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## Expression of human CD46 modulates inflammation associated with GalTKO lung xenograft injury

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### Abstract

Evaluation of lungs from GalTKO.hCD46 pigs, genetically modified to lack the galactose- $\alpha$ (1,3)-galactose epitope (GalTKO) and to express human CD46, a complement regulatory protein, has not previously been described. Physiologic, hematologic and biochemical parameters during perfusion with heparinized fresh human blood were measured for 33 GalTKO.hCD46, GalTKO (n=16), and wild type pig lungs (n=16), and 12 pig lungs perfused with autologous pig blood. Median GalTKO.hCD46 lung survival was 171 minutes compared to 120 for GalTKO (p=0.27) and 10 for wild type lungs (p<0.001). Complement activation, platelet activation, and histamine elaboration were significantly reduced during the first 2h of perfusion in GalTKO.hCD46 lungs compared to GalTKO ( C3a at 120' 812±230 vs. 1412±1047, p=0.02; CD62P at 120' 9.8±7.2 vs. 25.4±18.2, p<0.01; histamine at 60' 97±62 vs. 189±194, p=0.03). We conclude that, in addition to significant down-modulation of complement activation, hCD46 expression in GalTKO lungs diminished platelet and coagulation cascade activation, neutrophil sequestration and histamine release. Because GalTKO.hCD46 lung failure kinetics correlated directly with platelet and

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### Disclosure

Dr. Pierson serves on the Scientific Advisory Board of Revivacor.

### Author contributions

LB: concept/design, data analysis/interpretation, statistics, drafting article

TS: concept/design, data analysis/interpretation, approval of article

TZ: concept/design, approval of article

ER: concept/design, approval of article

AR: data analysis/interpretation, approval of article

CA: concept/design, approval of article

AL: concept/design, approval of article

XC: concept/design, approval of article

ES: concept/design, approval of article

GB: concept/design, approval of article

AN: concept/design, approval of article

CP: Providing of genetically modified pigs, approval of article

DA: Providing of genetically modified pigs, critical revision of article

AMA: concept/design, data analysis/interpretation, statistics, critical revision of article

RNP: concept/design, data analysis/interpretation, statistics, critical revision of article

neutrophil sequestration, coagulation cascade activation, and a rise in histamine levels within the first hour of perfusion, further progress will likely depend upon improved control of these pathways, by rationally targeted additional modifications to pigs and pharmacologic interventions.

## Keywords

lung; xenotransplantation; ex-vivo perfusion; complement; coagulation; platelet activation; leukocyte adhesion; GalTKO.hCD46

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## Introduction

Major advances have been achieved in survival and function of pig hearts, kidneys and liver xenografts in translational primate models based on genetic modifications to the pig (1–7). The same is true for cell and tissue xenotransplantation (8–10). Pig gene constructs tested to date include removal of the primary carbohydrate target on pig cells, galactose- $\alpha$ (1,3)-galactose, by “knockout” of the  $\alpha$ -galactosyl-transferase gene (GalTKO) or introduction of human complement regulatory proteins (hCRPs) such as human membrane cofactor protein (hMCP/hCD46) or human decay-accelerating factor (hDAF/hCD55). With either of these genetic modifications, the incidence of hyperacute rejection (HAR) and early graft failure (EGF) of xenogenic hearts, kidneys and livers is significantly reduced (11–12). In contrast, hCRP or GalTKO- expressing pig lung xenografts remain susceptible to injury (which we term hyperacute lung rejection, or HALR) within minutes to hours after ex vivo perfusion with human blood or and pig-to-baboon models (13–17).

Since GalTKO-transgenic cells are protected from preformed anti-Gal antibodies but can still be recognized by anti-non-Gal antibodies, in vitro experiments have proven that additional expression of hCRP adds further protection from xenogenic cell injury by inhibiting the complement cascade activation, triggered by antibody-antigen binding (18). Recent work from Westall et al. (19) demonstrated that GalTKO lungs expressing multiple genetic modifications (hCD55, hCD59) showed improved and sustained pulmonary function in a xenogenic perfusion model.

Here we test in a large experimental series how the GalTKO.hCD46 phenotype modulates HALR, and begin to reveal the mechanisms underlying GalTKO.hCD46 lung xenograft failure.

## Materials and Methods

### Animals

Genetically engineered pigs (BW 6–15 kg) lacking the alpha-Gal epitope (GalTKO) with or without human membrane cofactor protein (hCD46) were supplied by Revivicor (Blacksburg, VA). For GalTKO.hCD46 transgenic pigs, a human CD46 minigene construct, containing the endogenous hCD46 promoter and first two introns of genomic DNA fused to hCD46 coding sequence, was used to produce pigs with constitutive high level expression of the human complement inhibitor transgene (20). This hCD46 transgenic pig line was cross-

bred with homozygous GalTKO pigs (21) over more than 4 generations to produce a stable GalTKO.hCD46 line.

(The GalTKO lung results reported here were obtained using Revivicor pigs, and do not include previously reported results associated with GalTKO lungs evaluated in collaboration with MGH and Immerge Biotherapeutics Inc. (14–15)).

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine and were conducted in compliance with National Institutes of Health guidelines for the care and use of laboratory animals.

### Lung Harvest

Induction of anesthesia and surgical organ dissection was performed as previously described (14,18). Prior to flushing the lungs, 1-benzylimidazole (5 mg/kg BW; Sigma-Aldrich, St. Louis, MO, a thromboxane synthase inhibitor), and synthetic prostaglandin I<sub>2</sub> (0.03 mg/kg BW; Flolan; GlaxoSmithKline, Research Triangle Park, NC) were administered intravenously and allowed to circulate for several minutes.

### Lung Perfusion

The right and left lungs were separately perfused via the pulmonary artery using side-by-side circuits fashioned from silicon tubing and polyurethane connectors as previously described (13,22). Results associated with a variety of drug interventions to one lung of each pair will be reported separately. Pulmonary artery flow was measured and recorded with a flowmeter (Transonic Systems Inc., Model T206, Ithaca, NY). The Digimed System Integrator (Micro-Med, Louisville, KY) was used to provide a continuous measurement of both PA- and airway pressures via transducers integrated into the perfusion and ventilator circuits, respectively. Pulmonary vascular resistance (PVR) calculation and criteria defining “lung failure” prior to elective experimental termination at 4 hours have previously been described (14).

### Perfusate Preparation

**Xenogenic perfusions**—Approximately 450 ml of type A or O fresh whole blood was collected from two healthy human blood donors into a blood collection bag, containing 64 ml of CPDA-1. Two units (~240 ml each) of type-compatible thawed plasma were added for each unit of blood to obtain an initial perfusate volume of about two liters. Heparin (3 IU/ml blood, Heparin Sodium Injection, Sagent, Schaumburg, IL), calcium chloride (1.3 to 1.6 mg/ml blood, American Regent, Inc., Shirley, NY) to neutralize the CPDA chelating agent, and 8.4% sodium bicarbonate (~0.84 mg/ml blood, target pH of 7.4, Hospira, Inc., Lake Forest, IL) were added to the blood pool. All blood components were pooled and thoroughly mixed before circuit priming. Pre-perfusion hematocrit values were similar in all experimental groups (GalTKO.hCD46 16.3±0.4%; GalTKO 17.0±0.6%; WT 15.9±1.5%).

**Autologous perfusions**—One pint of blood was collected from the venous circulation by gravity drainage into a clinical blood collection bag over 5–15 minutes, with simultaneous peripheral volume replacement with crystalloid solution to minimize

hemodynamic perturbations prior to lung recovery. An equal volume (one pint) of saline was added to dilute the blood to a hematocrit of  $16.9 \pm 1.6\%$ . Finally, heparin, calcium chloride and  $\text{HCO}_3^-$  were added to the perfusate at similar dosages as described for the human blood preparation.

### Experimental groups

Human blood was perfused through GalTKO lungs (n=16) and GalTKO.hCD46 lungs (n=33). Historical and contemporaneous experiments using wild type (WT) pig lungs perfused with human blood (n=16, 10 of which were previously reported (14)) and pig lungs perfused with autologous pig blood (n=12, 10 of which were previously reported (14)) are reported for contextual analysis.

### Sampling regimen

Baseline blood samples were taken after the blood preparation (“pre” sample), and after circulating the blood in the perfusion circuit for at least 3 minutes (time 0 sample). Further samples were collected 5, 15, 30, 60, 120 and 240' after lung perfusion was initiated. All samples were stored at  $-70^\circ\text{C}$ .

Lung tissue samples were collected pre-perfusion and 10, 30, 60, 120 and 240' after perfusion was begun.

### Hematologic Analysis

Blood cell counts were enumerated by standard automated techniques (Antech Diagnostics, Rockville, MD) in blood samples collected in ethylenediaminetetraacetic acid (EDTA).

### Beta-thromboglobulin, Thrombin, Thromboxane B2 (TXB2), Histamine, and Complement Enzyme-Linked Immunosorbent Assays

Beta-thromboglobulin ( $\beta\text{TG}$ ) and prothrombin fragments 1+2 (F1+2) were measured by commercial enzyme-linked immunosorbent assay (ELISA) in plasma samples collected in CTAD tubes as previously described (14). EDTA plasma was used to measure histamine (ELISA kit Starfish, Gernusco, Italy) and C3a levels (14). Blood, collected at 3 time points (0', 15' and 60') in EDTA tubes containing 100  $\mu\text{l}$  (at 10  $\mu\text{g}/\text{ml}$ ) of meclofenamate (Sigma-Aldrich, St. Louis, MO), was analyzed to measure plasma levels of thromboxane (Thromboxane B2 EIA Kit, Cayman Chemical Company, Ann Arbor, MI; Catalog No. 519031).

### Measurement of Platelet Activation by flow cytometry

Blood samples were stained by monoclonal antibodies specific for CD41 (as a marker for platelets) (AbD Serotec, Raleigh, NC) and CD62P (expressed by activated platelets) (BD Pharmingen, San Diego, CA) and acquired on a FACSCalibur (BD Biosciences, San Jose, CA). Platelets and platelet aggregates, identified by size and by presence of CD41 staining, were analyzed for expression of CD62P by FACS analysis. Results were expressed as the percentage of CD62P-positive cells among CD41-positive cells.

## Measurement of Anti-non-Gal antibody levels by flow cytometry

IgM Antibody levels were measured in plasma samples collected before lung perfusion (“pre” sample) as previously described (14) with modifications.

## Histology and Immunochemistry

Lung biopsy specimens obtained during the perfusion and terminally samples were trisected, and processed as previously described (14). Frozen tissue sections from OCT-infused biopsy specimens (10' biopsies) were assessed by immunohistochemistry with monoclonal antibodies against human C5b-9 C5b-9at 1:50, rabbit anti-von Willebrand's factor antibody (both Dako, Carpinteria, CA), C4d (Quidel Corporation, San Diego, CA) at 1:50 and CD41 (Immunotech, Marseilles, France) at 1:100. The deposition of platelets and complement in lung tissue was quantified by staining intensity (from 0 to 3), taking into account both the extent (proportion of endothelial surface with detectable staining) and intensity of staining, relative to standard positive and negative “control” sections.

## GalTKO.hCD46 group analysis

The GalTKO.hCD46 lung cohorts was arbitrarily divided into three groups according to whether the lungs failed within 120 min (“early”), after 120 but before 240 min (“intermediate”), or did not meet failure criteria at four hours (“surviving” >240 min). The experimental groups was analyzed for complement cascade activation (C3a), thrombin generation (F1+2) and platelet activation ( $\beta$ TG, CD62P).

## Statistical analysis

Statistical analysis were performed as previously described (14) using a personal computer with the statistical package SigmaPlot for Windows (Version 11.0).

## Results

### Graft Survival and Function

Kaplan Meier survival analysis [Figure 1] shows that 8 of 12 pig lungs perfused with autologous blood reached elective termination after 240 minutes of perfusion. In contrast, as previously reported, all xenogenic-perfused lungs of wild type pigs failed within 36 minutes, in association with markedly elevated pulmonary vascular resistance. GalTKO lungs perfused with human blood survived for a median of 120 minutes (range 15–240 min) whereas organs from GalTKO.hCD46 animals had a median survival time (MST) of 171 minutes (range 5–240 min;  $p = 0.27$  vs. GalTKO). Three (of 16; 19%) of the GalTKO- and ten (of 33; 30%) of the GalTKO.hCD46 transgenic lungs survived to an elective termination. Although 4 of the 12 autologously-perfused lungs failed before reaching the elective termination time point, survival in this cohort was significantly prolonged compared to xenogenetically perfused groups ( $p = 0.04$  vs. GalTKO.hCD46). Gradual loss of pulmonary vascular barrier function, with tracheal edema, was the prevalent lung failure mode in this group. The lung failure mode associated with different lung phenotypes are listed in Table 1.

## Pulmonary Vascular Resistance

Autologously perfused pig lungs maintained very stable and low values over the time of perfusion whereas xenogenetic perfusion of wild type pig lungs led to a rapid extreme rise in PVR ( $284 \pm 50$  mmHg·min/L at 5') [Figure 2A]. Both GalTKO (+/-hCRP) groups showed an identical moderate rise in PVR to ~110–140 mmHg·min/L within the initial 30 minutes. At the subsequent measurement intervals, GalTKO.hCD46 transgenic lungs exhibited higher PVR values than with GalTKO organs, reaching statistical significance at 90 minutes of perfusion ( $p = 0.04$ ).

## Complement Activation

In contrast to the prolific elaboration of complement noted with wild type xenograft perfusion (C3a at 5'  $2019 \pm 377$  ng/ml), both GalTKO ( $63 \pm 29$ ,  $p < 0.001$ ) and GalTKO.hCD46 ( $33 \pm 10$ ,  $p < 0.001$ ) experimental groups exhibited statistically significantly reduced complement activation. C3a elaboration was not fully inhibited by the additional expression of hCD46, but was significantly reduced at 30, 60 and 120' time points when compared to the GalTKO group [Figure 2B].

## Platelet Sequestration and Activation

Platelet sequestration by GalTKO.hCD46 lungs was only significantly reduced at 5' when compared to GalTKO pig lung perfusions (% of initial platelets at 5'  $26 \pm 4$  vs.  $46 \pm 6$ ,  $p = 0.02$ ) [Figure 3A]. At later time points (1 to 4 h of perfusion) there was no statistical significant difference in platelet counts between GalTKO.hCD46 and GalTKO experiments. Activation of platelets measured by CD62P-expression on platelets was significantly decreased between 30 min and 2 h in the GalTKO.hCD46 group when compared to the GalTKO group (CD62 at 120'  $10.2 \pm 1.6$  vs.  $25.4 \pm 7.4$ ,  $p < 0.01$ ). In WT experiments, CD62P expression was significantly higher when compared to values of GalTKO.hCD46 lungs [Figure 3B].

Plasma thromboxane B2 levels remained lower in the GalTKO.hCD46 than in GalTKO group without reaching statistical significance ( $1919 \pm 483$  vs.  $4986 \pm 1736$  pg/mL,  $p = 0.17$ ) [Figure 3C]. Plasma beta-thromboglobulin ( $\beta$ TG) rose significantly within 5 minutes of perfusion in wild type lungs ( $\beta$ TG (IU/mL)  $1286 \pm 554$  vs. GalTKO  $144 \pm 36$ ,  $p = 0.01$  vs. GalTKO.hCD46  $62 \pm 15$ ,  $p < 0.001$ ). Although the initial rise of  $\beta$ TG level tended to be lower in GalTKO.hCD46 compared to GalTKO lungs during the subsequent hour (at 60':  $440 \pm 66$  vs.  $784 \pm 288$ ,  $p = 0.11$ ),  $\beta$ TG levels after 4 hours of perfusion were significantly lower in the three survivors among the GalTKO group when compared to ten survivors in the GalTKO.hCD46 group ( $483 \pm 54$  vs.  $1350 \pm 133$ ,  $p < 0.02$ ) [Figure 3D].

## Thrombin Formation

Relative to prolific, rapid thrombin generation with wild-type lungs at 30 min ( $F1+2$   $20.5 \pm 9$  nM), thrombin generation was significantly reduced in association with GalTKO ( $2.6 \pm 1.3$ ,  $p < 0.01$ ) or GalTKO.hCD46 lungs ( $2.1 \pm 0.4$ ,  $p < 0.001$ ) [Figure 4A]. Later in the experiment, GalTKO.hCD46 transgenic lungs showed reduced thrombin formation when compared to GalTKO lungs without reaching statistical significance ( $F1+2$  at 240':  $13.6 \pm 3.6$  vs.  $25.1 \pm 14.1$ ,  $p = 0.26$ ).

## Histamine levels

Histamine, as a marker for mast-cell driven innate immunologic response, showed a rapid increase in WT lungs at the 15' time point, significantly higher than in GalTKO.hCD46 lung perfusions ( $237\pm 113$  vs.  $49\pm 9$  nM,  $p < 0.01$ ). Levels in GalTKO experiments were higher than with GalTKO.hCD46, reaching statistical significance after one hour of perfusion ( $189\pm 61$  vs.  $97\pm 11$  nM,  $p < 0.03$ ) [Figure 4B]. Interestingly, the porcine blood, collected for the autologous perfusions, showed high histamine levels prior to the start of the perfusions whereas the human blood for the xenogenic perfusions had approximately 10-fold lower levels ( $96\pm 13$  vs.  $10\pm 1$  nM for GalTKO.hCD46).

## Leukocyte Sequestration

WT-, autologously-perfused- and GalTKO lungs all exhibited immediate neutrophils (PMN) sequestration with  $28\pm 13$ ,  $54\pm 22$  and  $45\pm 5$  percent of initial neutrophils remaining in the blood after 5' of perfusion, respectively. During the first half hour of perfusion, neutrophil counts in GalTKO.hCD46-lung perfusions showed significantly higher values when compared to control groups (% remaining at 30':  $59\pm 7$  vs. GalTKO  $26\pm 4$ ,  $p < 0.01$  vs. WT  $7\pm 3$ ,  $p < 0.01$ ) [Figure 5A]. At later time points there was no statistical difference in PMN sequestration between the groups.

Monocyte counts in GalTKO and WT experiments showed an immediate cell sequestration after perfusion initiation, whereas GalTKO.hCD46 lung perfusions demonstrated a cell number increase at 5' of perfusion ( $145\pm 31\%$  of initial monocyte counts) [Figure 5B]. Monocyte numbers showed no difference between the groups after 150' with about 50% of initial monocytes remaining in the perfusate; the relative contribution of pig and human monocytes was not enumerated in the course of the current work.

## Histology and Immunohistochemistry

WT lungs exhibited severe interstitial and alveolar hemorrhage that extended to the airway at failure, along with cellular infiltration and prevalent intravascular thrombosis [Figure 6B]. GalTKO lungs showed relatively preserved histology at 60', but infiltration by polymorphonuclear granulocytes, intravascular thrombosis and hemorrhage were prominent at lung failure [Figure 6D]. Only autologously-perfused and GalTKO.hCD46 lungs that had not met lung failure criteria showed normal microscopic anatomy with air-filled alveoli and thin inter-alveolar septae at an elective termination after 240' of perfusion [Figure 6F].

Classical pathway complement activation fragment C4d deposition in 10' biopsy samples showed the lowest scores in GalTKO.hCD46 tissue ( $0.94\pm 0.15$  vs. GalTKO  $1.3\pm 0.25$ ,  $p = 0.15$ ). C5b-9 membrane attack complex scores were very similar in GalTKO.hCD46 and GalTKO lungs but showed slightly lower values than measured in WT lungs (GalTKO.hCD46  $0.61\pm 0.11$  vs. WT  $1.0\pm 0.24$ ,  $p = 0.25$ ). CD41 staining, as a marker for platelet deposition, tended toward reduced intensity in GalTKO.hCD46 tissue ( $1.16\pm 0.23$  vs. GalTKO  $1.68\pm 0.32$ ,  $p = 0.19$ ) [Figure 7A–D].

### Anti-non-Gal antibody levels

Pre-perfusion anti-non-Gal IgM antibody levels were comparable in the perfusate used for GalTKO and GalTKO.hCD46 lung perfusions (GalTKO  $17.3 \pm 6.3$  vs. GalTKO.hCD46  $15.6 \pm 6.9$ ,  $p = 0.39$ ). Blood used for WT lung perfusions showed significantly lower anti-non-Gal antibody levels ( $9.2 \pm 2.1$ , vs. GalTKO.hCD46  $p < 0.01$ ). In neither group did the individual preformed antibody level correlate to survival time. C3a levels at 15' and 30' correlated in the GalTKO group to the level of anti-non-Gal antibody (at 15'  $r = 0.62$ ,  $p < 0.02$ ). This correlation was not significant in the GalTKO.hCD46 group (at 15'  $r = 0.28$ ,  $p = 0.16$ ).

### GalTKO.hCD46 group analysis

No difference in the kinetics of complement cascade activation (as C3a) was seen in association with early lung failure relative to longer-surviving lungs [Figure 8A]. Thrombin generation (F1+2 at 60'  $2.7 \pm 0.5$  vs.  $12.1 \pm 4.8$ ,  $p < 0.02$ ; at 120'  $5.9 \pm 1.1$  vs.  $14.0 \pm 1.8$ ,  $p < 0.02$ ) [Figure 8B] as well as platelet activation ( $\beta$ TG at 60'  $261 \pm 50$  vs.  $786 \pm 85$ ,  $p < 0.02$ ) [Figure 8C] was significantly lower in association with longer-surviving lungs when compared to lungs with short or intermediate survival times. CD62P-expression on platelets did not differ between groups [Figure 8D].

## Discussion

Previously published studies have demonstrated that wild type, hCRP, and GalTKO xenograft injury can be triggered or accelerated by anti-pig antibody (13–14,25–26), and implicate a combination of complement activation, coagulation cascade activation, and cellular adhesion and activation (27–29). The genetic modifications of adding a human complement pathway regulatory protein on the WT pig background or GalTKO alone led to a reduction of complement cascade activation and “survival” prolongation (14). Residual lung injury was attributed in part to binding of classical pathway complement activation triggered by anti-pig antibody (30–31) or anti-non-Gal antibody (14).

Membrane cofactor protein (CD46) catalyzes the degradation of C3b and C4b by Factor I, thereby inhibiting downstream complement pathway constituents (23–24) and should thus reduce GalTKO organ injury caused by the complement system. In support of this hypothesis, we find in our study that GalTKO lungs that additionally express human CD46 induced lower C3a generation in the first 2 hours of perfusion relative to GalTKO lungs.

We infer that the binding of preformed non-Gal antibodies to GalTKO xenograft, activating leukocytes and endothelial cells as well as the complement cascade, is presumably responsible for acute organ injury. The correlation between preformed non-GalT-antibody-levels and lung survival in the GalTKO, but not in the GalTKO.hCD46 group, suggests that the additional complement regulation by overexpression of hCD46 is effective to inhibit, if not prevent, lung injury caused by this mechanism.

Expression of hCD46 on GalTKO lungs modulates platelet activation, as CD41 deposition in the lung and CD62 expression on circulating platelets, and tends to lower thrombin generation. Since expression of human complement regulatory proteins is not thought to



directly inhibit clotting cascade amplification or the adhesion and activation of platelets, we infer that these are indirect effects through reduced complement-driven tissue and cell injury. For example, reduced complement activation may result in less vWF expression on endothelium and diminished vWF secretion into the blood and on circulating human platelets. Although the precise mechanism(s) is not defined by our studies to date, here we show that expression of hCRP on GalTKO organs confers anti-inflammatory, anti-coagulant, and anti-thrombotic effects that extend beyond the targeted pathway.

White blood cell and platelet sequestration is observable in all groups, but is greatly accelerated during pig lung perfusion by human blood. Platelet counts in autologous perfusions showed low-level sequestration with about 80% of initial platelets remaining in the perfusate after 2 hours of perfusion, illustrating the artifactual effects associated with the *ex vivo* lung perfusion model. In contrast, more than 50% of initial thrombocytes are sequestered within the first 15 minutes in xenogenic perfusions, demonstrating a drastic reaction to xenogenic stimuli. Neutrophil sequestration is associated with ischemia-reperfusion injury of allografts (32–33). We find a neutrophil sequestration in our autologously perfused lungs that is quantitatively similar to that observed in the GalTKO or WT lung perfusions. Interestingly, hCD46 expression on GalTKO led to a significant delay in neutrophil (and monocyte) sequestration when compared to other xenogenically- and autologously perfused experimental groups. We provisionally infer that complement activation plays an important role in cell sequestration in this early perfusion phase, as previously reported (34), and that it can be partly controlled by reduced complement activation seen in the context of genetic “overexpression” of complement regulatory molecules.

The significant rise in PVR in WT perfusions reflects one clinically important physiological consequence of the xenogenic immunologic reaction and the associated rapidly progressive lung injury. GalTKO and GalTKO.hCD46 lung perfusions generally do not exhibit as extreme PVR elevation. Although average PVR values among “survivors” in the GalTKO group remained lower than in surviving GalTKO.hCD46 lungs, this result is biased by the relatively low PVRs in 3 GalTKO lungs that survived to four hours. PVR data analysis of the GalTKO lungs revealed that several lungs were censored that just reached the PVR rejection criteria, reducing the average PVR for that group at subsequent time points. In contrast, a higher proportion of GalTKO.hCD46 organs remained in the “still-functioning” group by PVR (and all other) survival criteria, albeit with persistently high PVR, leading to the relatively elevated PVR values for the group.

Preliminary analysis suggests that “failure” of 4 out of 12 autologously-perfused lungs prior to elective termination was associated with surprisingly elevated pre-perfusion histamine-levels, which may adversely affect vascular barrier function.

GalTKO lungs expressing hCD46 exhibited better control of the complement cascade activation, as expected, but did not exhibit significantly prolonged survival compared to GalTKO lungs that do not express additional hCRP. As a hypothesis-generating strategy to begin to explain this observation, we compared biochemical indices of inflammation between three groups of GalTKP.CD46 lungs cohorted according to “survival time” of the

lung. The thrombin generation and platelet activation ( $\beta$ TG), but not complement activation, were significantly lower in association with longer-surviving lungs. We infer that the activation of platelets and the coagulation cascade in “non-surviving” lungs contributed importantly to the organ injury and determined outcome and survival time. In contrast, increased early complement activation (as C3a) was directly correlated with earlier graft failure for GalTKO lungs [Figure S1A]. GalTKO lungs that survived until the elective termination also showed a strong trend to reduced  $\beta$ TG levels when compared to earlier failing lungs [Figure S1C], suggesting to us that platelet activation is an important factor contributing to GalTKO lung injury, as for GalTKO.hCD46 lungs in our current report. Thus this post-hoc analysis of intra-group variability in outcome, comparing groups cohorted by arbitrarily selected survival duration intervals, and putative mechanistic indices of specific pathways associated with lung xenograft injury revealed expected (GalTKO) and hypothesis-generating (GalTKO.hCD46) data regarding what pathways remain important to progress toward clinically useful lung xenograft performance.

One limitation to our study is that we did not also study CD46 modification by itself. Thus, we have not formally excluded the possibility that the hCD46 construct used here might alone account for the observed effects. Previous work shows that hCD46 (hMCP) or hCD55 (hDAF) (17–18,35) exhibit incomplete protection, similar to the GalTKO results reported here. Based on two decades of work by us and many others, the GalTKO modification is widely viewed by scientists and regulators as an essential component of the pig genetic background needed for proceeding to clinical application most safely, while minimizing recipient immunosuppressive requirements. Repeating the extensive prior work by us and others with hCRP transgenic pig lungs was judged scientifically unnecessary to address our primary objectives: to determine whether hCD46 protects GalTKO lung from complement-driven injury; and to describe its influence on other HALR mechanisms.

In conclusion, GalTKO.hCD46 lungs exhibit biochemically significant and physiologically important protection from antibody- and complement-mediated HALR. Our data and analysis support our working hypothesis is that coagulation- and platelet-driven mechanisms are the principle mediators of PVR elevation and injury of GalTKO.hCD46 lungs in our model, although additional roles for residual complement activation, pulmonary intravascular macrophage activation, and human leukocytes (neutrophils, monocytes, NK cells) remain to be formally evaluated. We predict that efficient control of one or more among several known molecular incompatibilities regulating coagulation and platelet and leukocyte adhesion (37–44) will prove necessary and sufficient to prevent these well-described HALR phenomena, and yield consistent “survival” to four hours in this model, as well as life-supporting function of pig lungs in baboons and, eventually, in man.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## List of non-standardized abbreviations

$\beta$ TG                       $\beta$ -thromboglobulin

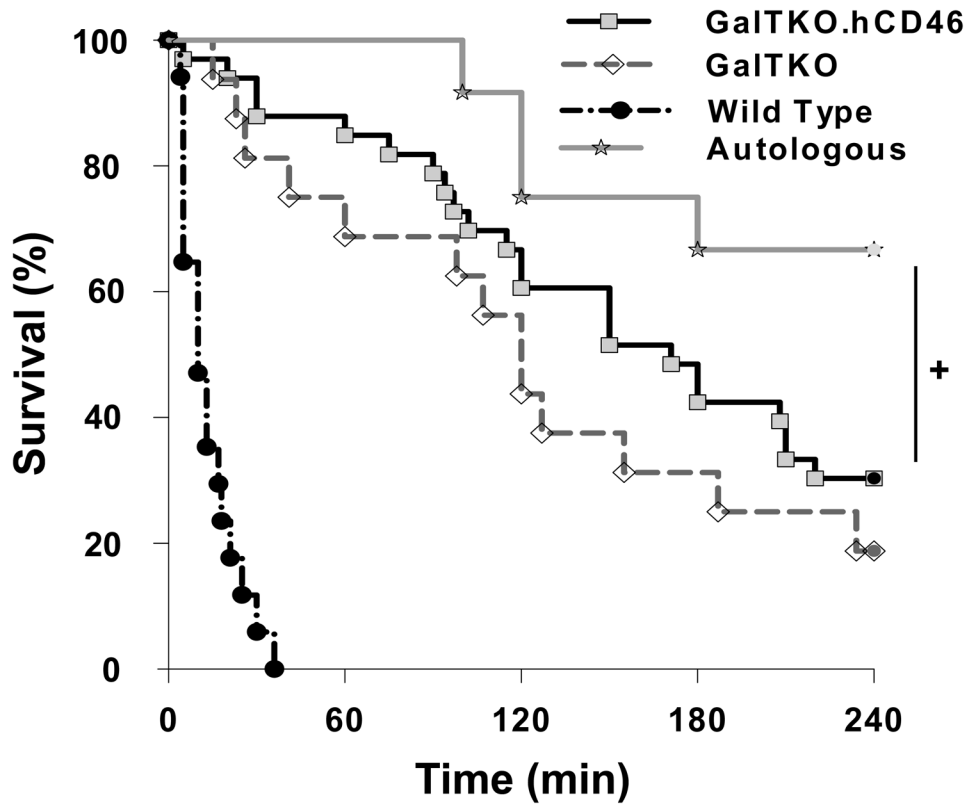
<b>CD62P</b>	P-selectin
<b>EGF</b>	early graft failure
<b>ELISA</b>	enzyme-linked immunosorbent assay
<b>F1+2</b>	Prothrombin fragments 1 + 2
<b>FACS</b>	Fluorescence-activated cell sorting
<b>GalTKO</b>	$\alpha$ 1,3-galactosyl transferase knockout
<b>HAR</b>	hyperacute rejection
<b>HALR</b>	hyperacute lung rejection
<b>hCD46</b>	human membrane cofactor protein, hMCP
<b>hCRP</b>	human complement regulatory protein
<b>hDAF</b>	human decay-accelerating factor, hCD55
<b>hMCP</b>	human membrane cofactor protein, hCD46
<b>MST</b>	Median survival time
<b>OCT</b>	optimal cutting temperature
<b>PMN</b>	polymorphonuclear leukocyte
<b>PVR</b>	pulmonary vascular resistance
<b>vWF</b>	von Willebrand Factor
<b>WT</b>	Wild type

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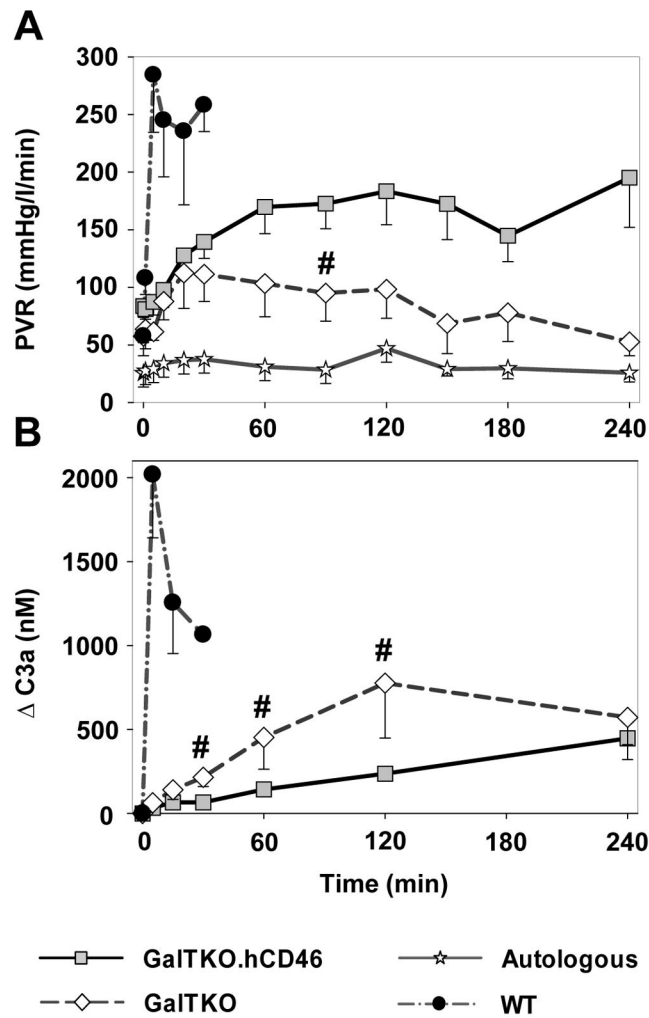
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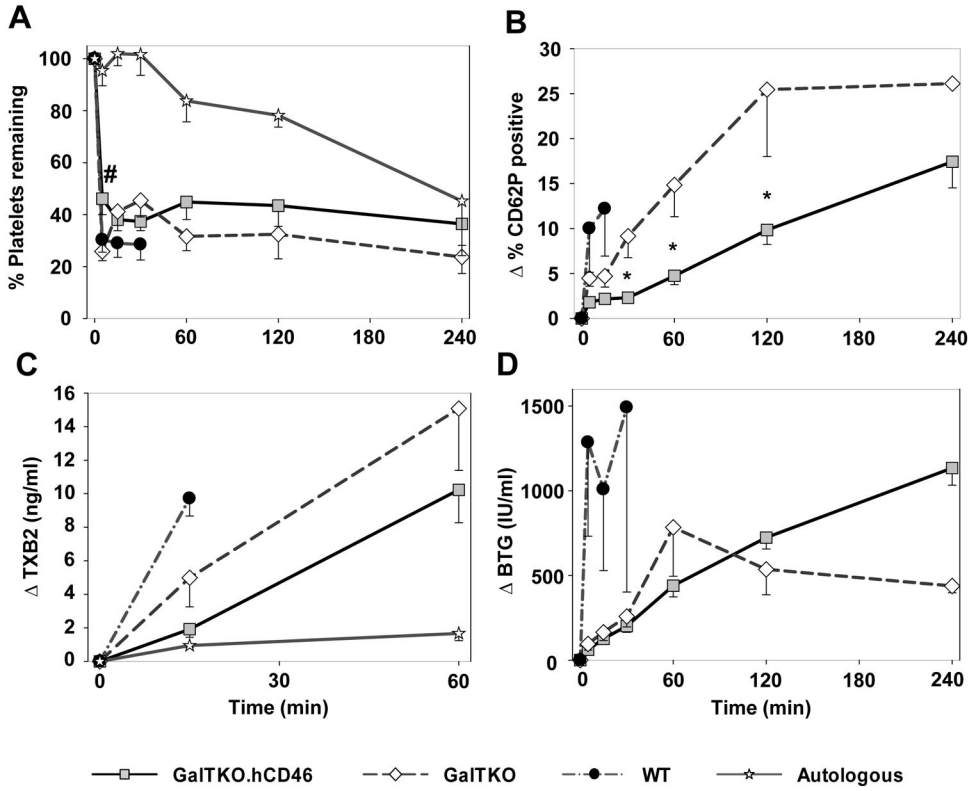


**Figure 1.**

Cumulative survival time of ex vivo perfused lungs. Lung “survival time” represents the time of perfusion at which a lung failure endpoint was met, based on the pre-defined criteria. Lung survival was significantly prolonged in GalTKO.hCD46 lungs compared to wild type ( $p < 0.001$ ) but not compared to GalTKO lungs ( $p = 0.27$ ). Autologously perfused lungs survived significantly longer than GalTKO.hCD46 transgenic lungs (+  $p = 0.04$ ).

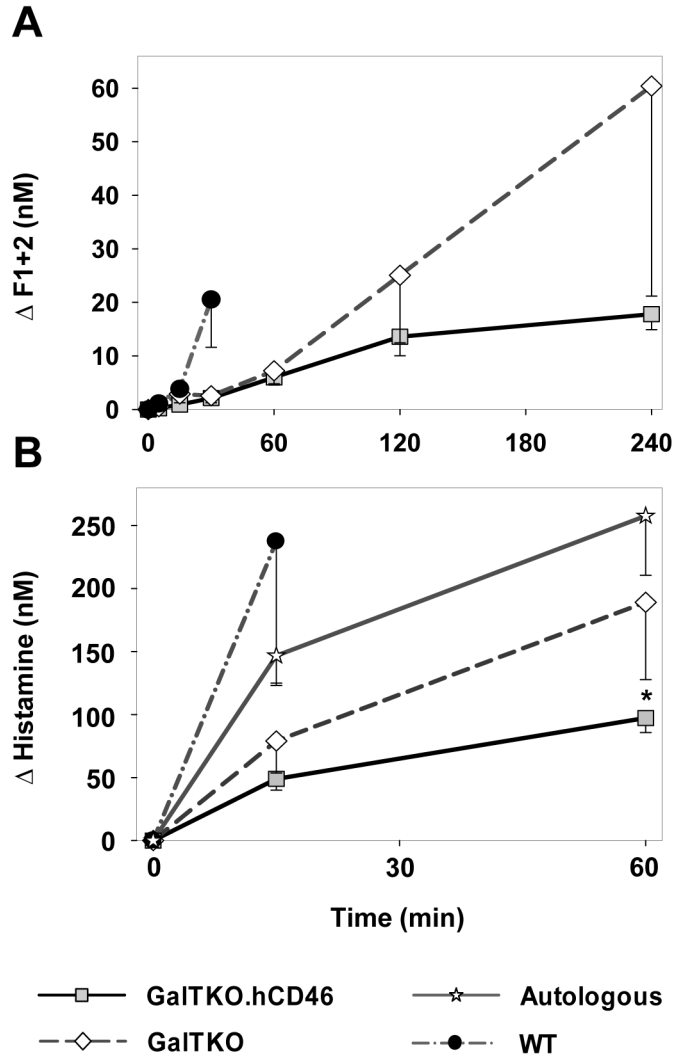


**Figure 2.** Pulmonary vascular resistance (PVR) and plasma levels of complement activation byproduct (C3a). (A) PVR expressed as a function of time for control and study groups. Time 0 represents measurements obtained during the first minute of lung perfusion. PVR after 4 hours of perfusion initiation was significantly higher in GalTKO.hCD46 lungs than GalTKO lungs (#  $p < 0.05$  for GalTKO.hCD46 vs. GalTKO). (B) Plasma C3a levels expressed as the amount of complement fragments produced above the pre-perfusion baseline. Complement activation was significantly lower in GalTKO pig lungs expressing hCD46 between 30 and 120 min of perfusion (#  $p < 0.03$  for GalTKO.hCD46 vs. GalTKO). All data are shown as the mean  $\pm$  SEM of all surviving experiments.

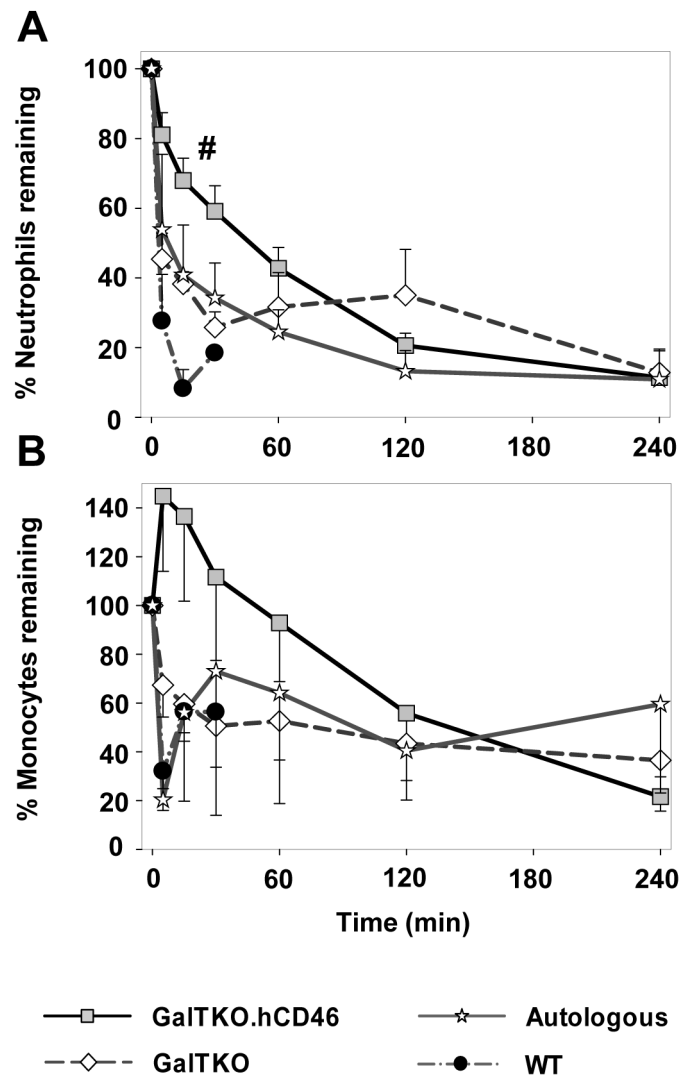


**Figure 3.** Platelet and coagulation cascade activation profile. **(A)** Blood platelet counts measured serially by automated counting. Platelet sequestration was expressed as the percentage of platelets remaining in the perfusate at each time point. # at 5 min,  $p = 0.02$  for GalTKO.hCD46 vs. GalTKO. **(B)** Platelet activation assessed by measuring the proportion of CD41+ platelets expressing CD62P by flow cytometry. \*  $p < 0.01$  for GalTKO.hCD46 vs. GalTKO. **(C)** Platelet activation assessed by plasma Thromboxane B2 levels. **(D)** Platelet activation assessed by plasma levels of  $\beta$ TG released from platelet granules. All values are shown as the mean  $\pm$  SEM of surviving experiments.

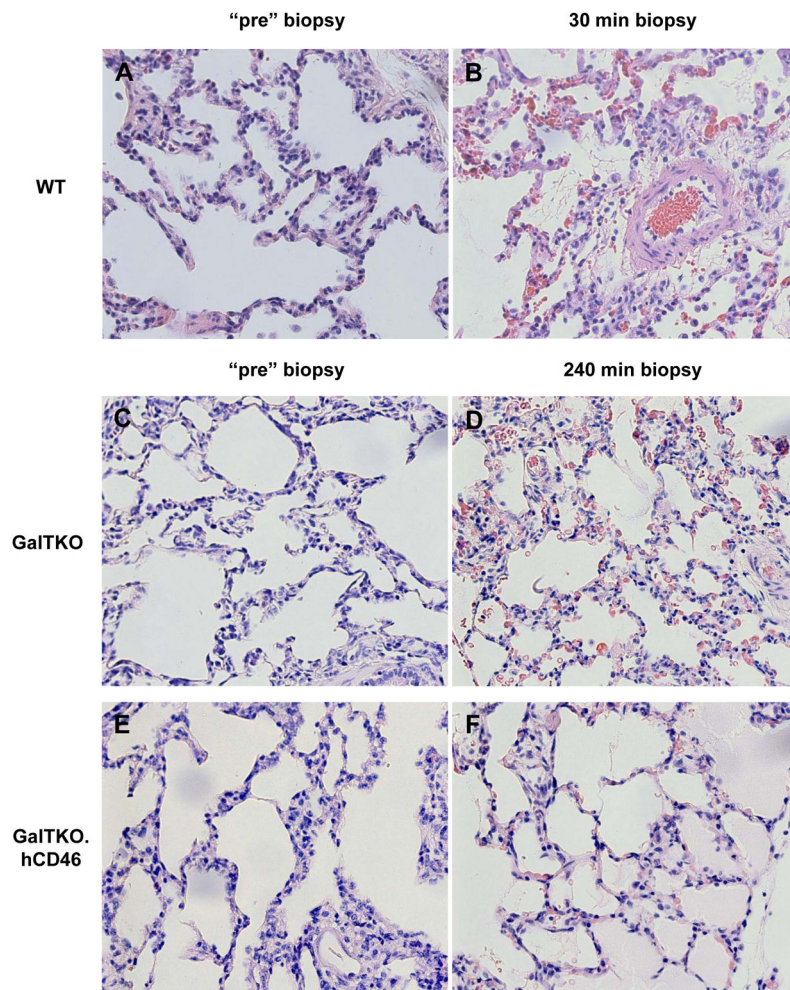




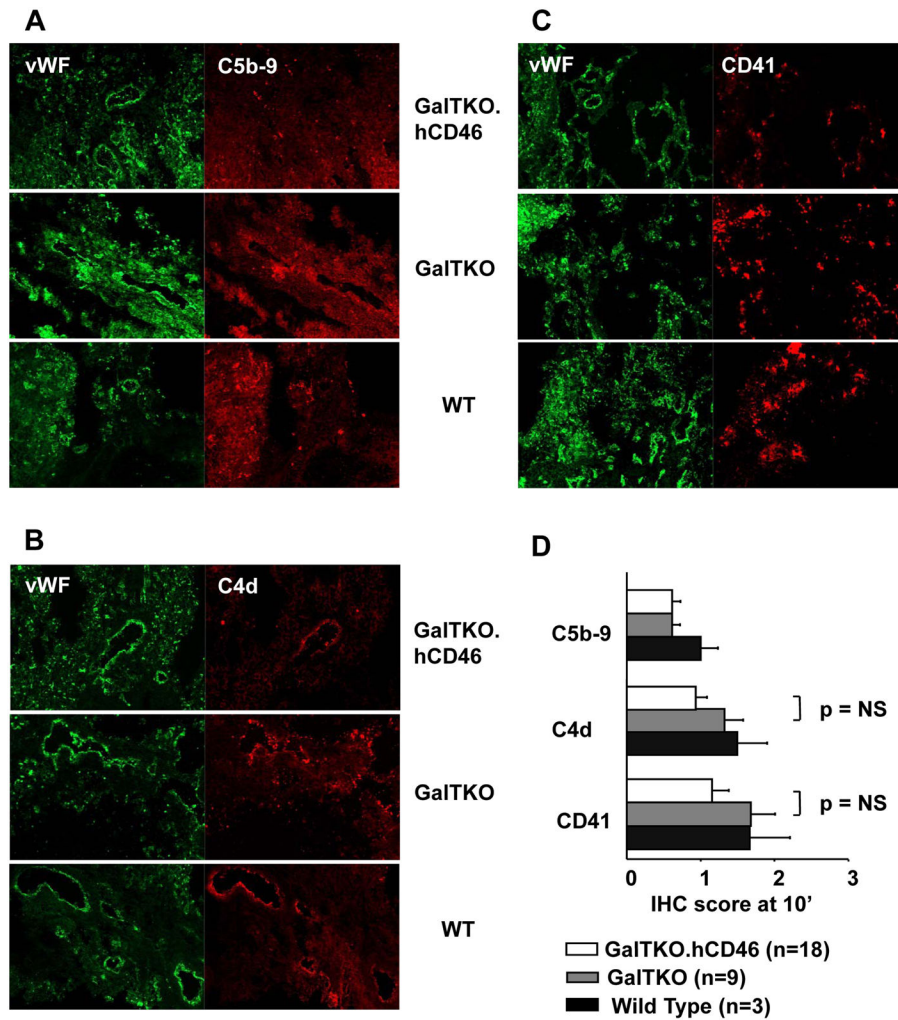
**Figure 4.** (A) Activation of the coagulation cascade was detected by the formation of thrombin measured by plasma levels of fragments F1+2. (B) Release of histamine was measured in blood samples collected during the first hour of perfusion. Histamine levels at 1h of perfusion reached significantly lower values in the GalTKO.hCD46 when compared to GalTKO group (\* p < 0.03). All data are expressed as change from the baseline and shown as the mean  $\pm$  SEM of surviving experiments.



**Figure 5.** 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. #  $p < 0.02$  GalTKO.hCD46 vs. GalTKO between 5 and 30 min.



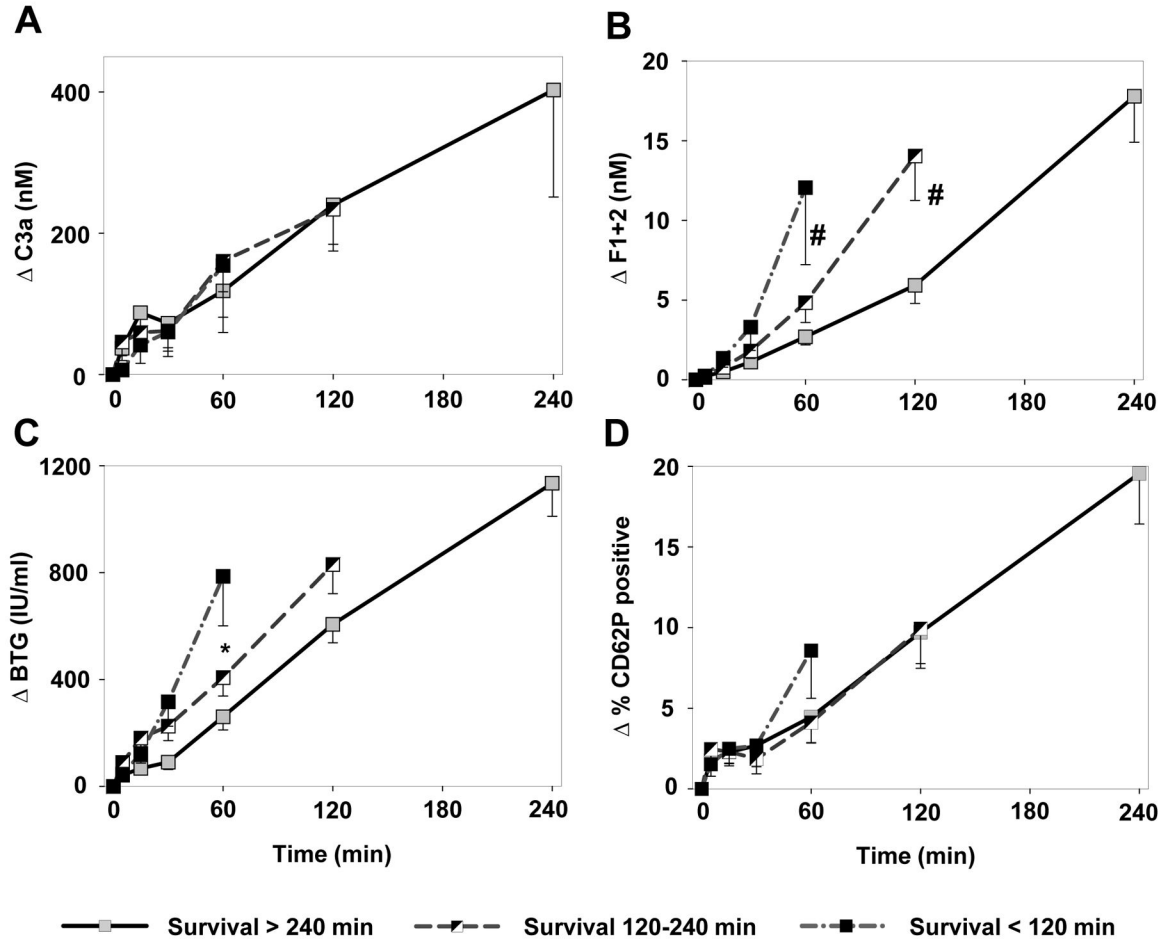
**Figure 6.** Pre-perfusion biopsies of Wild type (**A**), GalTKO (**C**) and GalTKO.hCD46 (**E**) show intact lung tissue. GalTKO.hCD46 lung tissue showed well preserved microscopic anatomy with air-filled alveoli and thin inter-alveolar septae and only little hemorrhage after 4 hours of xenogenic perfusion (**F**) whereas GalTKO biopsies (from a still functioning lung) exhibited infiltration by polymorphonuclear granulocytes, intravascular thrombosis and diffuse hemorrhage at the same time point (**D**). Wild type lung biopsies showed these findings already after 30 minutes of perfusion (**B**). H&E, 40X



**Figure 7.**

Two-color immunocytochemistry analysis of tissues from GalTKO.hCD46 and control perfused pig lungs. Pig lung tissue collected at identical time points (10 min after initiation of perfusion) was stained for von Willebrand Factor (green), identifying endothelium, and C5b-9 (A), C4d (B) and CD41 (C) (red). (D) Immunocytochemistry findings scored as the extent of tissue deposition as indicated in methods. GalTKO.hCD46 lungs showed non-significantly reduced C4d ( $0.9 \pm 0.1$  vs. GalTKO  $1.3 \pm 0.2$ ,  $p = 0.15$ ) and CD41 deposition ( $1.1 \pm 0.2$  vs. GalTKO  $1.7 \pm 0.3$ ,  $p = 0.19$  vs. GalTKO) in lung biopsies collected at 10 min when compared to GalTKO or Wild Type lungs. C5b-9 IHC-score was similar in GalTKO.hCD46 and GalTKO lungs.

### GalTKO.hCD46 lungs



**Figure 8.**

Analysis of complement and coagulation cascade and platelet activation within the GalTKO.hCD46 group. Perfusions were divided in experiments where lung failure occurred before 2 hours of perfusion (Survival < 120 min), between 2 and 4 hours (Survival 120–240 min) and experiments that were terminated electively at 4 hours (Survival > 240 min). **(A)** C3a levels in the groups were similar. There was no correlation between survival time and complement cascade activation. **(B)** Lungs that failed before the 2 hour time point and lungs that failed between 2 and 4 hours of perfusion showed significantly higher thrombin generation (# p < 0.02) when compared to surviving lungs at 1h and 2 h time points, respectively. **(C)** BTG levels at 1 h were significantly reduced in surviving lungs when compared to lungs failing before 2 hours (\* p < 0.01). **(D)** P-Selectin expression was identical in lungs surviving longer than 2 hours of perfusion. Experiments being terminated before the 2 hour mark showed increased P-Selectin values.

**Table 1**

Summary of lung failure mechanisms in GalTKO.hCD46 and control lungs. Lungs from Wild Type pigs failed almost all due to a loss of blood flow through the lungs (elevated PVR) whereas this mechanism is only responsible for about 50% of GalTKO.hCD46 lung failures. The development of trachea edema could be identified as the predominant reason for organ failure within the autologously perfused group.

Rejection reason	Experimental group n/% of failed lungs			
	GalTKO.hCD46	GalTKO	Wild type	Autologous
Trachea edema	7/30%	2/15%	2/12%	3/75%
Loss of perfusate	2/9%	2/15%	-	1/25%
Loss of blood flow	12/52%	7/54%	15/88%	-
Oxygenation failure	2/9%	2/15%	-	-