Published in final edited form as:

Xenotransplantation. 2018 March ; 25(2): e12385. doi:10.1111/xen.12385.

Interleukin-8 mediates neutrophil-endothelial interactions in pig-to-human xenogeneic models

Beth M. French¹, Selin Sendil¹, Krishna Mohan Sepuru², Jolene Ranek¹, Lars Burdorf¹, Donald Harris¹, Emily Redding¹, Xiangfei Cheng¹, Christopher Laird¹, Yuming Zhao¹, Benjamin Cerel¹, Krishna Rajarathnam², Richard N Pierson III, MD¹, and Agnes M. Azimzadeh^{1,#}

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

²Department of Biochemistry and Molecular Biology, Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555

Abstract

Background—Human neutrophils are sequestered by pig lung xenografts within minutes during *ex vivo* perfusion. This phenomenon is not prevented by pig genetic modifications that remove xeno-antigens or added human regulatory molecules intended to down-regulate activation of complement and coagulation pathways. The present study investigated whether recipient and donor interleukin-8 (IL-8), a chemokine known to attract and activate neutrophils during inflammation, is elaborated in the context of xenogeneic injury, and whether human or pig IL-8 promote the adhesion of human neutrophils in *in vitro* xenograft models.

Methods—Plasma levels of pig, human, or non-human primate (NHP) IL-8 from *ex vivo* pig lung perfusion experiments (n=10) and in-vivo pig-to-baboon lung transplantation in baboons (n=22) were analyzed by ELISA or Luminex. Human neutrophils stimulated with human or pig IL-8 were analyzed for CD11b expression, CD18 activation, oxidative burst, and adhesion to resting or TNF-activated endothelial cells (EC) evaluated under static and flow (Bioflux) conditions. For some experiments, human neutrophils were incubated with Reparixin (IL-8/CXCL8 receptor blocker) and then analyzed as in the *in vitro* experiments mentioned above.

Results—Plasma levels of pig IL-8 (~6,113 pg/ml) increased more than human (~1,235 pg/ml) between one and four hours after initiation of *ex vivo* lung perfusion. However, pig IL-8 levels remained consistently low (<60 pg/ml) and NHP IL-8 plasma levels increased by ~2,000 pg/ml after four hours in a pig-to-baboon lung xenotransplantation. In vitro, human neutrophils' CD11b expression, CD18 activation, and oxidative burst all increased in a dose dependent manner following exposure to either pig or human IL-8, which also were associated with increased

#Address correspondence to: Agnes Azimzadeh, PhD, Department of Surgery, University of Maryland Baltimore, 10 South Pine Street, MSTF Building, 434C, Baltimore, MD 21201, Phone: 410.706.0594, Fax: 410.706.0311, aazimzadeh@som.umaryland.edu. MISS BETH M FRENCH (Orcid ID : 0000-0002-6493-318X) DR. CHRISTOPHER T LAIRD (Orcid ID : 0000-0002-0733-8720)

Author contributions

Concept/design: AMA, BMF, KR, RNP; Data analysis/interpretation: BMF, AMA, LB, RNP, KR; Drafting article: BMF, AMA, RNP, SS, KR; Critical revision of article: BMF, AMA, RNP, KR; Approval of article: all; Statistics: BMF, AMA, LB; Funding secured by: RNP, AMA, KR; Data collection: BMF, SS, KMS, JR, LB, DH, ER, XC, CL, YZ, BC, AMA.

adhesion to EC in both static and flow conditions. Reparixin inhibited human neutrophil activation by both pig and human IL-8 in a dose-dependent fashion. At 0.1mg/ml, Reparixin inhibited the adhesion of IL-8-activated human neutrophils to pAECs by $84\pm 2.5\%$.

Conclusions—Pig IL-8 increased in an *ex vivo* model of pig-to-human lung xenotransplantation but is not detected *in vivo*, whereas human or NHP IL-8 is elevated to a similar degree in both models. Both pig and human IL-8 activate human neutrophils and increase their adhesion to pig aortic ECs, a process significantly inhibited by the addition of Reparixin to human neutrophils. This work implicates IL-8, whether of pig or human origin, as a possible factor mediating in lung xenograft inflammation and injury and supports the evaluation of therapeutic targeting of this pathway in the context of xenotransplantation.

Keywords

Xenotransplantation; neutrophils; interleukin-8; chemokines; cell adhesion; endothelial cells; lung

Introduction

The shortage of human organ donors remains a major limitation constraining the therapeutic potential in the transplantation field. Xenotransplantation, and in particular, the use of pig organs for human transplantation, is an attractive potential solution. Control of antibody- and complement-dependent rejection has been improved with $\alpha 1,3$ galactosyltransferase knockout (GalTKO) and transgenic human complement regulatory protein (such as CD46 or CD55) expression in genetically engineered (GE) pigs. However, despite these modifications, inflammation limits overall survival in preclinical large animal xenograft models¹, and lung xenografts prominently feature very rapid neutrophil sequestration^{2,3,4}.

Neutrophil adhesion and transendothelial migration have been well characterized in the context of inflammatory diseases and tissue injury from various causes including ischemia-reperfusion injury⁵. Classically, neutrophil adhesion to endothelial cells involves three discrete phases: rolling, firm attachment (arrest) and transcellular or paracellular migration. Rolling is mediated primarily by selectins, while arrest and transmigration both require activated CD18 integrins².

Interleukin 8 (IL-8, also known as CXCL8) is a potent pro-inflammatory chemokine that plays a key role in the recruitment and activation of neutrophils during inflammation in a variety of contexts^{6,7}. IL-8 gradients attract neutrophils towards sites of injury or infection, while also activating neutrophil integrins and increasing their selectin expression⁷. Neutrophil recruitment out of the blood stream into inflamed tissue is specifically dependent upon the expression, activation, and binding of the $\beta 2$ integrin family members LFA-1 (Lymphocyte function-associated antigen 1) and Macrophage-1 antigen (Mac-1, CD11b/CD18) which bind to intercellular adhesion molecule (ICAM-1). IL-8 enhances neutrophil recruitment by stimulating amplified expression of $\beta 2$ integrin complexes on neutrophils. In addition, IL-8 triggers $\beta 2$ integrin-mediated arrest of rolling neutrophils *in vivo* by stimulating increased expression of CD18 on the cell surface. Remarkably, neutrophil-endothelial cell adhesion receptor pair interactions generally function physiologically across the human-pig species barrier^{8,9,10}.

IL-8 bioactivity is mediated through two G-protein-coupled receptors, named CXCR1 and CXCR2. CXCR1 is used almost exclusively by IL-8 whereas CXCR2 is used by nearly all CXC chemokines¹¹. CXCR2 responds to low concentrations of IL-8 and is principally associated with chemotaxis and MMP-9 release. CXCR1, in contrast, responds to high concentrations of IL-8 and is associated with respiratory burst and phospholipase D2 activation. Thus, CXCR2 ligation induces leukocyte adhesion to activated vascular endothelium and migration to sites of inflammation, while CXCR1 ligation primes neutrophil antimicrobial activity¹². Under physiological conditions, IL-8 could exist as monomers, dimers, or a mixture of monomers and dimers. Therefore, both forms of IL-8 could interact with CXCR1 and CXCR2 with different affinities and potencies to mediate different cellular responses. Activation of both CXCR1 and CXCR2 can be inhibited by the noncompetitive allosteric inhibitor Reparixin ($IC_{50s} = 1$ and 100 nM, respectively)¹³.

Endothelial cell activation and neutrophil infiltration are features consistently associated with xenograft rejection between “discordant” species, which are typically characterized by presence of preformed ‘natural’ antibodies in the recipient’s blood that bind to donor antigens¹⁴. In vitro studies using endothelial cells from wild type (WT) pigs demonstrated that human serum, as a source of xeno-reactive natural antibodies and complement, induced endothelial cell activation with consequent neutrophil adhesion under static¹⁴ and flow^{15,16} conditions. Human neutrophils also directly recognize xenogeneic endothelium, independent of xeno-reactive natural antibody and complement. The reason behind this is not fully understood^{14,17,18}. Pig IL-8 mediates pig-to-human xenogeneic neutrophil interactions based on observations using WT pig endothelial cell supernatants in chemotaxis and intra-cellular calcium assays¹⁹. Interestingly, baboon IL-8 levels are elevated after pig-to-baboon artery patch transplantation, but not after pig heart or kidney organ xenotransplantation²⁰.

Sequestration of human neutrophils is a prominent finding after *ex vivo* perfusion of pig lungs²¹, and is not prevented despite multiple genetic modifications²². Here we evaluate the hypothesis that IL-8 elaboration is associated with, and potentially mediates, neutrophil sequestration in several pig-to-human lung xenotransplant models. We report that the chemokine IL-8, of primate (human or baboon) and pig origin, is elaborated in two different xenogeneic lung models. In addition, demonstrate the influence of human or pig IL-8 on CD11b expression, CD18 activation and oxidative burst by human neutrophils, and on adhesion of human neutrophils to pig and human endothelial cells under static and to pig aortic endothelial cells (pAECS) under flow conditions. Finally, we found that treatment of human neutrophils with the IL-8 receptor antagonist Reparixin prevents increased activation and adhesion to pAECs after human or pig IL-8 treatment. Our results suggest an important role for IL-8 during xenogeneic lung injury, one which has not previously been studied.

Materials and Methods

Animals

GE pigs lacking the alpha-Gal epitope and expressing human membrane cofactor protein (hCD46) (GalTKO.hCD46), additionally expressing the human (h) complement decay-accelerating factor protein (hCD55), human thrombomodulin (hTBM), tissue factor pathway inhibitor (hTFPI), endothelial protein C receptor (hEPCR), heme oxygenase 1 (HO-1),

integrin associated protein (hCD47), ecto-nucleoside triphosphate diphosphohydrolase (hCD39) or Tumor necrosis factor, alpha-induced protein 3 (A20) were provided by Revivicor (Blacksburg, VA, USA). Pigs of either gender were 5 to 10 weeks of age. Transgene expression in the lung was evaluated by immunohistochemistry and is documented in Table 1. All procedures were approved by The Institutional Animal Care and Use Committee (IACUC) at the University of Maryland, School of Medicine, and were conducted in compliance with The National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Lung harvest, *ex vivo* perfusion, and *in vivo* transplantation procedures

Anesthesia protocol and the harvest of the lung xenograft were performed as previously described²³. In brief, prior to flushing the lungs, heparin (500U/kg, MWI, Boise, ID), 1-benzylimidazole (BIA, 5 mg/kg BW; a thromboxane synthase inhibitor; Sigma-Aldrich, St. Louis, MO) and synthetic prostaglandin I₂ (Remodulin, 0.06mg/kg, United Therapeutics, Silver Spring, MD) were administered intravenously and allowed to circulate for several minutes. After procurement, the right and left lungs were surgically divided and perfused separately via the pulmonary artery using side-by-side circuits fashioned from silicon tubing and polyurethane connectors as previously described²³. GalTKO.hCD46 (n=5) and GalTKO.hCD46.hCD55 (n=5) transgenic pig lungs were perfused with heparinized (3U/ml perfusate) fresh human blood in an *ex vivo* circuit as previously described^{2,21,23}. Surgical aspects of the auto- (n=1), allo- (n=2) (non-xeno controls) and xeno-lung (n=19) *in vivo* transplantation procedures in baboons have been reported in detail²³. Xeno-lung *in vivo* transplantation utilized pig lungs that are genetically modified, either for 3-genes (GalTKO.hCD46.hTBM, n=2; GalTKO.hCD46.hEPCR, n=5) or for four added genes (including hCD55, hTBM, hTFPI, hEPCR, HO-1, hCD47, and hCD39 in various combinations n=6, seen in Table 1), all on the GalTKO.hCD46 background. Methylprednisolone (Solumedrol, starting at 10mg/kg and then tapered down to 0.5 mg/kg, MWI, Boise, ID), C1 esterase inhibitor (500IU, CSL Behring, King of Prussia, PA), and αGPIb (1mg/kg BW, University of Leuven, Leuven, Belgium) were given to all *in vivo* lung recipients. In later experiments, famotidine (anti-H2 histamine receptor antagonist; 0.5mg/kg, MWI, Boise, ID) and 1-Benzylimidazole (5 mg/kg BW, Sigma Aldrich, St Louis, MO) were also given to the recipients (Table 1). For all *in vivo* experiments, organ donor pigs were given Desmopressin (DDAVP, Diamond Back Drugs, Scottsdale, AZ) at 3μg/kg the day before surgery and on the day of surgery.

Hematologic analysis

Human blood perfusate and baboon blood samples were collected at protocol-driven time points into standard clinical blood collection tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulation. Complete blood cell count (CBC) measurements were measured using a clinical veterinary automated hemocytometer (Antech Diagnostics, Rockville, MD, USA), and values (except cell differential counts) independently corroborated using an in-house hemocytometer (Hemavet, Drew Scientific, Miami Lakes, FL). In two lung transplantation experiments (one autologous and one allogeneic non-xenogeneic control lung transplantations), total white blood cell counts were used as proxy

for neutrophil counts since the leukocyte differential was not reported from the clinical laboratory.

Quantification of IL-8 in plasma

Perfusion blood perfusate and baboon blood samples were collected at protocol-driven time points in EDTA blood tubes (Becton Dickinson, Franklin Lakes, NJ), centrifuged at 2,500 rpm for 15 min at 4°C and plasma samples were archived at -80 °C until analysis. Plasma levels of pig and human IL-8 were measured by ELISA using pig (R&D systems, cat. P8000) and human (ThermoScientific, cat. CHC1303) specific kits. ELISA plates were read on a Spectramax 3000. Plasma levels of baboon IL-8 were measured by Luminex using a MILLIPLEX MAP Non-Human Primate Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore Corporation, MA, USA) using a Luminex 100 analyzer (Luminex, Austin, Texas, USA) at the University of Maryland cytokine core laboratory.

Real-time RT-PCR

Total RNA was extracted from lung tissue before ("Pre-perfusion") and after 30 min and 4 hours of ex-vivo perfusion from untreated GalTKO.hCD46 lungs (n = 7) using the Qiagen (Valencia, CA) RNA isolation kit (RNeasy Mini kit) according to the manufacturer's protocol. RNA purity was established with the 260/280-nm absorption ratio. 2 µg total RNA was reverse-transcribed using Superscript IV VILO MasterMix (ThermoFisher, Halethorpe, MD). The resulting cDNA (20 ng) was amplified by using real-time PCR using ABI Prism 7900HT cycler using primers specific for pig IL-8, human IL-8 and pL32. Relative gene expression was calculated using the C_t method (C_t gene of interest- C_t housekeeping gene (pL32)), where C_t refers to the cycle number at which the PCR product for a particular gene is detected. The housekeeping gene, pRPL32, was used as an internal control.

Cloning, expression, and purification of recombinant IL-8

Pig IL-8 was expressed as a fusion protein from the plasmid vector pET32 Xa/LIC containing the pig IL-8 gene cloned at the ligation-independent cloning site. The plasmid was transformed into *Escherichia coli* BL(21)DE3 cells (Invitrogen). Transformants were grown in Luria broth (LB) media to an optimal A_{600} of 0.6 and induced with 0.25 mM IPTG for 16 h at 25°C. The cells were spun down and lysed by four freeze-thaw cycles followed by sonication. The supernatant containing the fusion protein was purified using a nickel-nitrilotriacetic acid column (Qiagen), dialyzed against a pH 7.4 buffer containing 50 mM Tris, 50 mM sodium chloride, and 2 mM calcium chloride, and cleaved using Factor Xa (Novagen, Madison, WI). The cleaved protein was purified using reverse-phase high-performance liquid chromatography, and fractions were checked for purity by SDS-PAGE and MALDI-TOF. We also measured the NMR spectrum to ensure that the protein is properly folded.^{24,25} (Data not shown). The protein fractions were lyophilized and stored at -20 °C until further use. Human IL-8 was expressed and purified essentially as described for pig IL-8. Both human and pig IL-8 used in our experiments came from a single batch. Purity of both chemokines was confirmed from mass spectrometry, and low endotoxin levels were verified as previously²⁶.

Endothelial cell isolation and culture

For static and flow neutrophil adhesion assays, primary pig aortic EC (pAECs) isolated from pig aortas were grown in culture medium (10% heat-inactivated FBS, 0.15mg/mL gentamicin, 25ng/ml fungizone, DMEM 1g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate). Cells were used after two to eight passages at 80%–95% confluence. HAECs (blood group O or B, Life Technologies, Carlsbad, CA) were cultured using a cell-specific growth factor kit (Lonza, Walkersville, MD) and culture medium (Medium 200, Lonza, Walkersville, MD). The endothelial cell phenotype was verified by expression of CD31 and cobblestone morphology.

Neutrophil isolation and activation

Human blood was collected from a peripheral vein in healthy human volunteers into EDTA tubes under a protocol approved by the University of Maryland Baltimore Institutional Review Board. Neutrophils were isolated by density gradient centrifugation through a mixture of sodium diatrizoate and Dextran 500 solution with density of 1.113 ± 0.001 g/ml (Lympholyte-poly, Cedarlane, Burlington, ON, Canada). Blood was layered over Lympholyte poly cell separation media and centrifuged at room temperature at 500g for 35 min with the brake off. The neutrophil layer was collected and residual red blood cells (RBC) were lysed for 5 min at room temperature using an RBC lysis buffer (Roche Applied Science, Basel, Switzerland). Neutrophils were then washed with room temperature Hank's Balanced Salt Solution (Gibco-Life Technologies, Waltham, Massachusetts, USA) without calcium chloride and magnesium chloride (HBSS) and then resuspended in HBSS at 5×10^5 neutrophils/ml (purity >90% and viability >95% as measured by flow cytometry (BD FACSVerse™, Becton Dickinson, Franklin Lakes, New Jersey, USA). Neutrophils were then incubated with calcein^{AM} (calcein-acetoxymethyl ester, 0.5 μ M in HBSS) (Corning, New York, USA) for 30 min with gentle agitation in the dark at 37°C. Labeled neutrophils were washed two times with HBSS. In some experiments, human neutrophils (5×10^5 neutrophils/ml in HBSS buffer with Ca^{2+} and Mg^{2+}) were activated with different concentrations (0.8, 8, 80 and 800 ng/ml) of pig or human IL-8 for 30 minutes at 37 °C, washed in HBSS and kept at 4°C until use. For some experiments, isolated neutrophils were incubated with different concentrations of Reparixin (MedChem Express, Monmouth Junction, NJ) before incubation with IL-8.

Flow cytometry staining

Resting or activated neutrophils were centrifuged, resuspended in HBSS (with Ca^{2+} and Mg^{2+}) buffer and 10^5 cells were stained with antibodies against human CD45 (Biolegend, San Diego, California, USA), CD11b (Becton Dickinson, Franklin Lakes, NJ, USA), CD18 activation epitope (eBioscience, San Diego, California, USA) and isotype controls for 30 minutes at 4°C. After washing, stained cells were resuspended in PBS and data was acquired on the same day on a BD FACSVerse. Flow cytometry data were analyzed using single cell analysis software (FlowJo, LLC, Ashland, OR, USA).

Neutrophil oxidative burst

To measure hydroxyl, peroxy and other reactive oxygen species (ROS) activity, freshly isolated neutrophils were centrifuged and resuspended in warm HBSS (with Ca^{2+} and Mg^{2+}) buffer. 1×10^5 cells were mixed with Vybrant CFDA-SE Cell Tracer (Invitrogen, Waltham, Massachusetts, USA, DMSO, final concentration: 1 μM) and incubated for 30 minutes at 37°C . Neutrophils were then washed with HBSS and incubated with different concentrations of pig or human IL-8 (0.8, 8, 80 and 800 ng/ml in warm HBSS buffer with Ca^{2+} and Mg^{2+}) for 30 minutes at 37°C . For some experiments, isolated neutrophils were incubated with different dosages of Reparixin before incubation with IL-8. After washing, stained cells were resuspended in PBS and data acquired the same day on a BD FACSVerse. Acquired data were analyzed using single cell analysis software FlowJo.

Static adhesion assays

GalTKO.hCD46 primary pAECs and human aortic endothelial cells (hAECs) were grown to confluent monolayers in 24 well culture plates. After washing endothelial cell monolayers with warm DMEM (1g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate, Sigma Aldrich, St. Louis, MO), freshly isolated calcein^{AM}-labeled human neutrophils (5×10^5 in warm HBSS buffer with Ca^{2+} and Mg^{2+}) were added at 10^4 neutrophils/ml per well and incubated for 30 min at 37°C . After washing unbound neutrophils with PBS at room temperature, attached neutrophils were quantified fluorometrically (excitation 485 nm, emission 530 nm) using a Spectramax 3000 plate reader (Sunnyvale, CA, USA). Neutrophil-EC adhesion was expressed as percent adhesion of adherent neutrophils compared to the fluorescent reading of 10^4 neutrophils/ml. In some instances, endothelial cell monolayers were treated with TNF (25 ng/ml for 4 hours at 37°C) to activate the endothelial cells, and neutrophils were activated with human or pig IL-8 (8 ng/ml for 30 min at 37°C) before incubation with AECs. Two replicate wells were analyzed in each experiment and results were expressed as the average of readings from 2–3 independent experiments performed with AECs from 2 different individual donors and 2–5 different human blood donors.

Bioflux flow adhesion assays

Primary pAECs expressing GalTKO.hCD46 were grown to confluent monolayers on microfluidic channel 24-well plates (Fluxion, South San Francisco, California, USA). Freshly isolated calcein^{AM}-labeled human neutrophils (10^4 neutrophils in 300 μL of HBSS with Ca^{2+} and Mg^{2+}) were introduced into the inflow wells of experimental channels and perfused at 1 dynes/cm² at 37°C for 20 minutes using a Bioflux 200 system (Fluxion). In some experiments, endothelial cells were pre-activated with human TNF (25 ng/ml in DMEM, 4 hr at 37°C , washed with DMEM) and neutrophils with human or pig IL-8 (8 ng/ml, 5×10^5 neutrophils/ml, 30 min at 37°C , washed with HBSS) prior to assessing neutrophil adhesion. For some experiments, isolated neutrophils were incubated with Reparixin (0.1mg/ml=350nM) before incubation with IL-8. Proximal [‘upstream’] and distal [‘downstream’] $10 \times$ images were obtained every 60 seconds of each channel’s perfusion chamber by fluorescent microscopy (Zeiss) using 100 ms exposure. Adhered neutrophils were counted after processing of the data using a counter system on the ImageJ system

(National Institute of Health, Bethesda, Maryland, USA). Counts from both fields of view were recorded in each chamber every minute for 20 minutes. Two fields from two replicate wells were analyzed in each experiment and results were expressed as the average of readings from three independent experiments performed with three different human blood donors.

Statistical analysis

Statistical analyses were performed using the statistical package INSTAT with GraphPad (La Jolla, CA, USA). Unless otherwise indicated, variables were expressed as the mean and the standard error of the mean (SEM). Variables that were normally distributed were assessed with a one-way analysis of variance and Student's t-test. Variables that were not normally distributed were assessed with Mann–Whitney test or Wilcoxon matched pairs test. P-values <0.05 were considered statistically significant.

Results

Plasma IL-8 increases during *ex vivo* lung perfusion

Human IL-8 levels were consistently low (<10pg/ml) in baseline human perfusate, and increased to ~25–100pg/ml after 60 minutes of *ex vivo* perfusion of GalTKO.hCD46 (p=0.02) and GalTKO.hCD46.hCD55 (p<0.0001) lungs (Figure 1, left panel). Pig IL-8 levels after 60 minutes of *ex vivo* perfusion remained low (~1–40pg/ml) and not significantly different from background levels measured at the start of perfusion (~1–20pg/ml) (Figure 1, middle panel). While both pig and human IL-8 were elaborated by 240 minutes, the increase in pig IL-8 (GalTKO.hCD46: 3,978±1,275 pg/ml, GalTKO.hCD46.hCD55: 8,249±1,805 pg/ml) was greater than the increase seen for human IL-8 (GalTKO.hCD46: 834±254 pg/ml, p=0.03; GalTKO.hCD46.hCD55: 1,637 ±227 pg/ml, p=0.005).

Xenogeneic pig lung perfusions exhibited dramatically decreased circulating neutrophil counts overtime. After the first hour, only 22% and 11% neutrophils were left in the human blood perfusate in association with GalTKO.hCD46 and GalTKO.hCD46.hCD55 pig lungs, respectively (Figure 1, right panel).

IL-8 mRNA increases during *ex vivo* lung perfusion

Pig IL-8 mRNA increased 2-fold at 30 minutes and 10–30-fold after 4 hours of *ex vivo* perfusion of GalTKO.hCD46 lungs with human blood (Supplemental Figure 2). Moreover, pig IL-8 transcripts were much more abundant (CT=3 and –1 at 30' and 240') than human IL-8 transcripts (CT=8 and 2 at 30' and 240').

Recipient IL-8 increases after *in vivo* lung transplantation

Baboon pre-operative values for plasma IL-8 levels (134±30 pg/ml, n=16) increased slightly throughout the follow-up interval for autologous and allogeneic lung transplant recipient baboons (combined non-xeno control final: 431±110 pg/ml) (Figure 2C). In contrast, baboon IL-8 increased significantly following xenogeneic *in vivo* lung transplantation experiments with 3 gene modifications (240min: 1,213±433 pg/ml, p=0.02, Figure 2A) and 6 gene

modifications ($2,234 \pm 423$ pg/ml, $p=0.009$, Figure 2B), with final values generally declining after the 4 hour time-point. Pig IL-8 levels in baboon plasma remain low throughout follow-up during *in vivo* lung transplantation. Specifically, levels of pig IL-8 after 4 hours of xenogeneic *in vivo* lung transplantation (3 gene: 21 ± 9 pg/ml; 6 gene: 26 ± 8 pg/ml) were not different from non-xeno control samples (26 ± 11) and baboon baseline values (12 ± 4 , $n=16$) ($p>0.05$) (Figure 2, middle panel). We asked whether neutrophil counts changed after *in vivo* lung transplantation as seen in *ex vivo* lung perfusions. Neutrophil counts decreased transiently within the first hour following xenogeneic lung transplantation (% PMN remaining at 30 min: 71 ± 8 for 3 gene lungs ($p=0.004$) and 70 ± 10 for 6 gene lungs ($p=0.01$) whereas counts remained stable after allo-/autogeneic lung transplantation (121 ± 3 , white blood cell counts for 2 non-xeno controls). One hour following lung transplantation, neutrophil counts progressively increased in the majority (~60%) of experiments to reach peak levels at the 4-hour time point (non-xeno: $170 \pm 45\%$ of pre-operative levels; 3 gene: $120 \pm 29\%$; 6 gene: $135 \pm 40\%$). At the final time point, neutrophil counts were very heterogeneous with 60% of xenogeneic recipients exhibiting decreased neutrophil counts levels and 40% showing higher counts relative to pre-operative levels (3 gene: $87 \pm 33\%$; 6 gene: $87 \pm 78\%$) (Figure 2, right panel).

Pig IL-8 activates human neutrophils

The sequence alignment of human, baboon and pig IL-8 reveal differences in amino acid residues that could influence binding of IL-8 to its receptors (Supplemental Figure 1). Whether human and pig IL-8 differentially modulate activation of human neutrophils or their adhesion to endothelium has not previously been described. Because pig IL-8 was elaborated in *ex vivo* lung perfusions, we explored the ability of pig IL-8 to activate human neutrophils. Both pig and human IL-8 increased expression levels of CD11b and activated CD18 on human neutrophils in a dose-dependent fashion (Figures 3B and C). Similarly, both human and pig IL-8 induced similar reactive oxygen species (ROS) levels in human neutrophils, measured as CFDA-SE staining, with significant ROS levels detected even with the lowest IL-8 concentration (Figure 3D).

IL-8 increases adhesion of human neutrophils to pAECs

Under static conditions, the adhesion of naïve human neutrophils to human or pig GalTKO.hCD46 aortic endothelial cells was similar (Figure 4, black vs. grey, left). When neutrophils were activated with human IL-8, cell adhesion was increased on both cell types; however, neutrophil adhesion was higher on pig compared to human AECs (Figure 4, $p<0.0001$). A similar pattern was seen when neutrophils were activated with human IL-8 and endothelial cells activated with TNF with a larger increase in adhesion on pig cells (human: 45 ± 7 , pig: 76.2 ± 3 , $p<0.0001$). Neutrophil activation with pig IL-8 similarly increased cell adhesion to both human and pig AECs, with a larger effect on pig AECs ($p<0.001$). When neutrophils were activated with pig IL-8 and endothelial cells activated with TNF there was a larger increase in percent adhesion (72.3 ± 1) than with activation of neutrophils or endothelial cells alone; however, that difference did not reach significance. Finally, activation of human or pig AECs with TNF alone also significantly increased neutrophil adhesion on hAECs ($p<0.01$) and pAECs ($p<0.0001$) and the effect was again more pronounced on pig AECs ($p<0.001$) (Figure 4).

Under flow conditions, when neutrophils were activated with human or pig rIL-8, the rate of adhesion and number of neutrophil adhesion events per field at 20 min increased compared to untreated neutrophils (Figure 5 and C). Activation of endothelial cells with TNF increased adhesion of resting human neutrophils to pAECs, and adhesion was further increased when neutrophils were activated with human or pig IL-8 (hIL-8: 85.5 ± 4 , pIL-8: 76.5 ± 3) (Figure 5B and 5D). Pig IL-8 tended to be less potent than human IL-8 on both resting (20min: $p < 0.0001$) and TNF-activated (20min: $p < 0.0001$) endothelial cells (Figure 5C and 5D).

IL-8 mediates neutrophil activation, oxidative burst, and adhesion to pAECs

To determine the effect of IL-8 on neutrophil function, isolated human neutrophils were incubated with Reparixin before activation with pig or human IL-8 (8 ng/ml). As shown in Figure 6, neutrophil activation, as CD11b, CD18 staining and ROS release, were decreased by Reparixin in a dose-dependent fashion (Figure 6A–C). Inhibition of human IL-8-induced activation was quasi maximal at about 0.1 mg/ml (350nM) of Reparixin for all three parameters, and the percent inhibition was highest for CD11b expression ($72 \pm 2\%$) and CD18 activation ($72 \pm 2\%$), followed by ROS ($67 \pm 3\%$). Similar results were observed for pig IL-8 activation except for a lower inhibition of CD11b expression ($62 \pm 3\%$) compared to IL-8 ($p < 0.001$).

We next assessed the efficacy of Reparixin in more stringent conditions, using 10-fold higher concentrations of IL-8 (80 ng/ml, Figure 6 D–F). Similar to activation with 8 ng/ml, Reparixin treatment decreased activation and ROS release in a dose dependent manner when neutrophils were subsequently activated with either human or pig IL-8. Specifically, the concentration of 0.1 mg/ml, Reparixin inhibited human IL-8 induced neutrophil activation for all three parameters: CD11b expression ($57 \pm 0.3\%$), CD18 activation ($76 \pm 3\%$) and ROS ($63 \pm 10\%$). Similar results were seen with activation of human neutrophils by pig IL-8; however, there was slightly lower inhibition of CD11b expression ($49 \pm 1\%$) and CD18 activation (69 ± 7) whereas ROS was inhibited to the same level.

Finally, the adhesion of human neutrophils activated with 8ng/ml of pig or human IL-8 to either resting (Fig 7A) or TNF-activated pAECs (Fig 7B) was inhibited by pretreatment with Reparixin (0.1 mg/ml) under flow conditions. Inhibition was higher with activation by human IL-8 (resting: 87 ± 2 , TNF: 75 ± 2) vs. pig IL-8 (resting: 80 ± 2.5 , TNF: 70 ± 2) ($p < 0.0001$). These results show that IL-8 mediates the activation and xenogeneic adhesion of human neutrophils.

Discussion

In this study, we find that IL-8 is elaborated during *ex vivo* perfusion of genetically modified pig lungs with human blood, and is largely of pig origin. The elaboration of pig IL-8 is low one hour after *ex-vivo* perfusion is initiated, and is markedly increased by four hours. In contrast, pig IL-8 levels remain low in the *in vivo* lung transplant model. Meanwhile, “recipient” (baboon or human) IL-8 increases over time in both *ex vivo* and *in vivo* lung transplant models.

The host's innate immune system perceives the presence of "discordant" organ xenograft as "non-self", and accordingly releases multiple cytokines and chemokines that recruit leukocytes⁷, a response teleologically designed to eliminate the insult and initiate tissue repair. Moreover, it is well established that multiple chemokines including IL-8, are released in response to organ reperfusion injury and recruit neutrophils^{5,7,27}, a proinflammatory response which is presumably detrimental and contributes to xenograft tissue damage. Although the rapid kinetics of neutrophil sequestration during *ex vivo* lung perfusion occur before significant IL-8 elaboration is detected in plasma, our findings suggest that IL-8 may play a role in neutrophil sequestration and acute injury after lung xenotransplantation, and offer a new therapeutic target to prevent mechanisms of xenogeneic lung injury.

In order to better understand the role of IL-8, we next characterized the influence of human and pig IL-8 on human neutrophil activation and adhesion to pAECs *in vitro*. We find that human and pig IL-8 are both highly potent, and function across species, even at concentrations as low as 0.8 ng/ml, as evidenced by upregulation of CD11b, CD18 activation, and ROS release. Both also promote human neutrophil adhesion to human and pAECs under both static and flow conditions. After activation with pig or human IL-8, the greater increase in human neutrophil adhesion to pig compared to human endothelial cells suggests that IL-8 may exert an even more important role in a xenogeneic than in allogeneic conditions.

Since lung injury phenotype and kinetics have been highly similar between our *ex vivo* and *in vivo* lung models,²³ we presumed that the upstream triggers for IL-8 elaboration are similar in both models and expected to detect similar IL-8 chemokines profiles in both models. During xeno-associated lung inflammation, resident tissue macrophages release a variety of cytokines and chemokines and are therefore likely to be a major source of pig IL-8. IL-8 is also produced by a number of other cells including monocytes, endothelial cells, and neutrophils themselves^{28,29}. However, pig IL-8 levels were surprisingly very low *in vivo*. We speculate that pig IL-8 elaborated in the *ex vivo* model is either synthesized in response to immune injury, or that pig IL-8 elaboration overwhelms receptor binding and other scavenging mechanisms in the *ex vivo* model, but not *in vivo*. The low levels of pig IL-8 *in vivo* could also be a result of the anti-inflammatory drugs given to the recipients and donors (Table 1). Drugs such as steroids and C1 Inhibitor have well-studied anti-inflammatory activities.^{30,31,32} The difference in outcomes between the *ex vivo* perfusion and the *in vivo* transplantation experiment points to a possible explanation of a local (organ-associated) versus a systemic (whole body) effect. Alternatively, the upstream triggers for pig IL-8 elaboration may be significantly stronger in the pig-to-human lung perfusion model due to the presence of anti-Neu5Gc antibodies present in human blood, but not in baboons.

The reason for the relative heterogeneity of IL-8 levels between individual experiments is not clear. All pig lungs were harvested using standard surgical procedures with approximately the same ischemia time, except for one animal, for which lungs were harvested off site. Since IL-8 levels were in the lower range for that experiment, differences in ischemia do not appear to drive differences in IL-8 levels. *In vivo*, some variability could in theory come from the transgenes expressed by the lung, but this explanation is unlikely since pig IL-8 levels were low in all experiments. All together, the variability between

experiments is most likely related to differences between individual human blood donors or animals, as reported previously.¹⁷

It is difficult to reconcile the rapid kinetics of neutrophil sequestration after lung perfusion or transplantation with the low levels of IL-8 detected in plasma at the one-hour time-point. One might speculate that IL-8 may play a biological role after rapid binding to cellular receptors well before significant levels are detectable in plasma. Low levels of human IL-8 were detectable after 1 hour whereas pig IL-8 was not detectable at that time-point, suggesting that IL-8 of human origin may be important early on. In contrast, IL-8 of pig origin was more abundant after 4 hr of *ex vivo* perfusion suggesting that pig IL-8 may trigger late inflammatory events. Alternatively, and perhaps more likely, initial neutrophil sequestration may be driven by IL-8 independent mechanisms, such as P-selectin, platelet-activating factor (PAF)^{19,33}, Fas ligand³⁴, or sialidase-mediated adhesion.³⁵ Recombinant pig IL-8 induced chemotaxis only in porcine but not human neutrophil in prior studies¹⁹. In contrast, we found that pig IL-8 was almost as potent as human IL-8 to induce the activation and adhesion of human neutrophils from several blood donors. We conclude that further studies are warranted to delineate the contribution of IL-8 to neutrophil activation and recruitment in the context of a pig lung perfusion or transplantation.

Reparixin is a noncompetitive inhibitor of IL-8 binding through CXCR1 and CXCR2 chemokine receptor blockade. It blocks a range of activities related to IL-8 signaling, including leukocyte recruitment and other inflammatory responses, without affecting receptor activation induced by other CXCR1 and CXCR2 agonists¹³. In a variety of animal studies, it was found that Reparixin decreases ischemia–reperfusion injury (IRI) in various organ systems by inhibiting inflammatory responses such as neutrophil recruitment^{27,36,37}. To the best of our knowledge, Reparixin has never been tested in a xenotransplantation setting. We found that Reparixin decreased the activation of human neutrophils by IL-8 of either human or pig origin. CXCR1/2 receptor blockade with Reparixin also decreased the adhesion of IL-8 activated human neutrophils to TNF-activated pAECs. We infer that blockade of the IL-8 pathway may offer a potentially important new therapeutic target to blunt xenogeneic lung injury.

Our observations extend that of other researchers who studied IL-8 levels in the context of xenotransplantation. David Cooper's group found no increase in baboon IL-8 after *in vivo* GalTKO ($\pm$ hCD46) heart (days 21, 26 and 28) or kidney (days 6, 9 and 10) xenotransplantation.²⁰ Interestingly, they observed high levels of baboon IL-8 pre-transplantation (200–2,000 pg/ml), presumably associated with their treatment regimen, and not attributable to the xenotransplant procedure, or related to immune events. In contrast, we found generally lower baseline levels (30–500pg/ml) and a consistent and significant increase 4 hours after lung transplantation (600–3,000 pg/ml). Interestingly, baboon IL-8 declined at later time-points, suggesting that IL-8 elaboration is more prominent in the peri-operative period. Consistent with our current findings, we previously described increased plasma levels of human and pig IL-8 after *ex-vivo* GalTKO lung perfusions²¹. Similarly, in an *ex vivo* perfusion model of WT or GalTKO.hCD46.HLA-E pig forelimbs, Bongoni et al reported low levels of IL-8 at baseline which increased after 12 hours to a peak of 1,000–

15,000 pg/ml³⁸, levels similar to those we observe at 4 hours in our *ex vivo* lung perfusion model.

In lung allotransplantation studies, a direct correlation was shown between increased IL-8 levels in bronchoalveolar lavage (BAL) and the amount of donor lung injury at the time of transplant, as well as an inverse correlation with quality of postoperative graft performance³⁹. The robust production of IL-8 we observed illustrates the intensity of the pro-inflammatory milieu elicited in association with the interaction between human blood and pig lung. The current retrospective observational analysis of samples from our *ex vivo* lung perfusion model is not well suited to explore whether increased IL-8 is quantitatively associated with, or causally related to, intensity of lung injury. While our *ex vivo* findings implicate IL-8 in the pathogenesis of lung xenograft injury, further work will be required to test whether its elaboration is pathogenic, or a bystander phenomenon, and to define the role of pig IL-8 in neutrophil behavior.

In conclusion, IL-8 primarily of pig origin is rapidly elaborated in an *ex vivo* model of pig-to-human lung xenotransplantation. Pig IL-8 potently induced upregulation and activation of CD11b and CD18 and ROS in human neutrophils. Further, both human and pig IL-8 mediate increased adhesion of human neutrophils to pAECs in an *ex vivo* Bioflux model. Blocking of the IL-8 receptor *in vitro* with Reparixin prevented the induced activation of the neutrophils with pig and human IL-8 and the increased adhesion to pAECs. Although we speculate that the initial neutrophil sequestration seen in our *ex vivo* model is probably not primarily mediated by IL-8, our data suggest that later events may well be modulated at least in part by IL-8 of pig or primate origin elaborated within hours after exposure of pig lungs to either human or baboon blood. As such, this work identifies the IL-8 pathway as an important pathway potentially pivotal to xenograft inflammation and injury and as a candidate for therapeutic interventions such as blockade of IL-8 or its receptor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

These studies were supported by U19 AI090959, P01 HL107152 and by unrestricted educational gifts from and sponsored research agreements with United Therapeutics' subsidiary, Lung Biotechnology LLC. This work was supported by the University of Maryland Clinical Translational Science Institute and the University of Maryland General Clinical Research Center.

Abbreviations

BAL	Brochoalveolar lavage
Calcein^{AM}	Calcein-acetoxymethyl ester
CFDA-SE	5,6-Carboxyfluorescein Diacetate, Succinimidyl Ester
EDTA	Ethylenediaminetetraacetic acid
GalTKO	α1,3 galactosyltransferase knockout

Xenotransplantation. Author manuscript; available in PMC 2019 March 01.

GE	Genetically engineered
hAECs	Human aortic endothelial cells
HBSS	Hank's Balanced Salt Solution
hCD46	Human membrane cofactor protein
hCD55	Human complement decay-accelerating factor protein
IACUC	The Institutional Animal Care and Use Committee
IL-8	Interleukin-8
NIH	National Institutes of Health
NMR	Nuclear magnetic resonance
pAEC	Pig aortic endothelial cell
PVR	Pulmonary vascular resistance
ROS	Reactive oxygen species
WT	Wild type

References

1. Cooper DK, Ekser B, Ramsoondar J, et al. The role of genetically engineered pigs in xenotransplantation research. *The Journal of Pathology*. 2016; 238(2):288–99. [PubMed: 26365762]
2. Burdorf L, Stoddard T, Zhang T, et al. Expression of human CD46 modulates inflammation associated with GalTKO lung xenograft injury. *American Journal of Transplantation*. 2014; 14(5): 1084–1095. [PubMed: 24698431]
3. Platt JL, Fischel RJ, Matas AJ, et al. Immunopathology of hyperacute xenograft rejection in a swine-to-primate model. *Transplantation*. 1991; 52(2):214–220. [PubMed: 1871792]
4. Ezzelarab M, Garcia B, Azimzadeh A, et al. The innate immune response and activation of coagulation in α 1,3-galactosyltransferase gene-knockout xenograft recipients. *Transplantation*. 2009; 87(6):805–812. [PubMed: 19300181]
5. Ioannou A, Lucca JD, Tsokos GC. Immunopathogenesis of ischemia/reperfusion-associated tissue damage. *Clinical Immunology*. 2011; 141(1):3–14. [PubMed: 21839685]
6. Wolff B, Burns AR, Middleton J, et al. Endothelial cell “memory” of inflammatory stimulation: human venular endothelial cells store interleukin 8 in Weibel-Palade bodies. *The Journal of Experimental Medicine*. 1998; 188(9):1757–1762. [PubMed: 9802987]
7. Bickel M. The role of interleukin-8 in inflammation and mechanisms of regulation. *Journal of Periodontology Online*. 1993; 64:456–60.
8. Warrens AN, Simon AR, Theodore PR, Sykes M. Human-porcine receptor-ligand compatibility within the immune system: relevance for xenotransplantation. *Xenotransplantation*. 1999; 6(2):75–8. [PubMed: 10431783]
9. Cardozo LA, Rouw DB, Ambrose LR, Midulla M, et al. The neutrophil: the unnoticed threat in xenotransplantation? *Transplantation*. 2004; 78(12):1721–8. [PubMed: 15614144]
10. Robinson LA, Tu L, Steeber DA, Preis OA, et al. *Journal of Immunology*. 1998; 161(12):6931–8.
11. Stillie R, Farooq SM, Gordon JR, et al. The functional significance behind expressing two IL-8 receptor types on PMN. *Journal of Leukocyte Biology*. 2009; 86:529–543. [PubMed: 19564575]
12. Nasser MW, Raghuwanshi SK, Grant DJ, et al. Differential activation and regulation of CXCR1 and CXCR2 by CXCL8 monomer and dimer. *Journal of Immunology*. 2009; 183(5):3425–3432.

13. Bertini R, Allegretti M, Bizzarri C, et al. Noncompetitive allosteric inhibitors of the inflammatory chemokine receptors CXCR1 and CXCR2: Prevention of reperfusion injury. *Proceedings of the National Academy of Sciences*. 2004; 101(32):11791–11796.
14. Holgersson J, Ehrnfelt C, Hauzenberger E, et al. Leukocyte endothelial cell interactions in pig to human organ xenograft rejection. *Veterinary Immunology and Immunopathology*. 2002; 87(3–4): 407–415. [PubMed: 12072266]
15. Morigi M, Zoja C, Colleoni S, et al. Xenogeneic human serum promotes leukocyte adhesion to porcine endothelium under flow conditions, possibly through the activation of the transcription factor NF- κ B. *Xenotransplantation*. 1998; 5(1):57–60. [PubMed: 9507734]
16. Ehrnfelt C, Serrander L, Holgersson J. Porcine endothelium activated by anti- α -GAL antibody binding mediates increased human neutrophil adhesion under flow. *Transplantation*. 2003; 76(7): 1112–1119. [PubMed: 14557762]
17. Al-Mohanna F, Collison K, Parhar R, et al. Activation of naive xenogeneic but not allogeneic endothelial cells by human naive neutrophils: a potential occult barrier to xenotransplantation. *American Journal of Pathology*. 1997; 151(1):111–20. [PubMed: 9212737]
18. Sheikh S, Parhar R, Al-Mohanna F. Rapid static adhesion of human naïve neutrophil to naïve xenoendothelium under physiologic flow is independent of Gal α 1,3-gal structures. *Journal of Leukocyte Biology*. 2002; 71(6):932–40. [PubMed: 12050177]
19. Gilli UO, Schneider MK, Loetscher P, et al. Human polymorphonuclear neutrophils are recruited by porcine chemokines acting on CXCR2, and platelet-activating factor. *Transplantation*. 2005; 79(10):1324–1331. [PubMed: 15912099]
20. Ezzelarab MB, Ekser B, Azimzadeh A, et al. Systemic inflammation in xenograft recipients precedes activation of coagulation. *Xenotransplantation*. 2014; 22(1):32–47. [PubMed: 25209710]
21. Nguyen B-NH, Azimzadeh AM, Schroeder C, et al. Absence of gal epitope prolongs survival of swine lungs in an ex vivo model of hyperacute rejection. *Xenotransplantation*. 2011; 18(2):94–107. [PubMed: 21496117]
22. Harris DG, Quinn KJ, French BM, et al. Meta-analysis of the independent and cumulative effects of multiple genetic modifications on pig lung xenograft performance during ex vivo perfusion with human blood. *Xenotransplantation*. 2014; 22(2):102–111. [PubMed: 25470239]
23. Burdorf, L., Azimzadeh, AM., Pierson, RN. *Xenotransplantation Methods in Molecular Biology*. New York: Costa, Cristina, Máñez, Rafael; 2012. Xenogeneic lung transplantation models; p. 169-189.
24. Rajarathnam K, Clark-Lewis I, Sykes BD. 1H NMR solution structure of an active interleukin-8 monomer. *Biochemistry*. 1995; 34:12893–12990.
25. Rajarathnam K, Clark-Lewis I, Dewald B, et al. 1H NMR evidence that Glu-38 interacts with the N-terminal domain in Interleukin-8. *FEBS Lett*. 1996; 399:43–46. [PubMed: 8980116]
26. Das ST, Rajagopalan L, Guerrero-Plata A, et al. Monomeric and Dimeric CXCL8 Are Both Essential for In Vivo Neutrophil Recruitment. *PLoS ONE*. 2010; 5(7)
27. Cugini D, Azzollini N, Gagliardini E, et al. Inhibition of the chemokine receptor CXCR2 prevents kidney graft function deterioration due to ischemia/reperfusion. *Kidney International*. 2005; 67(5): 1753–1761. [PubMed: 15840022]
28. Yoshimura T, Matsushima K, Tanaka S, et al. Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proceedings of National Academy of Sciences*. 1987; 84:9233–7.
29. Matsushima K, Morishita K, Yoshimura T, et al. Molecular cloning of a human monocyte-derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin 1 and tumor necrosis factor. *Journal of Experimental Medicine*. 1988; 167:1883–93. [PubMed: 3260265]
30. Barnes PJ. How corticosteroids control inflammation: Quintiles Prize Lecture 2005. *British Journal of Pharmacology*. 2006; 148(3):245–54. [PubMed: 16604091]
31. Thorgeresen EB, Ludviksen JK, Lambris JD, et al. Anti-inflammatory effects of C1-inhibitor in porcine and human whole blood are independent of its protease inhibition activity. *Innate Immunology*. 2010; 16(4):254–264.

32. Saethre M, Schneider MK, Lambris JD, Magotti P, et al. Cytokine secretion depends on Galalpha (1,3)Gal expression in a pig-to-human whole blood model. *Journal of Immunology*. 2008; 180(9): 6346–53.
33. Cruzado JM, Torras J, Riera M, Lloberas N, et al. Effect of a platelet-activating factor (PAF) receptor antagonist on hyperacute xenograft rejection; evaluation in a pig kidney-human blood xenoperfusion model. *Clinical Experimental Immunology*. 1998; 113(1):136–44. [PubMed: 9697996]
34. Dupont PJ, Warrens AN. Fas ligand exerts its pro-inflammatory effects via neutrophil recruitment but not activation. *Immunology*. 2007; 120(1):133–9. [PubMed: 17233740]
35. French BM, Sendil S, Pierson RN III, Azimzadeh AM. The Role of Sialic Acid in Xenotransplantation. *Xenotransplantation*. 2017 In press.
36. Cavalieri B, Mosca M, Ramadori P, et al. Neutrophil Recruitment in the Reperfused-Injured Rat Liver was Effectively Attenuated by Repertaxin, a Novel Allosteric Noncompetitive Inhibitor of Cxcl8 Receptors: A Therapeutic Approach for the Treatment of Post-Ischemic Hepatic Syndromes. *International Journal of Immunopathology and Pharmacology*. 2005; 18(3):475–486. [PubMed: 16164828]
37. Souza DG, Bertini R, Vieira AT, et al. Repertaxin, a novel inhibitor of rat CXCR2 function, inhibits inflammatory responses that follow intestinal ischaemia and reperfusion injury. *British Journal of Pharmacology*. 2004; 143(1):132–142. [PubMed: 15302676]
38. Bongoni AK, Kiermeir D, Jenni H, et al. Complement dependent early immunological responses during ex vivo xenoperfusion of hCD46/HLA-E double transgenic pig forelimbs with human blood. *Xenotransplantation*. 2014; 21(3):230–243. [PubMed: 24635052]
39. Fischer K, Hamza A, Eismann R, et al. Differential diagnostic use of interleukin patterns in patients being monitored after transplantation. *Clinica Chimica Acta*. 2001; 310(1):71–80.

Figure 1. IL-8 release during *ex-vivo* lung perfusion

Plasma levels of human (*left*) and pig (*middle*) IL-8 for *ex vivo* xenogeneic perfusion of lungs from either GalTKO.CD46 homozygous (n=5) (*top*, A) or GalTKO.CD46.CD55 (n=6) (*bottom*, B) pigs. Each symbol represents an individual pig lung perfusion experiment.

Results are shown as the mean $\pm$ SD from two individual ELISA experiments. Blood neutrophil counts (*right*) were expressed as the percent neutrophils remaining relative to pre-perfusion levels. Symbols represent the same pig lung perfusion experiments as for IL-8.

*p=0.02, vs. time 0, ** p<0.0001 vs. time 0, #p=0.03 vs. human IL-8, ##p=0.005 vs. human IL-8.

Figure 2. IL-8 release during *in-vivo* lung transplantation

Plasma levels of baboon (*left*) and pig (*right*) IL-8 from *in vivo* orthotopic left lung transplant recipients. Lungs were procured from GalTKO.hCD46 pigs that express either one or two additional complement, coagulation, or anti-inflammatory genes (*top*, A) (3-gene GalTKO.hCD46.hTBM, n=2; GalTKO.hCD46.hEPCR, n=5) or four added genes (*middle*, B) (including hCD55, hTBM, hTFPI, hEPCR, HO-1, hCD47, and hCD39 in various combinations: 6-gene pigs, n=6) as described in Methods and Table 1. Three non-xenogeneic controls (*bottom*, C) include 1 autologous lung transplant (dashed line) and 2 allogeneic lung transplants (solid line) in baboons. Blood neutrophil counts (*right*) were expressed as the percent neutrophils relative to pre-transplant levels (white blood cell counts were used as proxy for 1 autologous and 1 allogeneic control). *p=0.02, vs. time 0, **p<0.001 vs. time 0.

Figure 3. Human and pig IL-8 activate human neutrophils

Isolated neutrophils were stained for CD45, CD11b, and CD18 activation epitope or analyzed for oxidative burst (CFDA-SE) after activation with different doses of human or pig recombinant IL-8. A) Representative flow cytometry diagrams showing cell and expression profiles after stimulation with 8 ng/ml of human or pig IL-8. B–D) Pig and human IL-8 both increased levels of CD11b and CD18 expression and ROS levels in a dose-dependent manner. Data represent the mean $\pm$ SD from 5 independent experiments each done with fresh neutrophils and 5 different blood donors for human neutrophils. * $p < 0.001$ vs. no activation for both hIL-8 and pIL-8; ** $p < 0.005$ vs. no activation for both hIL-8 and pIL-8.

Figure 4. IL-8 increases adhesion of human neutrophils to pAECs

The adhesion of human neutrophils to resting or TNF-activated human (black bars) or pig (grey bars) aortic endothelial cells was measured under static conditions. Two replicate wells with the same endothelial cell source and same human blood donor were analyzed in each experiment. Results were expressed as the mean $\pm$ SD of percent neutrophil adhesion from duplicates wells readings in 2–3 independent experiments performed with AECs from 2 different individual donors and human blood from 2–5 different donors. * $p < 0.0001$ vs. hAECs, $^{\wedge}p < 0.0001$ vs. medium and monotherapy, $^{\vee}p < 0.01$ vs. medium, $^{\vee\vee}p < 0.01$ vs. hIL-8.

Figure 5. IL-8 pretreatment increases human neutrophil adhesion to pAECs under flow conditions

Resting or IL-8 pre-activated (hIL-8 or pIL-8; 8 ng/ml for 30 min) human neutrophils were flowed over resting (A, C) or TNF-pre-activated (B, D) (25 ng/ml for 4 hr) GalTKO.hCD46 pAECs monolayers at 1 dyne/cm² for 20 minutes. Neutrophils were counted repeatedly over 20 minutes in two microscopic fields from two duplicate channels in each experiment.

Results were expressed as the mean±SD of the number of neutrophils per image field from three independent experiments using GalTKO.hCD46 pAECs and fresh neutrophils from one of three different human blood donors. The relative number of adherent neutrophils per high power field was enumerated at the end of the 20 minute perfusion interval, and results summarized in panels C and D. Each experiment was performed with fresh neutrophils. *p<0.0001 pIL-8 vs. hIL-8.

Figure 6. Reparixin inhibits the activation of human neutrophils by pig and human IL-8
Isolated neutrophils were stained similar to Figure 3. Neutrophils were incubated with different concentrations of Reparixin and then activated with pig or human IL-8 (8 ng/ml (A–C) or 80 ng/ml (D–F)). Reparixin treatment decreased levels of CD11b and CD18 expression and ROS levels in a dose-dependent manner after both human or pig IL-8 activation. Results were expressed as the mean and SEM from four independent experiments using fresh neutrophils from one of three different human blood donors. * $p < 0.001$ vs. no reparixin, ** $p < 0.002$ vs. no reparixin, # $p < 0.0001$ vs. no reparixin.

Figure 7. Reparixin decreases human neutrophil adhesion to pAECs under flow after IL-8 activation

Isolated neutrophils were activated with 8 ng/ml of human or pig IL-8 and flowed over resting (A) or TNF-activated (B) pAEC monolayers similar to Figure 6. Some neutrophils were incubated with 0.1 mg/ml of Reparixin before IL-8 activation. Results were expressed as the mean $\pm$ SD of the number of neutrophils per image field from three independent experiments using GalTKO.hCD46 pAECs and fresh neutrophils from one of three different human blood donors. The relative number of adherent neutrophils per high power field was enumerated at the end of the 20-minute perfusion interval. * $p < 0.0001$ vs. control without reparixin.

Table 1Pig genetics and drug treatments used for *in-vivo* lung transplants

Pig genotype (GalTKO.hCD46) plus:	Number of transplants	Treatment given to recipients	Treatment given to donors	Mean life-supporting time (Hours)
hEPCR	5	Prednisolone, C1-inhibitor, αGP1b	Desmopressin, Remodulin, BIA	6.7
hTBM *	2	Prednisolone, C1-inhibitor, αGP1b	Desmopressin, Remodulin, BIA	5
hCD55, hEPCR, TFPI, hCD47	2	Prednisolone, C1-inhibitor, αGP1b, Famotidine, BIA	Desmopressin, Remodulin, BIA	16
hCD47, hTBM *, hCD39, A20 *	2	Prednisolone, C1-inhibitor, αGP1b, Famotidine, BIA	Desmopressin, Remodulin, BIA	6
hTBM, hCD55, hEPCR, hCD39	2	Prednisolone, C1-inhibitor, αGP1b, Famotidine, BIA	Desmopressin, Remodulin, BIA	10.5

* Expression of those human transgenes could not be found or validated in the pig lung tissue by immunochemistry