts. Pigs are favoured as a potential xenograft donor source because this use of pigs causes little ethical controversy and the actual risk of retrovirus transmission to humans is thought to be substantially lower than originally perceived [2]. The major initial immunological obstacle to “discordant” pig-to-human solid organ transplantation is hyper-acute rejection (HAR). Many breakthroughs in concept and practice have significantly advanced the understanding of HAR over the past several decades [2–4]. As organs from unmodified pigs were evaluated in animal models, HAR was found to be primarily a consequence of the recipient’s pre-formed anti-pig antibodies binding on porcine vascular endothelial cells leading to complement activation, thrombosis and graft failure [5]. This process occurs within minutes to hours of human blood perfusion in porcine organs. Consequently, conventional strategies to delay or prevent hyper-acute rejection include anti-xenograft antibody removal and complement inhibition [6–11].

As the carbohydrate structure Galactose- α (1,3)-Galactose (Gal) is recognized by over 80% of anti-pig antibodies found in man, genetically modified galactosyl transferase knock-out (GalTKO) pig organs have been developed [12,13]. Endothelium and parenchymal cells from GalTKO animals lack the Gal α 1,3Gal epitope. As predicted, pilot studies using heart and kidney [14–16] and orthotopic lung [17] transplants in baboons showed that the GalTKO phenotype is associated with decreased antibody binding and reduced activation of the complement cascade. Here, we report results from ex vivo perfusion of lungs with human blood, which allow us to describe for the first time the mechanisms of organ failure associated with GalTKO lungs, compared with historical wild-type, hDAF-expressing and autologous reference groups.

Materials and methods

Animals

GalTKO pigs (n = 5, 20 to 60 kg) were supplied by Immerge Biotherapeutics, Inc. (Boston, MA, USA). The lungs were harvested at the Transplant Biology Research Center in Boston as described previously [18,19] and below, and transported to University of Maryland with a cold ischemic time of four to 6 h. Results using transgenic pigs homozygous for human decay-accelerating factor (hDAF^{+/+}, Novartis Pharma, Basel, Switzerland, n = 13, 12 to 18 kg) [11] and some of the wild-type (WT) and autologous lungs tested using the same model [20] have previously been reported. All procedures were approved by the University of Maryland Animal Care and Use Committee, and in compliance with guidelines from NIH publication 86 to 23, the *Guide for the Care and Use of Animals*.

Lung harvest

Animals were anesthetized with ketamine (10 mg/kg) (Ketaset; Fort Dodge Animal Health, Fort Dodge, IA, USA) and xylazine (1 mg/kg) (Rompun; Bayer Pharmaceuticals, Shawnee Mission, KS, USA), and kept under general anesthesia (isoflurane 0.5 to 3%) throughout the surgical procedure. After intubation, median sternotomy, and heparinization (500 units/kg), 0.5 mg prostaglandin E₁ (PGE₁) (ProstinVR Pediatric; Pfizer; New York, NY, USA) was administered directly into the right heart. The main pulmonary artery was cannulated and lungs were flushed with 50 ml/kg Perfadex (Vitrolife, Goteburg, Sweden) containing 0.5 mg PGE₁/l at 4 °C). The heart-lung block was removed and placed on iced normal saline. The lungs were transported on ice in a partially inflated state.

Ex vivo lung perfusion model

The ex vivo pig lung perfusion was performed as described in detail previously [11], with the modification that a single lung (right or left) was selected for ex vivo perfusion with human or pig perfusate (~1 l/lung) prepared as described below. As previously reported [11,18,19,21] blood flow, pulmonary artery and airway pressures were monitored continuously and PVR was calculated using the equation: $PVR \text{ (mmHg/ml/min)} = \text{pulmonary artery pressure/measured flow}$. Blood gas samples were collected from the pulmonary veins to assess for oxygenation throughout the experiments. A pop-off valve was installed and set at 50 cm H₂O to avoid excessive pulmonary artery pressure during periods of high PVR. The lungs were ventilated (Harvard Apparatus, South Natick, MA, USA) with a balanced air mix (21% O₂, 5% CO₂) 12 to 15 times per minute. Tidal volumes of 10 ml/kg were set initially and adjusted visually for adequate inflation while avoiding peak inspiratory pressure over 14 cm H₂O.

The study endpoint was arbitrarily set at 4:00 h, and experiments “surviving” for this interval were terminated after final sample collection. Lung failure was defined as loss of transpulmonary blood flow (<3 ml/kg/min), development of gross tracheal oedema (prohibiting lung ventilation or associated with lack of oxygenation) or loss of perfusate volume (>85% of starting reservoir volume) by massive intraparenchymal sequestration.

Perfusate preparation

Human blood (type O) was collected from volunteer donors by the General Clinical research Center (GCRC) of University of Maryland in accordance with protocols approved by the Committee for the Protection of Human subjects. Approximately, 450 ml of fresh human blood from one donor was collected in blood collection bags (Boin Medica Corporation, Seoul, Korea) containing 100 ml citrate phosphate dextrose adenine solution (CPDA). Two units of pooled fresh-frozen plasma from blood type O were added to reach a baseline perfusion volume of 900 ml. For autologous pig lung perfusions, autologous pig blood was collected from the pig donor prior to organ procurement and stored in CPDA until use. In historical pig lung perfusions, pig perfusate was obtained by mixing pig blood with an equal volume of saline (n = 7). In three contemporaneous pig lung perfusions, pig blood was mixed with commercial allogenic pig plasma (n = 2; Per-Freez Biologicals, Rogers, AK, USA) or 50/50 Ringer’s Lactate/Hetastarch (n = 1). Heparin was added to CPDA whole blood and plasma to achieve a plasma concentration of 2 IU/ml (Elkins-Sinn, Cherry Hill, NJ, USA), calcium chloride (1.3 mg/ml, Fujisawa, Deerfield, IL, USA) was added to restore normal ionized calcium levels in CPDA banked blood products (to achieve 8.5 to 10.5 mg/dl) and the pH was adjusted to physiologic values using sodium bicarbonate 8.4% (American Pharmaceutical Partners, Los Angeles, CA, USA) based on blood gas results. The perfusate was recirculated at 37 °C for approximately 10 min before baseline blood samples were collected prior to initiation of lung perfusion.

Complement, βTG, and thrombin (F1 + 2) enzyme-linked immunosorbent assays (ELISA)

Complement C3a was measured by a commercial ELISA (C3a EIA; Quidel, San Diego, CA, USA) in plasma samples collected in ethylenediamine tetra-acetic acid (EDTA). Prothrombin fragments 1 + 2 (F1 + 2) and β-thromboglobulin (βTG) were measured by ELISA (Asserachrome βTG, Diagnostica Stago, Parsippany, NJ and Enzygnost F1 + 2; Dade Behring/Siemens, Deerfield, IL, USA) in plasma samples collected in CDTA blood collection tubes (Becton Dickinson, Franklin Lakes, NJ, USA).

Hematologic and flow cytometry analysis

White blood cells and platelets were enumerated by standard automated techniques in blood samples collected in EDTA.

The proportion of activated platelets was quantified by flow cytometry by expression of CD62P as follows: blood samples collected in CDTA tubes were immediately fixed with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffered saline +1% paraformaldehyde to prevent in vitro activation and stored at 4 °C for 1 to 3 days until staining. Fixed blood cells were stained with FITC-labelled anti-human CD41 (Serotec, Raleigh, NC, USA) and PE-labelled anti-human CD62P (BD Pharmingen, San Diego, CA, USA) monoclonal antibodies according to the manufacturer's instructions (BD Pharmingen; Source Book Section 2.19). Samples were acquired on a FACs Calibur and data analyzed using Cell Quest (BD Biosciences, San Jose, CA, USA). Results were expressed as the percent of CD41+ events (platelets) expressing CD62P, after subtraction of baseline levels.

Histology and immunochemistry

Serial lung biopsies after reperfusion and multiple terminal samples were trisected and processed as follows. Formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin (H&E) for light microscopy and analyzed qualitatively for the presence of intravascular thrombosis, and interstitial, alveolar or larger airway oedema and haemorrhage. Two other pieces were snap frozen in liquid nitrogen, one without further manipulation, and the other one after gentle instillation of diluted optimal cutting temperature (OCT) compound into visible airways. Sections from OCT-frozen tissues were assessed by immunohistochemistry as previously described [21,22]. Briefly, 6 µm sections were fixed in acetone (5 min at -20 °C) and stained with rabbit anti-von Willebrand factor antibody (Dako, Copenhagen, Denmark; 1:50) and green fluorescent Cy2-labelled goat-anti-rabbit IgG antibody (Jackson Laboratories, West Grove, PN, USA) to identify blood vessels. Individual sections were stained with monoclonal antibodies against human IgM (BD Pharmingen, San Diego, CA, USA; 1:50), C4d (Quidel, Santa Clara, CA, USA; 1:50), CD41 (Immunotech Coulter, Westbrook, ME, USA; 1:100) or no primary antibody (negative control) and red fluorescent Cy3-labelled goat-anti-mouse IgG (Jackson Laboratories). Staining intensity was scored on a scale from 0 to 3 by two investigators (AA and RNP) blinded with respect to study group, using the following scale: 0, absence of staining; 1, staining in <10% of vessels or weak diffuse; 2, staining in 10–50% of vessels or mild diffuse; 3, staining in >50% of vessels or intense diffuse.

Complement-dependent cytotoxicity (CDC)

The amount of antibodies inducing complement-dependent lysis of GalTKO cells was measured as previously described [23], with modifications [24]. Briefly, peripheral blood mononuclear cells isolated from wild-type and GalTKO pigs were incubated with heat-inactivated serum followed by complement, and cytotoxicity was quantified at various dilutions (1:2 to 1:512) by staining with 7-amino-actinomycin D (7-AAD). Results were expressed as the per cent of cell lysis determined at the 1:2 serum dilution, corrected for background cytotoxicity in the absence of complement.

Measurement of anti-Gal and non-Gal antibodies by flow cytometry

Primary pig aortic endothelial cells (PAEC) were isolated from WT and GalTKO pig aortas as previously described. [9]. Cells were used after four to six passages at 80 to 95% confluence. The endothelial cell phenotype was assessed by expression of CD31 using mouse anti-pig CD31 antibody (Serotec, Raleigh, NC, USA; # MCA 1746F). Absence of expression of Gal residues by GalTKO cells was verified by staining with the lectin from

Bandeiraea Simplicifolia BSI-B₄ (Sigma, St. Louis, MO, USA; # L-2895). Fifty microliter of WT or GalTKO PAEC suspensions (2×10^6 cells/ml) were incubated with an equal volume of heat-inactivated serum samples (diluted 1:10 for anti-Gal and 1:2 for anti-non-Gal) in staining buffer (phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA)). Antibody binding was revealed using PE-labelled goat anti-human IgM (Fc μ specific) antibodies (Invitrogen, San Diego, CA, USA), or biotin-labelled goat anti-monkey IgG (Fc γ specific) antibodies (Nordic, Accurate, Westbury, NY, USA) followed by PE-labelled streptavidin (BD Pharmingen). All incubations were performed at 4 °C. Five thousand events were acquired on a FACs Calibur and data analyzed using Cell Quest. Results were expressed as the median fluorescence intensity (MFI).

Measurement of anti-Gal and non-Gal antibodies by ELISA

Maxisorp microtiter plates (Nalgene Nunc Int, Rochester, NY, USA) were coated with 5 μ g/ml of one of the following antigens: Gal, Galactose- α (1,3)-Galactose disaccharide conjugated to polyacrylamide (PAA-Bdi); LacNAc-PAA, Nace-tyllactosamine conjugated to PAA (Gal β 1,4Glc-Nac β 1-PAA, 08-022) and PAA, polyacrylamide conjugate backbone (all from GlycoTech Corporation, Rockville, MD, USA); and NeuGc; *N*-glycolylneuraminic acid or Hanganutziu-Deicher antigen (NeuGc α 2,3Gal β 1,4GlcNac β 1-R (Alberta Research Center, Ontario, Canada). After incubation overnight at 4 °C, wells were blocked with PBS containing 0.05% Tween 20 (PBS-T) for 1:00 h. Next, serum samples and human AB serum pool (H4522 Sigma, Milwaukee, WI, USA) in PBS-T were added to coated wells in duplicate and incubated for 2:00 h at room temperature. Binding of IgM and IgG was revealed using affinity-purified alkaline phosphatase (AP)-conjugated goat anti-human Fc μ (Jackson Laboratories) or biotinylated goat anti-monkey Fc γ (Nordic) followed by AP-conjugated streptavidin (Jackson). Optical density was read at 405 nm after addition of paranitro-phenylphosphate substrate solution. Results were expressed as arbitrary units (AU) calculated from the linear part of a standard curve generated using the human serum pool (defined as equivalent to one unit), corrected for specific individual values detected on the polyacrylamide backbone (PAA) used as specificity control.

Measurement of cytokines and other inflammatory mediators using protein arrays

Levels of porcine and human cytokines/mediators were quantified in plasma samples taken before the start of perfusion and at the time of lung failure using the SearchLight Chemiluminescent Protein Array service available through Endogen (Rockford, IL, USA). This evaluation was only done for GalTKO lungs. Results were expressed as fold increase over baseline levels before initiation of perfusion.

Correction for hemoconcentration

Hemoconcentration was detected in some experiments as an unexpected rise in anti-Gal antibody levels measured by ELISA after lung perfusion. The degree of hemoconcentration correlated directly with the subjective severity of fluid sequestration in the lung. To correct for the degree of protein enrichment in plasma, total immunoglobulin IgM and IgG and total protein serum levels were measured in our local clinical laboratory. The percent increase in total IgM or IgG level was used to correct ("normalize") post-perfusion IgM or IgG antibody levels for the perfusate hemoconcentration typically seen during hyperacute lung rejection; and results were expressed as normalized MFI or normalized units. Similarly, cytokine/mediator levels were normalized using percent change in plasma total protein levels.

Statistical analysis

Unless otherwise indicated, continuous variables were expressed as the mean plus standard error of the mean (SEM). Variables that were not normally distributed (C3a, β TG, F1 + 2, blood cell differential counts, histology scores) were analyzed using the Mann-Whitney non-parametric test. Those that were normally distributed (PVR) were assessed with the student *t*-test. Lung perfusion survival time was graphed using the Kaplan–Meier method and the log-rank test was used to compare survival time between groups. Plasma cytokines before and after GalTKO lung perfusions were compared using the paired student *t*-test. P-values <0.05 were considered statistically significant. All statistical analyses were performed on a personal computer with the statistical package SPSS for Windows XP (version 11.0; SPSS, Chicago, IL, USA) or GraphPad InStat (version 5.1; GraphPad Software, San Diego, CA, USA).

Results

Lung survival and function

GalTKO lungs survived 132 ± 52 min compared with 10 ± 9 min for wild type lung ($P < 0.001$) and 45 ± 60 min for hDAF^{+/+} lungs ($P = 0.18$) (Fig. 1). GalTKO lungs displayed stable physiologic flow and pulmonary vascular resistance (43 ± 9.8 mmHg/L/min at 5 min) until shortly before graft demise, similar to autologous lung perfusion, and unlike wild-type (297 ± 46 mmHg/L/min at 5 min, $P < 0.01$) and hDAF^{+/+} lungs (110 ± 25 mmHg/L/min at 5 min, $P = 0.12$) (Fig. 2A). PVR of GalTKO lungs remained significantly lower than that of wild-type lungs at 10 and 20 min ($P < 0.05$). Whereas the mechanism of lung failure for wild-type lungs was consistently loss of transpulmonary blood flow due to high PVR, only two of five GalTKO lungs failed because of high PVR, at 65 and 132 min, compared with <20 min with wild-type lungs. Other GalTKO lungs failed due to pulmonary interstitial and intra-alveolar fluid sequestration and loss of perfusate volume at 56, 136 and 180 min.

Complement activation

The increase in plasma C3a at 15 and 60 min after initiation of lung perfusion was significantly lower in association with GalTKO lungs than with hDAF^{+/+} lungs (320 ± 192 ng/ml vs. 1544 ± 247 ng/ml at 15 min, $P = 0.015$; 463 ± 157 ng/ml vs. 1537 ± 319 ng/ml at 60 min, $P = 0.01$) (Fig. 2B) and wild-type lungs ($P < 0.05$ at 5 and 15 min).

Blood cells counts

Over ninety percent of neutrophils were sequestered from perfusate within the first 5 min of perfusion of wild-type lungs (Fig. 3A). Neutrophils were sequestered to a similar extent in association with GalTKO lung perfusion, although with delayed kinetics: at 15 min, significantly more neutrophils remained in the circulation with Gal-TKO lungs ($28 \pm 7\%$), than with wild-type lungs ($4 \pm 1\%$; $P = 0.03$ at 5 and 15 min). Neutrophil counts were also significantly higher with GalTKO lungs than with hDAF^{+/+} lungs at 15 min ($P = 0.006$).

There was a rapid decrease in circulating monocytes with perfusion of both wild-type ($27 \pm 9\%$ at 5 min) and hDAF^{+/+} ($58 \pm 4\%$ at 5 min) lungs, which was delayed in GalTKO lungs ($109 \pm 36\%$ at 5min, $P = 0.029$ vs. WT) (Fig. 3B). Cell numbers above 100% between 10 and 30 min reflect the appearance of porcine cells in the perfusate (unpublished observations).

Platelet sequestration and activation

GalTKO lung perfusion was associated with platelet sequestration kinetics very similar to that for wild-type and hDAF^{+/+} lungs, with 70% decline in platelets during the first 5 min

that persisted through the experiments (Fig. 4A). However, the proportion of platelets remaining in the perfusate that were activated (expressing CD62P) was consistently lower with GalTKO at early time points ($2.8 \pm 0.3\%$ in GalTKO vs. $12 \pm 5\%$ for wild-type and $13 \pm 11\%$ for hDAF^{+/+} at 5 min) (Fig. 4B). Similarly, compared with wild-type lungs, β TG levels in association with GalTKO lungs were significantly lower than in WT and hDAF^{+/+} lungs in the first 15 min, and remained low, but did not reach significance at later time-points (Fig. 4C).

Coagulation activation marker-F1 + 2 and thrombin formation

Coagulation activation at 15 min, measured by thrombin formation (F1 + 2), was the highest in GalTKO lungs (25 ± 11 nM) compared with wild-type (4.4 ± 0.8 nM; $P = 0.43$) and hDAF^{+/+} lungs (3.5 ± 1.5 nM; $P = 0.06$) and remained higher than in reference groups at all time-points (Fig. 4D).

Histology and immunohistochemistry

Autologous lungs showed a normal microscopic anatomy with air-filled alveoli, thin inter-alveolar septae, and no intravascular thrombosis at elective termination after 4 h of perfusion (Fig. 5A). Wild-type lungs perfused with human blood exhibited prevalent, severe interstitial and alveolar haemorrhage that extended to the airway at failure, along with cellular infiltration and prevalent intravascular thrombosis (Fig. 5B). hDAF^{+/+} and GalTKO lungs showed relatively preserved histology at 60 min, but infiltration by polymorphonuclear granulocytes, intravascular thrombosis and haemorrhage were prominent at lung failure (Fig 5C,D).

Whereas both wild-type and hDAF lungs exhibited intense IgM and classical pathway complement activation fragment C4d deposition at 10 min after initiation of perfusion, GalTKO lungs showed limited IgM and complement deposits at that time point. Both WT and hDAF^{+/+} lungs showed intense platelet deposition (CD41 staining) 10 min after initiation of lung perfusion. In contrast, although the kinetics of decline in platelet counts in the perfusate were similar (Fig. 4A), platelet detection in GalTKO lungs was delayed, as CD41 deposition was remarkably rare in GalTKO lungs in the 10 min biopsies ($P = 0.057$ vs. WT) (Fig. 6). At GalTKO lung failure, there was a prominent platelet deposition in association with alveolar septal endothelium, similar to that observed terminally in lungs from other groups.

Anti-Gal and non-Gal antibody levels

As expected, both anti-pig IgM and IgG reactive with Gal+ (WT) swine PAEC cells by flow cytometry largely disappeared from the circulation after perfusion of WT lungs (Fig. 7A). After perfusion of GalTKO lungs, anti-pig IgM levels appeared to increase in some experiments when assessed on Gal+ cells (Fig. 7B left); we attribute this phenomenon to hemoconcentration linked to progressive lung oedema, as it was not apparent when antibody levels were corrected for total IgM or IgG protein levels ("normalized" MFI Fig. 7B right). Similarly, when assessed on GalTKO cells, a modest decline in anti-pig antibody levels after perfusion of GalTKO lungs (Fig. 7C left) was more readily detectable when results were normalized for immunoglobulin protein levels (Fig. 7C right). Overall, the decrease in anti-pig antibody titers with GalTKO lungs was less consistent or profound than with hDAF or WT lungs, con-firming that Gal is the major antigenic target of human natural antibodies. Cytotoxicity toward GalTKO PAECs was reduced following WT ($67 \pm 3\%$ inhibition) and a lesser extent following GalTKO ($43 \pm 19\%$) lung perfusion, indicating that anti-pig antibodies absorbed to antigens other than Gal in lung tissue were able to fix complement and activate the complement cascade (Fig. 7D).

As expected in ELISA testing, after normalization, levels of antibody against Gal α 1,3Gal were similar before and after perfusion of Gal-TKO lungs (Fig. 7E left panel), in contrast to a sharp decrease after perfusion of WT lungs [25]. Before perfusion, levels of antibody reactive with two previously described [26–28] non-Gal porcine carbohydrate xenoantigens, NeuGc and LacNAc, varied considerably between experiments (each perfusate represents pooled plasma from six human blood donors) (Fig. 7E middle and right panels, respectively). Anti-NeuGc IgG decreased in all the five experiments, while anti-NeuGc IgM and anti-LacNAc antibody levels decreased during a minority of GalTKO lung perfusions.

Release of cytokines and other inflammatory mediators

Pig interleukin (IL)8 and human IL6 and IL8 protein levels detected by chemiluminescent protein array technology were increased in plasma at the time of failure of GalTKO lungs in most experiments; pig IL6, human IL1B, and interferon gamma (IFNG) exhibited proportionately smaller increases at that interval (Fig. 8A,B). A number of proteins associated with platelet and coagulation cascade activation (soluble CD40 ligand, soluble P-selectin, and tissue factor) were increased in 3 to 4 of five GalTKO lung experiments, with the notable exception of von Willebrand factor (VWF) levels, which tended to be lower ($P = 0.15$) (Fig. 8C). Protein mediators released by neutrophils [myeloperoxidase (MPO), $P = 0.009$], but not by monocytes (sCD14, $P = 0.29$) were also increased (Fig. 8D).

Discussion

This study demonstrates that, compared with wild-type and hDAF^{+/+} lungs, GalTKO lungs subjected to ex vivo perfusion with human blood were associated with significant delay in the acute lung injury observed in association with HAR of solid organs, and with a consistent trend toward improved survival. Failure of GalTKO lungs to sequester preformed human anti-Gal antibody provides compelling indirect evidence that Gal antigen is absent and that this antigen has a major role in HAR. Decline in the titer of antibodies directed against non-Gal pig antigens after lung perfusion implicates such “non-Gal” antibodies in GalTKO lung injury, as does the associated reduction in CDC. The relatively low level of plasma C3a elaboration demonstrates that intrinsic porcine complement regulatory mechanisms are preserved when the relatively high titer of anti-Gal antibody is rendered irrelevant, in accordance with in vivo GalTKO lung transplants [17], showing that GalTKO lung xenografts are partially protected with respect to complement activation. However, the delayed rise in pulmonary vascular resistance after the first hour, coupled with progressive perfusate sequestration demonstrate that physiologically important injury mechanisms do occur in the absence of Gal, and thus are not entirely Gal-dependent [20,25,29]. Antibody (non-Gal) deposition remained detectable, and is presumably pathogenic; pre-perfusion adsorption of human plasma against GalTKO organ endothelium is warranted to test this hypothesis. Similarly, an important question not addressed by the present experiment is whether membrane-bound or soluble-phase complement activation contributes to GalTKO lung injury [21].

Intravascular thrombosis, platelet and coagulation cascade activation, and progressively intense tissue platelet deposition in association with Gal-TKO lung failure strongly implicate coagulation pathways in ultimate GalTKO lung demise, as previously described for WT, human DAF- and membrane cofactor protein (MCP)-expressing lungs [20,25,30]. Decreased complement activation and reduced β TG levels in association with Gal-TKO lungs during the first hour of perfusion suggest that initial platelet activation is in part driven by anti-Gal-antibody and classical pathway complement activation, as previously shown for hDAF-expressing lungs [11]. Complement components such as C1q and membrane attack complex can directly activate platelets [31,32]. In addition, complement can activate platelets indirectly by causing injury to endothelial cells; injury to endothelium has several

procoagulant effects [33,34]. In addition to complement, Fc on bound antibody, thrombin, adenosine diphosphate (ADP), thromboxane, von Willebrand factor, and collagen are exposed on and adjacent to injured, activated, retracted swine endothelial cells; each provides a potent stimulus to platelet activation [5,35]. Gal positive pig lung xenografts have been shown to rapidly shed vWF after reperfusion [36]. Although healthy endothelium normally prevents “physiologically inappropriate” intravascular thrombosis in allografts, Robson, Dorling, and colleagues have pioneered work showing that specific molecular incompatibilities exist in coagulation pathway regulation across discordant species [37–40]. To the extent that one or more of these mechanisms is found to dominate the induction of GalTKO lung injury, targeting that mechanism will likely offer additional protection necessary to facilitate consistent, durable life-supporting GalTKO lung xenograft function.

Platelets disappear from the perfusate at early time-points after GalTKO lung perfusions (Fig. 4A), but early tissue deposition is minimal (Fig. 6). This observation could be explained by the decreased formation of procoagulant platelet-endothelial cell aggregates [41] but not platelet-neutrophil aggregates [42,43] and suggests that some of the early platelet agonists formed after pig lung perfusion by human blood were suppressed with GalTKO lungs and thus are Gal-dependent. Thrombin formation is particularly prolific in association with perfusion of GalTKO lungs. The fact that some (3/5) GalTKO lungs elaborated high levels of thrombin (F1 + 2) at all time points probably reflected a consequence of better trans-pulmonary blood flow in GalTKO lungs, and proportionately increased exposure of blood to porcine lung endothelium where the consequence of physiologic incompatibilities between coagulation pathway molecules of these species are amplified. In our estimation, it is less likely that pig endothelium lacking the α Gal antigen more efficiently supports direct prothrombinase activity by GalTKO endothelial cells relative to hDAF or WT endothelium.

Limitations of our study include the lack of some data in the autologous control group. Complement and coagulation assays do not cross-react in the pig species. Platelet and neutrophil counts were not recorded in historical autologous pig lung perfusion experiments. In three contemporaneous pig lung perfusions, platelet counts decreased transiently in one experiment, and dropped by 70% in the other when autologous pig blood was diluted with random allogenic pig plasma, but not when pig blood was diluted in Ringer’s Lactate/Hetastarch. Importantly, in vivo, neutrophils’ and platelets’ blood counts dropped in xeno-, but not in allo-lung perfusions [17]. Taken together, our studies acknowledge the difficulty in obtaining rigorous autologous pig control data in our model and suggest that the drop in neutrophil and platelet counts is related to xenogeneic interactions. Another potential limitation is the difference in size and ischaemia time between GalTKO and other groups. In our experience, taking together data from a large cohort of lungs from pigs with various backgrounds, we found no obvious correlation between the animal size and the intensity or tempo of xenogeneic rejection. An ischaemia time of up to 6 h was imposed by the transportation of scarce GalTKO organs for this study; however, this time remains within the limits of clinical practice. Recent studies using GalTKO pig lungs from a different source and shorter ischaemia times confirm the Gal-independent failure of pig lungs and suggest vWF as a key factor in platelet loss (Burdorf, manuscript in preparation). We infer that the ex vivo model is useful for studying mechanisms of rejection, before interventions are tested in the expensive and labour-intensive in vivo model.

The nature of pig antigens recognized by non-Gal antibodies remains largely unresolved [26,44]. Two carbohydrate candidates include the NeuGc and NAcetylactosamine carbohydrate antigens [26–28]. Although absorption from plasma was detected by ELISA, presumably reflecting adsorption to these antigens in the GalTKO lung, detection of these antibodies was not consistent among different human blood pools, and their titer did not

correlate closely with lung behaviour or with non-Gal antibodies measured on pig cells by flow cytometry or CDC. Ongoing work by us and by others seeks to identify physiologically important non-Gal antigens.

Although, anti-non-Gal antibody, complement and coagulation (including platelets) seem to play a major role in the mechanism driving GalTKO hyperacute lung rejection, our previous study showed the relevance of porcine pulmonary intravascular macrophages and thromboxane elaboration in the rise of PVR associated with hyperacute lung rejection [45]. In addition, Waddell and colleagues showed that human monocytes adhere to pig endothelial cells by binding to the Gal antigen, which not only causes monocytes sequestration in the tissue but also acts as a trigger to up-regulation of endothelial cells' adhesion molecule, cytokine release and tissue factor expression [46]. It is interesting that we observed only a transient delay in human monocyte sequestration in the context of GalTKO lungs, suggesting that galectin-3-mediated interactions [47] or coagulation pathway components may mediate monocyte adhesion to GalTKO endothelium. Moreover, an increase in pro-inflammatory cytokines, chemokine and other mediators was readily detectable as early as 1 h after perfusion of GalTKO lungs, and increased levels of the chemokine IL8 were associated with neutrophil sequestration. Targeting pulmonary intravascular macrophages [45,48] and inflammatory cell adhesion and activation [49] are therefore additional logical steps that may prove necessary to prevent hyperacute lung rejection.

Conclusion

In summary, GalTKO lungs exhibit prolonged survival compared with wild-type and transgenic hDAF^{+/+} lungs in a clinically relevant ex vivo perfusion model. Although antibody adsorption and complement activation are significantly reduced and pulmonary vascular physiology better protected during initial perfusion relative to WT or hDAF-expressing lungs, GalTKO lungs still succumbed to injury within hours in association with prevalent platelet sequestration and prolific thrombin activation as well as intravascular thrombosis and inflammation by polymorphonuclear granulocytes. Targeting coagulation pathway dysregulation as well as platelet and neutrophil adhesive interactions represent logical next targets in our ongoing effort to further understand and control the mechanisms behind lung injury in the xenogeneic setting.

Acknowledgments

This work was supported by: NIH U01 AI 066335 and a VA Merit Award (Pierson), NIH R01 HL67110 (Allan); NIH F32 HL079818 and a TSFRE resident research award (Nguyen); a Deutsche Forschungsgemeinschaft research award (Schroeder); and an Other Tobacco Related Diseases research grant from the Maryland Cigarette Restitution Fund Program. The work is also supported by the General Clinical research Center (GCRC) of University of Maryland for nursing staff and financial support in collecting blood from human donors for ex-vivo lung perfusion experiments. We thank Immerge Biotherapeutics for supplying GalTKO lungs and Dr. David Cooper for kindly providing the NeuGc antigen. We also thank Xiangfei Cheng, Qi Feng and Nitin Sangrampurkar for their valuable technical assistance.

Abbreviations

7-AAD	7-amino-actinomycin D
A.U	arbitrary units
ADP	adenosine diphosphate
AP	alkaline phosphatase
ATA	aurintricarboxylic acid

BSA	bovine serum albumin
CDC	complement-dependent cytotoxicity
CPDA	citrate phosphate dextrose adenine solution
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
F1 + 2	prothrombin fragments
F3	tissue factor
FACS	fluorescence activated cell sorting
fgl-2	fibrinogen-like protein 2
Gal	galactose- α (1,3)-galactose
GalTKO	galactosyl transferase gene knock-out
H&E	hematoxylin and eosin
HAEC	human aortic endothelial cells
HAR	hyperacute rejection
hDAF	human decay accelerating factor-transgenic
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethanesulfonic acid
IFNG	interferon gamma
IL	interleukin
LacNAc-PAA	Nacetyllactosamine conjugated to PAA
MCP	membrane cofactor protein
MFI	median fluorescence intensity
min	minute
MPO	myeloperoxidase
NeuGc	<i>N</i> -glycolylneuraminic acid or Hanganutziu-Deicher antigen
OCT	optimal cutting temperature (compound)
PAA	polyacrylamide (backbone)
PAEC	pig aortic endothelial cells
PBS	phosphate-buffered saline
PVR	pulmonary vascular resistance
sCD14	soluble CD14
sCD40LG	soluble CD40 ligand
SD	standard deviation
SELP	soluble P-selectin
SEM	standard error of the mean
TNF	tumor necrosis factor alpha
VWF	von Willebrand factor

β TG

β -thromboglobulin

References

1. Cooper DKC. Xenografting: how great is the clinical need? *Xeno.* 1993; 1:25–26.
2. Pierson RN III. Current status of xenotransplantation. *JAMA.* 2009; 301:967–969. [PubMed: 19258587]
3. Cooper, DKC.; Ye, Y.; Rolf, JRLL.; Zuhdi, YYEN. The pig as potential organ donor for man. In: Cooper, DKC.; Kemp, E.; Reemtsma, K.; White, DJG., editors. *Xeno-Transplantation.* Berlin: Springer-Verlag; 1991. p. 481-500.
4. Pierson RN III, Dorling A, Ayares D, et al. Current status of xenotransplantation and prospects for clinical application. *Xenotransplantation.* 2009; 16:263–280. [PubMed: 19796067]
5. Pierson RN III. Antibody-mediated xenograft injury: mechanisms and protective strategies. *Transpl Immunol.* 2009; 21:65–69. [PubMed: 19376229]
6. Leventhal JR, John R, Fryer JP, et al. Removal of baboon and human antiporcine IgG and IgM natural antibodies by immunoadsorption. *Transplantation.* 1995; 59:294–300. [PubMed: 7839454]
7. Cozzi E, White DJG. The generation of transgenic pigs as potential organ donors for humans. *Nature Med.* 1995; 1:964–966. [PubMed: 7585226]
8. White DJG, Langford GA, Cozzi E, Young VK. Production of pigs transgenic for human DAF: a strategy for xenotransplantation. *Xenotransplantation.* 1995; 2:213.
9. Azimzadeh A, Meyer C, Watier H, et al. Removal of primate xenoreactive natural antibodies by extracorporeal perfusion of pig kidneys and livers. *Transpl Immunol.* 1998; 6:13–22. [PubMed: 9640624]
10. Fiane AE, Videm V, Johansen HT, et al. C1-inhibitor attenuates hyperacute rejection and inhibits complement, leukocyte and platelet activation in an ex vivo pig-to-human perfusion model. *Immunopharmacology.* 1999; 42:231–243. [PubMed: 10408384]
11. Schroder C, Pfeiffer S, Wu G, et al. Effect of complement fragment 1 esterase inhibition on survival of human decay-accelerating factor pig lungs perfused with human blood. *J Heart Lung Transplant.* 2003; 22:1365–1375. [PubMed: 14672751]
12. Phelps CJ, Koike C, Vaught TD, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science.* 2003; 299:411–414. [PubMed: 12493821]
13. Kolber-Simonds D, Lai L, Watt SR, et al. Production of alpha-1,3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations. *Proc Natl Acad Sci U S A.* 2004; 101:7335–7340. [PubMed: 15123792]
14. Tseng YL, Kuwaki K, Dor FJ, et al. Alpha1,3-Galactosyltransferase gene-knockout pig heart transplantation in baboons with survival approaching 6 months. *Transplantation.* 2005; 80:1493–1500. [PubMed: 16340796]
15. Kuwaki K, Tseng YL, Dor FJ, et al. Heart transplantation in baboons using alpha1,3-galactosyltransferase gene-knockout pigs as donors: initial experience. *Nat Med.* 2005; 11:29–31. [PubMed: 15619628]
16. Yamada K, Yazawa K, Shimizu A, et al. Marked prolongation of porcine renal xenograft survival in baboons through the use of alpha1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. *Nat Med.* 2005; 11:32–34. [PubMed: 15619627]
17. Nguyen BN, Azimzadeh AM, Zhang T, et al. Life-supporting function of genetically modified swine lungs in baboons. *J Thorac Cardiovasc Surg.* 2007; 133:1354–1363. [PubMed: 17467457]
18. Pierson RN III, Pino-Chavez G, Young VK, et al. Expression of human decay accelerating factor may protect pig lung from hyperacute rejection by human blood. *J Heart Lung Transplant.* 1997; 16:231–239. [PubMed: 9059935]
19. Pierson RN, Kasperkonig W, Tew DN, et al. Hyperacute lung rejection in a pig-to-human transplant model – the role of anti-pig antibody and complement. *Transplantation.* 1997; 63:594–603. [PubMed: 9047157]

20. Pfeiffer S, Zorn GL III, Zhang JP, et al. Hyperacute lung rejection in the pig-to-human model. III. Platelet receptor inhibitors synergistically modulate complement activation and lung injury. *Transplantation*. 2003; 75:953–959. [PubMed: 12698079]
21. Azimzadeh A, Zorn GL III, Blair KS, et al. Hyperacute lung rejection in the pig-to-human model. 2. Synergy between soluble and membrane complement inhibition. *Xenotransplantation*. 2003; 10:120–131. [PubMed: 12588645]
22. Zhang JP, Blum MG, Chang AC, et al. Immunohistologic evaluation of mechanisms mediating hyperacute lung rejection, and the effect of treatment with K76-COOH, FUT-175, and anti- Gal column immunoadsorption. *Xenotransplantation*. 1999; 6:249–261. [PubMed: 10704068]
23. Wong BS, Yamada K, Okumi M, et al. Allosensitization does not increase the risk of xenoreactivity to alpha1,3-galactosyltransferase gene-knockout miniature swine in patients on transplantation waiting lists. *Transplantation*. 2006; 82:314–319. [PubMed: 16906027]
24. Patel R, Terasaki PI. Significance of the positive cross-match test in kidney transplantation. *N Engl J Med*. 1969; 280:735–739. [PubMed: 4886455]
25. Pfeiffer S, Zorn GL III, Blair KS, et al. Hyperacute lung rejection in the pig-to-human model 4: evidence for complement and antibody independent mechanisms. *Transplantation*. 2005; 79:662–671. [PubMed: 15785372]
26. Ezzelarab M, Ayares D, Cooper DK. Carbohydrates in xenotransplantation. *Immunol Cell Biol*. 2005; 83:396–404. [PubMed: 16033535]
27. Miwa Y, Kobayashi T, Nagasaka T, et al. Are *N*-glycolylneuraminic acid (Hanganutziu-Deicher) antigens important in pig-to-human xenotransplantation? *Xeno-transplantation*. 2004; 11:247–253.
28. Zhu A, Hurst R. Anti-*N*-glycolylneuraminic acid antibodies identified in healthy human serum. *Xenotransplantation*. 2002; 9:376–381. [PubMed: 12371933]
29. Nguyen BH, Zwets E, Schroeder C, et al. Beyond antibody-mediated rejection: hyperacute lung rejection as a paradigm for dysregulated inflammation. *Curr Drug Targets Cardiovasc Haematol Disord*. 2005; 5:255–269. [PubMed: 15975038]
30. Zorn, GLI.; Pfeiffer, S.; Azimzadeh, AM.; Pierson, RN, Iii. Thrombin inhibition protects the pulmonary xenograft from hyperacute rejection; *Surgical Forum*. 2000. p. 338-340.
31. Sims PJ, Wiedmer T. The response of human platelets to activated components of the complement system. *Immunol Today*. 1991; 12:338–342. [PubMed: 1755945]
32. Peerschke EI, Ghebrehiwet B. Platelet receptors for the complement component C1q: implications for hemostasis and thrombosis. *Immunobiology*. 1998; 199:239–249. [PubMed: 9777409]
33. Goepfert C, Imai M, Brouard S, et al. CD39 modulates endothelial cell activation and apoptosis. *Mol Med*. 2000; 6:591–603. [PubMed: 10997340]
34. Benatui L, Fernandez AZ, Romano E. Aggregation of human platelets in plasma by porcine blood cells in vitro is probably mediated by thrombin generation. *Xenotransplantation*. 2003; 10:454–459. [PubMed: 12950988]
35. Cowan PJ, D'apice AJ. Complement activation and coagulation in xenotransplantation. *Immunol Cell Biol*. 2009; 87:203–208. [PubMed: 19153592]
36. Holzkecht ZE, Coombes S, Blocher BA, et al. Immune complex formation after xenotransplantation : evidence of type III as well as type II immune reactions provide clues to pathophysiology. *Am J Pathol*. 2001; 158:627–637. [PubMed: 11159199]
37. Robson SC, Cooper DK, D'apice AJ. Disordered regulation of coagulation and platelet activation in xenotransplantation. *Xenotransplantation*. 2000; 7:166–176. [PubMed: 11021661]
38. Chen D, Riesbeck K, Kembell-Cook G, et al. Inhibition of tissue factor-dependent and - independent coagulation by cell surface expression of novel anticoagulant fusion proteins. *Transplantation*. 1999; 67:467–474. [PubMed: 10030296]
39. Cowan PJ, D'apice AJ. The coagulation barrier in xenotransplantation: incompatibilities and strategies to overcome them. *Curr Opin Organ Transplant*. 2008; 13:178–183. [PubMed: 18685300]
40. Schulte AMEJ, Cruz MA, Siegel JB, et al. Activation of human platelets by the membrane-expressed A1 domain of von Willebrand factor. *Blood*. 1997; 90:4425–4437. [PubMed: 9373253]

41. Lin CC, Chen D, Mcvey JH, et al. Expression of tissue factor and initiation of clotting by human platelets and monocytes after incubation with porcine endothelial cells. *Transplantation*. 2008; 86:702–709. [PubMed: 18791452]
42. Fletcher MP, Stahl GL, Longhurst JC. C5a-induced myocardial ischemia: role for CD18-dependent PMN localization and PMN-platelet interactions. *Am J Physiol*. 1993; 265:H1750–H1761. [PubMed: 7902015]
43. Itoh S, Takeshita K, Susuki C, et al. Redistribution of P-selectin ligands on neutrophil cell membranes and the formation of platelet-neutrophil complex induced by hemodialysis membranes. *Biomaterials*. 2008; 29:3084–3090. [PubMed: 18452987]
44. Byrne GW, Stalboerger PG, Davila E, et al. Proteomic identification of non-Gal antibody targets after pig-to-primate cardiac xenotransplantation. *Xenotransplantation*. 2008; 15:268–276. [PubMed: 18957049]
45. Collins BJ, Blum MG, Parker RE, et al. Thromboxane mediates pulmonary hypertension and lung inflammation during hyperacute lung rejection. *J Appl Physiol*. 2001; 90:2257–2268. [PubMed: 11356791]
46. Peterson MD, Jin R, Hyduk S, et al. Monocyte adhesion to xenogeneic endothelium during laminar flow is dependent on alpha-Gal-mediated monocyte activation. *J Immun*. 2005; 174:8072–8081. [PubMed: 15944315]
47. Jin R, Greenwald A, Peterson MD, Waddell TK. Human monocytes recognize porcine endothelium via the interaction of galectin 3 and alpha-GAL. *J Immun*. 2006; 177:1289–1295. [PubMed: 16818789]
48. Cantu E, Gaca JG, Palestro D, et al. Depletion of pulmonary intravascular macrophages prevents hyperacute pulmonary xenograft dysfunction. *Transplantation*. 2006; 81:1157–1164. [PubMed: 16641602]
49. Parish CR. The role of heparan sulphate in inflammation. *Nat Rev Immunol*. 2006; 6:633–643. [PubMed: 16917509]

Fig. 1. Cumulative survival time of ex vivo perfused lungs. GalTKO, α galactosyl transferase knock-out pig lungs; WT, wild-type pig lungs; or hDAF^{+/+}, hDAF transgenic pig lungs, were perfused with human blood; autologous, pig lungs from various sources perfused with autologous pig blood. Lung “survival time” represents the time of perfusion until which physiologic lung failure was determined, based on pre-defined criteria. #P = 0.0002 vs. WT; P = 0.178 vs. hDAF^{+/+}; P = 0.0003 vs. autologous.

Fig. 2. Pulmonary vascular resistance (PVR) and plasma levels of complement activation byproduct (C3a). (A) PVR expressed as a function of time for control and study groups. Time 0 represents measurements obtained during the first minute of lung perfusion, after circulation of the blood through the circuit for 10 min. PVR of GalTKO lungs was significantly lower than WT lungs and very similar to autologous lungs for the first 30 min. After 1 h, PVR of GalTKO lungs remained stable but higher than autologous lungs and then individual experiment levels usually rose sharply before failure. (B) Plasma C3a levels expressed as the amount of complement fragments produced above the pre-perfusion baseline (Δ). Complement activation was significantly curtailed in pig lungs lacking Gal. All data are shown as the mean $\pm$ SEM of all surviving experiments in one group at each time.

Fig. 3. Neutrophils (A) and monocytes (B) sequestration from circulation. Blood cell numbers measured serially by automated counting are expressed as percent remaining in circulation. Data expressed as change from the baseline and shown as the mean $\pm$ SEM of surviving experiments. *P < 0.05 vs. WT; #P < 0.05 vs. hDAF^{+/+}.

Fig. 4. Platelet and coagulation cascade activation profile. (A) Blood platelet counts measured serially by automated counting. Platelet sequestration was expressed as the percentage of platelets remaining in the perfusate at each time. (B) Platelet activation assessed by measuring the proportion of CD41+ platelets expressing CD62P by flow cytometry. (C) Platelet activation assessed by plasma levels of β TG released from platelet α granules. (D) Activation of the coagulation cascade was detected by the formation of thrombin measured by plasma levels of fragments F1 + 2. All data are expressed as change from the baseline (Δ) and shown as the mean $\pm$ SEM of surviving experiments. * $P < 0.05$ vs. WT; # $P < 0.05$ vs. hDAF^{+/+}.

Fig. 5. Histologic analysis of tissues from GalTKO and control perfused pig lungs. (A) An autologous control lung showed normal lung histology with thin alveolar septa and no pulmonary edema or intravascular thrombosis at 60 min. Mild cellular infiltration and endothelial cell activation is detected at elective termination after 300 min of perfusion. (B) WT lungs perfused with human blood showed severe interstitial/alveolar haemorrhage extended to the airway, cellular infiltration by polymorphonuclear granulocytes as well as severe intravascular thrombosis, in the cases illustrated at 13 and 21 min after reperfusion. (C) hDAF^{+/+} lungs showed largely preserved lung histology with thin alveolar septa, and limited congestion of capillaries and fibrin formation (not shown) at 60 min in a long survivor. In contrast, intravascular thrombosis and hemorrhage are prominent at lung failure (final, 165 min after reperfusion). (D) GalTKO lungs showed mild intravascular thrombosis, septal vascular congestion and edema at 60 min which were accentuated at lung failure (132 min after reperfusion).

Fig. 6.

Two-color immunocytochemistry analysis of tissues from GalTKO and control perfused pig lungs. (A) Pig lung tissues collected at the indicated times were stained for IgM antibody deposition, classical complement activation fragment (C4d), or platelets (CD41) (red); co-staining for von Willebrand factor (VWF) identifies the endothelium (green insert). Strong staining of IgM on the endothelium in WT and hDAF^{+/+} groups was less prominent in GalTKO lungs at 10 min. Complement activation fragment C4d was associated with IgM deposits in capillaries, but much less frequently in large vessels. Both WT and hDAF^{+/+} lungs showed intense platelet deposition at 10 min. In contrast, platelet deposition was rare in GalTKO lungs at that 10 min time-point. However, at lung failure 60 min after reperfusion, there was prominent platelet deposition in association with alveolar septal endothelium in the GalTKO lungs. (B). Immunocytochemistry findings scored as the extent of tissue deposition as indicated in Methods. *There is a strong statistical trend towards decreased platelet deposition at 10 min in GalTKO lungs ($P = 0.057$ vs. WT). Original magnification $\times 200$.

Fig. 7. Anti-Gal and non-Gal antibody levels. (A–C) Antibody binding to Gal-sufficient (Gal+, A, B) or Gal-deficient (GalTKO, C) PAEC cells was measured by flow cytometry before and after perfusion of a Gal-sufficient (WT, A) or Gal-deficient (GalTKO, B,C) pig lung. Serum dilution was 1:10 for Gal+ cells and 1:4 for GalTKO cells. Results were expressed as MFI (median fluorescence intensity), or normalized MFI (MFI corrected for total immunoglobulins levels as indicated in Methods). (D) Anti-non Gal antibody levels were measured in a complement-dependent cytotoxicity (CDC) assay using GalTKO peripheral blood mononuclear cells. Results were expressed as the percent lysis at 1:2 serum dilution. (E) Antibody levels to defined Gal and non-Gal carbohydrate antigens were measured by enzyme-linked immunosorbent assay and expressed as arbitrary units using a human serum pool as standard (1 A.U.). Arbitrary units were then normalized to total IgM or IgG levels as indicated in Methods. The percent change in antibody titer after lung perfusion for each condition is indicated as mean $\pm$ SD on each quadrant in panels A--D. The proportion of lungs showing decreased antibody levels is indicated in panel E. The legend indicates the individual GalTKO lungs subjected to ex vivo perfusion, ranked by increasing survival time (indicated in parenthesis), with assigned symbols and line characteristics.

Fig. 8. Release of cytokines and other inflammatory mediators. Fold change in pig (A) and human (B) cytokines and IL8 chemokine, human platelet and coagulation activation markers (C), and monocyte-associated mediators (sCD14) and human neutrophil-(MPO) (D) measured at the time of lung failure in the perfused plasma by chemiluminescent protein array. Results were expressed as fold increase relative to the concentration of target protein pre-perfusion after normalizing the final concentration to total protein levels as described in Methods. Each symbol represents one individual GalTKO lung subjected to ex vivo perfusion. IL, interleukin; TNF, tumor necrosis factor alpha; IFNG, interferon gamma; sCD40LG, soluble CD40 ligand; SELP, soluble P-selectin; VWF, von Willebrand factor; F3, tissue factor; MPO, myeloperoxidase; sCD14, soluble CD14. #Human assays from Panel C cross-react with pig targets. The legend indicates the individual GalTKO lung perfusion experiments, ranked by increasing survival time (indicated in parenthesis), with assigned symbols characteristics. The dashed line represents no change in protein levels.