STUDY OF PHYSIOLOGIC AND IMMUNOLOGIC INCOMPATIBILITIES OF PIG TO HUMAN TRANSPLANTATION

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ABSTRACT

Ray K. Chihara

STUDY OF PHYSIOLOGIC AND IMMUNOLOGIC INCOMPATIBILITIES OF PIG TO HUMAN TRANSPLANTATION

Solid organ transplantation is limited by available donor allografts. Pig to human transplantation, xenotransplantation, could potentially solve this problem if physiologic and immunologic incompatibilities are overcome. Genetic modifications of pigs have proven valuable in the study of xenotransplantation by improving pig to human compatibility. More genetic targets must be identified for clinical success. First, this study examines platelet homeostasis incompatibilities leading to acute thrombocytopenia in liver xenotransplantation. Mechanisms for xenogeneic thrombocytopenia were evaluated using liver macrophages, Kupffer cells, leading to identification of CD18, β2 integrin, as a potential target for modification. When disruption of CD18 was accomplished, human platelet binding and clearance by pig Kupffer cells was inhibited. Further, human and pig platelet surface carbohydrates were examined demonstrating significant differences in carbohydrates known to be involved with platelet homeostasis. Carbohydrate recognition domains of receptors responsible for platelet clearance Macrophage antigen complex-1 (CD11b/CD18) and Asialoglycoprotein receptor 1 in pigs were found to be different from those in humans, further supporting the involvement of platelet surface carbohydrate differences in xenogeneic

thrombocytopenia. Second, immunologic incompatibilities due to antibody recognition of antigens resulting in antibody-mediated rejection were studied. Identification of relevant targets was systematically approached through evaluation of a known xenoantigenic protein fibronectin from genetically modified pigs. N-Glycolylneuraminic acid, a sialic acid not found in humans, was expressed on pig fibronectin and was identified as an antigenic epitope recognized by human IgG. These studies have provided further insight into xenogeneic thrombocytopenia and antibody-mediated rejection, and have identified potential targets to improve pig to human transplant compatibility.

Christopher Burlak, Ph.D., Chair

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Chapter 1: Introduction

Transplantation of solid organs has transformed medicine for end stage organ failure, yet shortage of donor allografts continues to limit the use of these lifesaving medical treatments. The transplant waitlist has over 100,000 patients in the United States and grows each year while the number of patients transplanted has been relatively stagnant demonstrating a worsening shortage of donor organs despite high selectivity for enlistment (Figure 1-1). Xenotransplantation, transplant across species, has been proposed as a potential solution to the shortage of donor organs. However, barriers to xenotransplantation must be overcome for successful clinical application. Our research focuses on understanding the underlying mechanisms behind these barriers to identify and develop methods to overcome the interspecies incompatibilities faced in xenotransplantation.

History of Xenotransplantation

The concept of xenotransplantation dates back thousands of years and is seen in multiple cultural and religious studies. For example, the ancient Egyptians built the Great Sphinx of Giza around 2500BC (1), a statue with lion's body with a human head. The Lamassu a protective deity on the cover of the Xenotransplantation journal (2) was depicted by a winged lion or bull with a human head accredited most often to the ancient Assyrians. Although many other recognizable mythical figures with combined features of several animal species exist such as the chimera and griffon, the first specific written description of xenotransplantation most likely comes from the story of Ganesha the god of wisdom who obtained the iconic elephant head through transplantation after decapitation by Shiva the god of destruction according to Hindu mythology and the Puranas, ancient Hindu texts, dated around 600BC (Figure 1.2) (3).

The xeno- portion of word xenotransplantation comes from Greek origins meaning strange or foreign (4, 5), albeit the use of xenotransplantation in science is neither strange nor foreign. Transplantation across species has been widely utilized experimentally to study immunology, graft rejection and cancer. For example, injection of human cancer cells into NOD-*scid IL2r*γ^{null} mice has greatly enhanced knowledge in cancer biology and treatment (6).

The modern concept of clinical xenotransplantation generally refers to the transplant of living nonhuman biological material into humans for treatment of disease. Decellularized animal products such as biological mesh for abdominal wall hernias, skin grafts and cardiac valves for aortic stenosis are usually not

included in the clinical definition for xenotransplantation. The field is further divided into several subcategories, which include living cell, tissue and solid organ transplantation. Use of xenogeneic living cells and tissues are being explored for treatment of Parkinson's disease (neurons), diabetes mellitus (pancreatic islets), anemia (red blood cells), burns (skin) and cataracts (cornea) (7). Xenogeneic mesenchymal stem cells may provide a source of omnipotent cells for regenerative medicine. Solid organ xenografts could be utilized as a treatment for end stage organ failure. Potential xenotransplant related medical uses are summarized in Figure 1-3 (7).

Xenografts are categorized into concordant or closely related and discordant or distantly related nonhuman organs. Limited clinical successes have been obtained through concordant xenotransplantation of the liver, heart and kidney. Dr. Starzl performed two liver xenotransplants in 1992-1993 using baboon grafts with patient survival of 70 and 26 days (8). Dr. Bailey performed an orthotopic cardiac xenotransplant using a baboon graft on a female neonate, baby Fae, with hypoplastic left heart in 1984 resulting in a survival of 20 days (9). The most notable success was Dr. Reemtsma's concordant xenotransplant studies in 1963-1964, an era prior to routine use of hemodialysis and the immunosuppressant cyclosporine, when several chimpanzee kidneys were transplanted into patients with survival up to 9 months (10). Although concordant renal xenotransplant trials demonstrated great potential, the advent of hemodialysis, concerns of zoonosis, ethical dilemmas and impracticality for large-scale production prevented further use of nonhuman primate organs.

While hemodialysis has greatly impacted treatment of end stage renal disease, kidney transplantation is sought after because of advantages such as reduced mortality, reduced cardiac events and improved quality of life (11).

Porcine organs, discordant xenografts, have been considered the most viable option, since pigs have relatively similar physiology, shorter gestation time, reproduce in liters of 5-12 and have decreased risks for zoonosis. Pigs are also considered more ethically acceptable, since over 100 million pigs are sacrificed per year as a source of dietary protein and in several medical applications for example mesh for hernias, cardiac valves and medicines including heparin for anticoagulation and insulin for diabetes historically. Although discordant xenograft trials in humans have resulted in failure mainly due to antibody-mediated rejection with maximal survival of pig liver and cardiac xenotransplant patients at 26 and 23 hours respectively, organs transplanted were from domestic pigs (12, 13). Genetically modified pig organs have not been attempted in humans due to governmental restrictions, but have shown promising results in pig-to-nonhuman primate xenotransplant models. Whether or not genetically modified pig-to-human transplantation will yield successes similar or greater to historical concordant clinical xenotransplant trials is yet to be determined.

Strategies for Improving Pig and Human Compatibility

Recent xenotransplant studies have focused on organ and tissue rejection and physiologic incompatibilities resulting from cross-species transplantation in order to understand and bridge the compatibility gap between pigs and humans. Research in humoral immunity, accommodation, tolerance, coagulation and their relationship to physiologic incompatibilites and the organ rejection process has advanced through use of xenotransplant models (14-16). Strategies utilized to improve compatibility can be categorized as changes to either the host or donor. Host modification strategies include porcine bone marrow transplantation for a chimeric recipient immune system (17, 18), porcine thymus transplantation (19), plasmapheresis and various immunosuppressive drugs. Donor modifications include production of pig-human chimeras (20) and genetic modifications to pigs. Since immunosuppression, plasmapheresis, porcine bone marrow and thymus transplantation involve further procedures to the host increasing the likelihood of sequelae, donor modification would be preferred unless changes to the host are highly specific with minimal risks. Ethical concerns with production of pig-human chimeras through use of human stem cell injections into fetal pigs and increased risks of zoonosis (20) have made genetic modification to pigs the most appealing method of improving pig-to-human compatibility.

The ability to genetically alter pigs highlights a key difference between allo- and xenotransplantation. In allotransplantation, bloodtype, crossmatch and human leukocyte antigen testing must be completed between an available donor and recipient to determine compatibility, whereas the donor may be directly

modified to become compatible with the recipient in xenotransplantation. The product of porcine genetic modifications that overcome immunologic and physiologic barriers could be directly utilized clinically.

Genetic Engineering and Modifications

Pigs have demonstrated good genetic engineering potential by successful transgenesis of several human genes and knockout of a pig gene using sperm-mediated gene transfer, homologous recombination and cloning techniques (21-23). Pigs transgenic for human complement regulation, apoptosis, coagulation, inflammation and platelet activation genes have been generated (7, 24-31). Transgenic pigs expressing human complement regulatory proteins CD46, CD55 and CD59 have demonstrated reduced incidence of hyperacute rejection (32-34). The knockout of α1,3-galactosyltransferase gene (GTKO) lead to substantial improvements in antibody-mediated rejection under clinically acceptable levels of immunosuppression (22). Transgenesis of human anti-apoptosis, anti-inflammatory and anti-coagulation genes require further investigation after combination with pigs of GTKO background in order to fully determine clinical significance in organ xenotransplantation.

Major technological advancements in genetic engineering have come mainly from zinc finger nucleases, which are engineered DNA sequence-binding modules combined usually with a Fok1 non-specific DNA cleavage domain (Figure 1-4) (35-37). Recently, zinc finger nucleases have demonstrated efficient and straightforward methods to alter the pig genome by successful generation of knockout pigs (38, 39). The use of zinc finger nucleases to knock-in genes has been utilized in mice (40). Targeted knock-in of a gene into a specific locus has the advantage of simultaneous knockout of an endogenous gene resulting in replacement rather than introduction of a gene. Similarly, transcription activator-

like effector nucleases (Talens) and meganucleases could be used to modify the porcine genome. Further development of genetic modification techniques through use of zinc fingers nucleases, Talens and meganucleases would allow for quick and efficient modification of pigs. The best combination of genetic modifications and other novel genetic targets must be identified to utilize recent advancements in genetic engineering.

Current Perspectives on Organ Specific Barriers to Xenotransplantation

Since solid organs have varying function and structure, it is not surprising to find varying organ specific barriers to xenotransplantation. The maximum survival time of pig organs vary from 5 days for lung, 8 days for liver, 57 days for orthotopic heart and 90 days for kidney in genetically modified pig-to-nonhuman primate xenotransplant models (7). Coagulation dysfunction for lung and ensuing vasculature thrombosis (29, 41), thrombocytopenia for liver and resultant hemorrhage (42, 43) and antibody-mediated rejection for heart and kidney (44-46) are the current major organ specific obstacles to clinically applicable xenotransplantation. Coagulation dysfunction and thrombocytopenia issues for lung and liver respectively are thought as immediate physiologic barriers demonstrating substantially decreased xenograft survival compared to heart and kidney where non-immediate immunologic reasons are considered the primary barrier (Figure 1-5) (7).

Among these organs, the liver stands out, because there are no clinically available options to temporarily sustain patients with acute liver failure as a bridge to allotransplantation. Dialysis, left ventricular assist and extracorporeal membrane oxygenation exists for kidney, heart and lung respectively and has been successfully utilized as a bridge to allotransplantation (47, 48). Bioartificial liver devices, currently undergoing clinical trials, and extracorporeal porcine liver perfusions would only work with select patients due to failure in dealing with portal hypertension commonly associated with acute on chronic liver failure. The liver may also have immune compatibility advantages, since allotransplantation

studies have shown the liver to be resistant to hyperacute rejection which may be explained by a unique type of immune privilege known as liver tolerance (49). If acute thrombocytopenia issue could be resolved, liver xenotransplantation may be utilized as a bridge to allotransplantation (42, 50).

Thrombocytopenia in Liver Xenotransplantation

GTKO pig liver to nonhuman primate xenotransplant studies resulted in thrombocytopenia and bleeding within hours of reperfusion (42). Since these studies did not demonstrate signs of rejection (51), xenogeneic thrombocytopenia is now considered the immediate barrier to clinically applicable liver xenotransplantation. Two general hypotheses have been proposed and evaluated for the observed thrombocytopenia, which are platelet aggregation with circulating monocytes and neutrophils (52) and clearance by liver sinusoidal cells (Figure 1-6) (53). Platelet clearance by the liver more readily explains the immediate loss of platelets seen in nonhuman primate models compared to platelet aggregation, although a combination of both maybe responsible for overall thrombocytopenia. Our studies with ex vivo porcine liver perfusion with human platelets demonstrated platelet phagocytosis by liver sinusoidal cells (Figure 1-7) (53). Determination of mechanisms behind platelet phagocytosis would lead to genetic targets for attenuating thrombocytopenia.

For insights into causes of xenogeneic platelet clearance, platelet homeostasis studies were examined leading to several potential mechanisms for exploration. Transfusion medicine research demonstrated that platelets must be stored at room temperature to avoid rapid clearance from circulation seen when platelets stored under cold conditions are transfused (54, 55). Asialoglycoprotein receptor 1 (ASGR1) expressed by hepatocytes and macrophage antigen complex 1 (Mac-1) were implicated in clearance of platelets that have lost sialic acid carbohydrates exposing beta glucans (Figure 1-8) (56-59). Porcine liver

sinusoidal endothelial cells (LSEC) were isolated and determined to express the ASGR1 receptor (60). Porcine LSEC involvement with human platelet binding and phagocytosis was identified (53). Inhibition and siRNA experiments revealed ASGR1 as a receptor involved with xenogeneic platelet clearance (60). Porcine Kupffer cells (KC), liver macrophages, were found to express the Mac-1 receptor and were evaluated for human platelet phagocytosis in Chapter 2.

Since thrombocytopenia was known to occur when platelets were exposed to cold and septic conditions, platelet differences were evaluated leading to discovery of platelet carbohydrate changes under specific conditions. Platelets exposed to the cold have increased galactose β1-4 glucosamine (Gal β1-4 GlcNac) carbohydrate expression (57, 61). Thrombocytopenia caused by streptococcus pneumoniae sepsis was attributed to neuraminidase, an enzyme that cleaves off neuraminic / sialic acids a common terminal carbohydrate on oligosaccharides, released by bacteria resulting in neuraminic acid removal from platelets resulting in increased exposure of Gal β1-4 GlcNac (56). Since ASGR1 and Mac-1 have carbohydrate recognition domains with affinity towards Galβ1-4 GlcNac, carbohydrate changes in platelets were implicated as a cause of platelet clearance. To determine if the same principles applied to the xenogeneic thrombocytopenia, human and pig ASGR1, Mac-1 receptors and platelet carbohydrates were evaluated and compared in chapter 3.

Xenoantigens and Antibody-Mediated Rejection

Antibody-mediated rejection is considered the primary barrier to heart and kidney xenotransplantation, although antibody-mediated rejection maybe a barrier for long-term liver xenografts once thrombocytopenia is overcome. The transplant rejection process occurs when the host immune system identifies the donor graft as non-self. Rejection has been classically linked to host immune recognition methods of pathogens that continually invade our innate immune barriers, skin and oral mucosa. Although the rejection process is not completely understood, GTKO pig-to-nonhuman primate xenotransplant models have shown antibodies recognizing antigens on xenografts as a major factor in graft rejection (19, 44-46, 63-66).

The removal of the xenoantigen galactose α1-3 galactose carbohydrate (αGal) from pig glycoproteins through generation of GTKO pigs lead to greatly reduced incidence of hyperacute xenograft rejection demonstrating the importance of the humoral immune system in the rejection process. Although removal of αGal resulted in decreased antibody binding, complement deposition and cell lysis, acute vascular antibody-mediated rejection remained for renal and cardiac GTKO pig-to-nonhuman primate studies from remaining xenoantigens (44-46, 67). The proposed model for acute vascular antibody-mediated rejection begins with preformed and induced host antibodies that bind antigens on the xenograft endothelium initiating a cascade of events. The antibodies cause complement activation and deposition resulting in activation and lysis of xenograft endothelial cells exposing basement membranes. Activation of the

endothelium combined with exposed underlying extracellular matrix and collagen triggers the activation and degranulation of platelets, activation of the coagulation cascade and thrombosis of vasculature leading to downstream ischemia and eventual graft failure (Figure 1-9) (62, 68). Antibody-dependent cell-mediated injury to the xenografts may also contribute to rejection through activation of NK cells by antibodies bound to xenograft endothelium resulting in the release of cytokines that promote endothelial cell activation and eventual apoptosis (69). GTKO pig-to-nonhuman primate xenotransplant models demonstrate the important role antibody recognition of xenoantigens play in xenograft rejection.

Further modifications of donor antigenic epitopes and/or additional recipient treatment strategies appear necessary to prevent antibody-mediated rejection which led to generation of xenoantigen lists though proteomic studies requiring validation (44, 46, 70). Increasing knowledge in the normal homeostatic role of naturally occurring auto-antibodies to endothelial cells, normal and altered intracellular, cell surface and plasma proteins (71-73) and relation to damage associated molecular patterns (74) calls into question whether or not mere existence of antibodies towards the xenogeneic version of proteins is enough to determine pathologic significance. In addition preformed antibodies recognizing both autologous and xenogeneic forms of a protein may not share the same antigenic epitopes and consequently cannot be ruled out as pathologically irrelevant. Another confounding problem is the use of denaturing agents in protein analysis. The presence of preformed antibodies to misfolded or altered autologous proteins complicate immunogenic analyses of denatured

GTKO proteins due to possible exposure of normally hidden physiologic antigenic epitopes (Figure 1-10a) used probably for waste clearance (72). This concept can be clearly illustrated through evaluation of preformed human antibodies to native-globular and denatured-reduced forms of purified human serum albumin. The presence of preformed human IgG and IgM antibodies recognizing denatured-reduced and not the native-globular form of allogeneic albumin are demonstrated in (Figure 1-10b). Nonetheless, denatured proteins are studied because of experimental difficulties associated with native proteins including solubility, interfering protein-protein interactions, protein separation and resolution. While many of the above issues have been avoided through study of induced antibodies after pig-to-nonhuman primate xenotransplantation, this approach is limited because this strategy cannot be used to identify preformed antibodies recognizing xenoantigens (44, 45). In addition, induced antibodies may develop secondary to cellular and tissue damage caused by the primary preformed antibody-mediated immune response resulting in exposure of intracellular proteins normally hidden from the endovascular compartment (75). Even after determination of an antigenic protein, many potential antigenic epitopes need to be considered on glycoproteins including peptides, glycosylations and glycosylated peptides. Relevant antigenic non-glycosylated proteins may be humanized or knocked out if noncritical for survival, however the same may not hold true for glycoproteins because antigenic glycosylations may be added post-translationally to humanized protein peptide sequences. Therefore, understanding pathologic human antibody recognition of antigenic

epitopes on xenogeneic glycoproteins is key to discovery of relevant genetic targets for modification.

A glycoprotein xenoantigen candidate for study is GTKO pig fibronectin because of relative abundance, ubiquitous expression in tissues, production by endothelial cells that line blood vessels and ability to induce antibodies in a xenotransplant model (44). Studying human fibronectin in parallel with GTKO pig fibronectin has several advantages. Since protein isolation and storage conditions may lead to denaturation and exposure of physiologic antigenic epitopes (76), evaluation of the human form of fibronectin as a control would alleviate xenoantigen analysis problems associated with conformation and protein degradation dependent antibody recognition. Additionally, various useful glycoprotein purification methods used have the potential to generate protein misfolding and therefore, must be compared back to the autologous human fibronectin treatment to discern differences between pathologic and physiologic antibody recognition. Simultaneous comparison of the GTKO pig and human fibronectin and endothelial cells address many challenges associated with identification of pathological antigenic epitopes, which are described in chapter 4.

The Hanganutziu-Deichter (HD) antigen, N-glycolylneuraminic acid (Neu5Gc) containing antigens, has been described as a possible antigenic carbohydrate epitope in xenotransplantation. Nonhuman mammals including baboons and chimpanzees express the sialic acid Neu5Gc whereas humans express only N-acetylneuraminic acid (Neu5Ac) due to a mutation in the cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) gene (77).

Known carbohydrate antigen comparisons between domestic pigs, GTKO pigs and humans are summarized in Figure 1-11. The CMAH enzyme converts CMP-Neu5Ac to CMP-Neu5Gc in the cytosol and then transported into the golgi to be conjugated to oligosaccharides on glycoproteins (78). Human serum was determined to have antibodies towards Neu5Gc (79) and CMAH / α1,3-galactosyltransferase double knockout mice studies demonstrated cytotoxicity of human antibodies towards Neu5Gc (80). Despite these studies, the importance of the Neu5Gc carbohydrate antigen continues to be controversial due to the clinical successes obtained in concordant Neu5Gc positive xenografts trials. GTKO pig-to-nonhuman primate xenotransplant models continue to demonstrate antibody-mediated rejection where the HD antigen is present in both host and recipient suggesting the presence of other antigenic epitopes. The Neu5Gc carbohydrate as an antigenic epitope for GTKO pig fibronectin was investigated in chapter 4.

Objectives Addressed

Our studies focus on understanding, evaluating and identifying possible genetic targets for modification to attenuate liver xenotransplant associated thrombocytopenia and antibody-mediated rejection necessary to make clinically applicable organ xenotransplantation a reality. The immediate obstacle to liver xenotransplantation has been identified as thrombocytopenia. Platelet clearance due to liver sinusoidal cell phagocytosis, pig and human platelet receptors and platelet carbohydrate differences were explored as mechanisms for xenotranplant associated thrombocytopenia. Potential genetic targets to attenuate thrombocytopenia were evaluated. Antibody-mediated rejection continues to be a barrier to heart and kidney transplantation despite generation of GTKO pigs. Focused initial evaluation of a known GTKO pig glycoprotein xenoantigen, fibronectin, was completed following methodologies required to validate and examine pathologic antigenic epitopes. The HD carbohydrate antigen involvement in GTKO pig fibronectin antigenicity was explored.

Figure 1-1. Comparison of the number of patients on the waiting list to patients transplanted from 1995-2010. Greater growth in number of patients added on to the waiting list (solid line) compared to number of patients transplanted (dashed line). (United Network of Organ Sharing database)

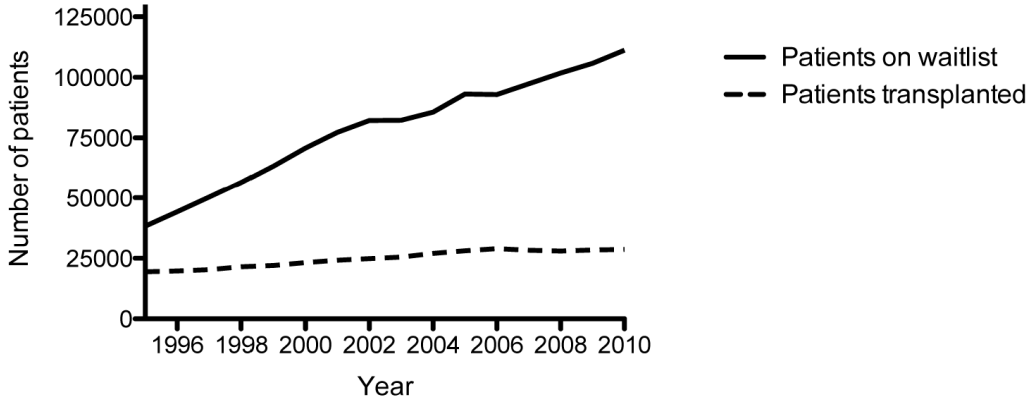


Figure 1-2. Statue of Ganesha the god of wisdom depicted with the characteristic elephant's head acquired through xenotransplantation based on Hindu mythology.

Ganesha Stele. 11th century. Portland Art Museum, Portland, Oregon. March 8, 2013. http://www.portlandartmuseum.org.

Location: Bihar or Bengal, India, or Bangladesh.

Time Period: 11th century Pala Period

Medium: gray schist.

Dimensions: 35 3/4 in x 18 in x 6 in.

Collection: Asian Art.

Type of art: Sculpture.

Museum Purchase: Funds provided by exchange through gifts of Mr. and Mrs. Hugh McCall

Statement of Use: Materials are included under the fair use exemption and are restricted from further use.

Figure 1-3. Summary of potential xenotranplantion treatments for a variety of diseases from Ekser 2012 (7).

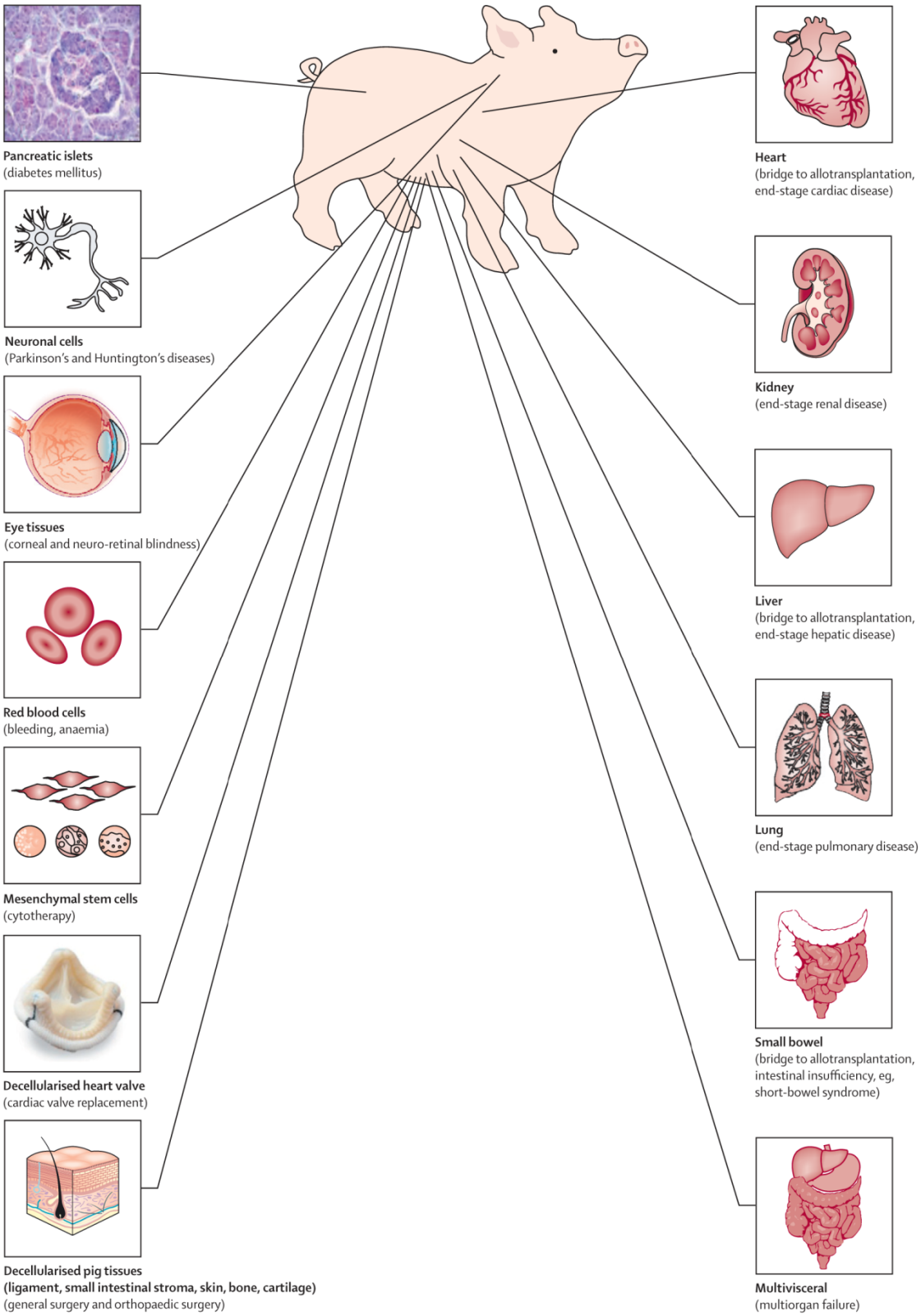


Figure 1-4. Zinc finger nuclease diagram from Maeder 2009 . The zinc finger array is designed to bind a specific nucleotide sequence to target a specific gene. When combined with the Fok1 non-specific DNA cleavage domain, a double strand DNA break may occur leading to deletion or insertion of base pairs following non-homologous end joining.

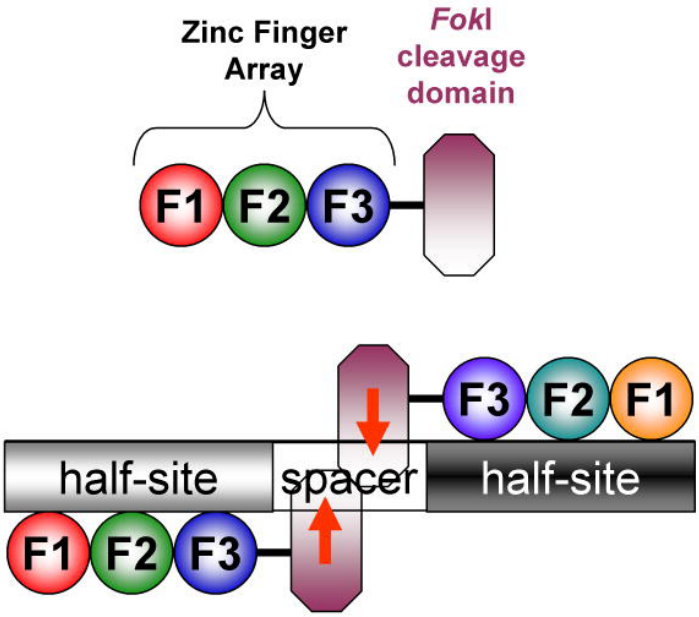


Figure 1-5. Comparison of pig-to-nonhuman primate xenograft longest survival per organ type in number of days. Lung and liver xenografts have distinctly lower survival compared to heart and kidney.

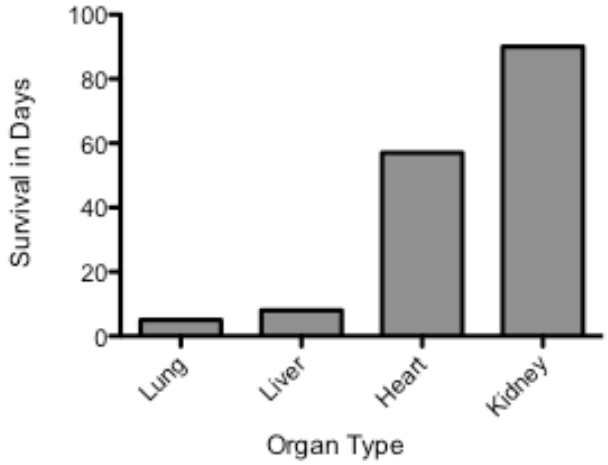
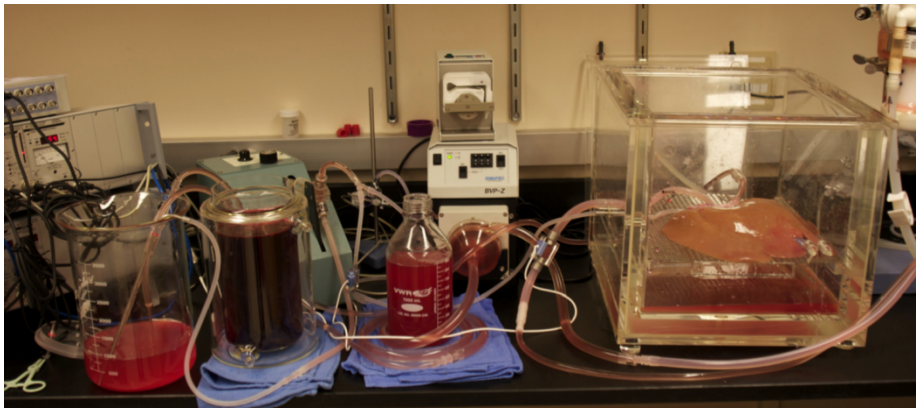


Figure 1-6. Ex vivo porcine liver perfusion circuit and human platelet perfusion graph from Burlak 2010 (53). Domestic porcine livers were harvested and connected to the circuit shown below. Human and pig platelets were circulated through the pig liver demonstrating rapid clearance of human platelets within minutes after perfusion.



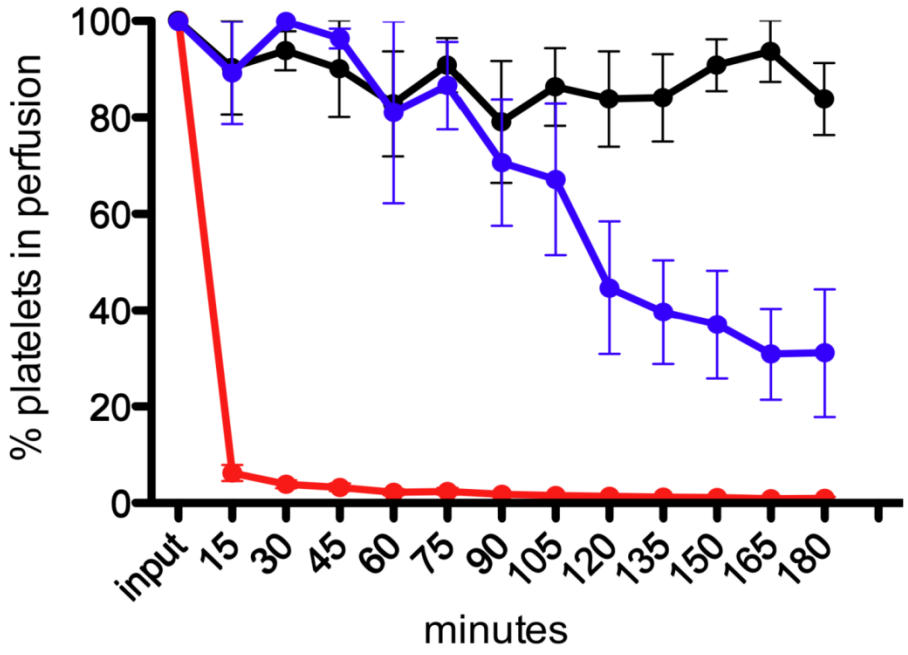
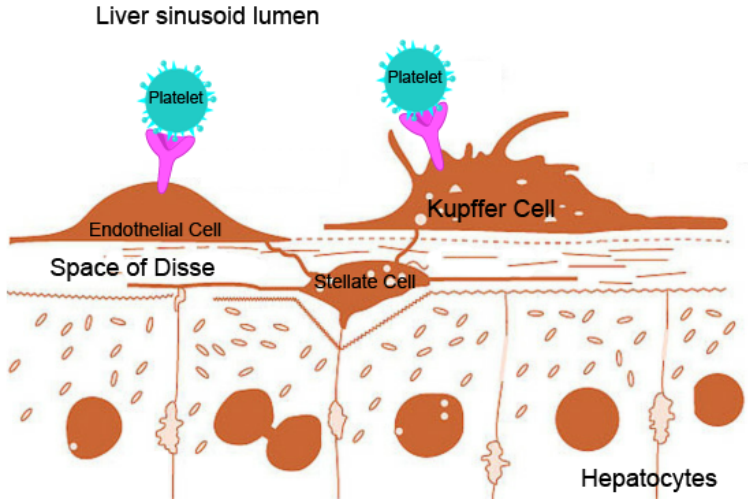


Figure 1-7. Diagram of the liver sinusoidal cells and platelet recognition and confocal images of domestic pig liver biopsies at various time intervals after perfusion of labeled human platelets using ex vivo circuit from Burlak 2010 (53). Labels are as follows: CD31 (red) labeling endothelial cells, Carboxyfluorescein succinimidyl ester (CFSE)-labeled human platelets (green), and CD107a (blue) denoting lysosomes. Human platelets begin to bind to the liver sinusoidal surface (white arrows) with subsequent fusion with lysosomes to form phagosomes (yellow arrows) and eventual movement of CFSE into hepatocytes (red arrow).



Adapted from MIT OpenCourseWare liver sinusoidal cells diagram under the creative commons license agreement: http://ocw.mit.edu/terms/

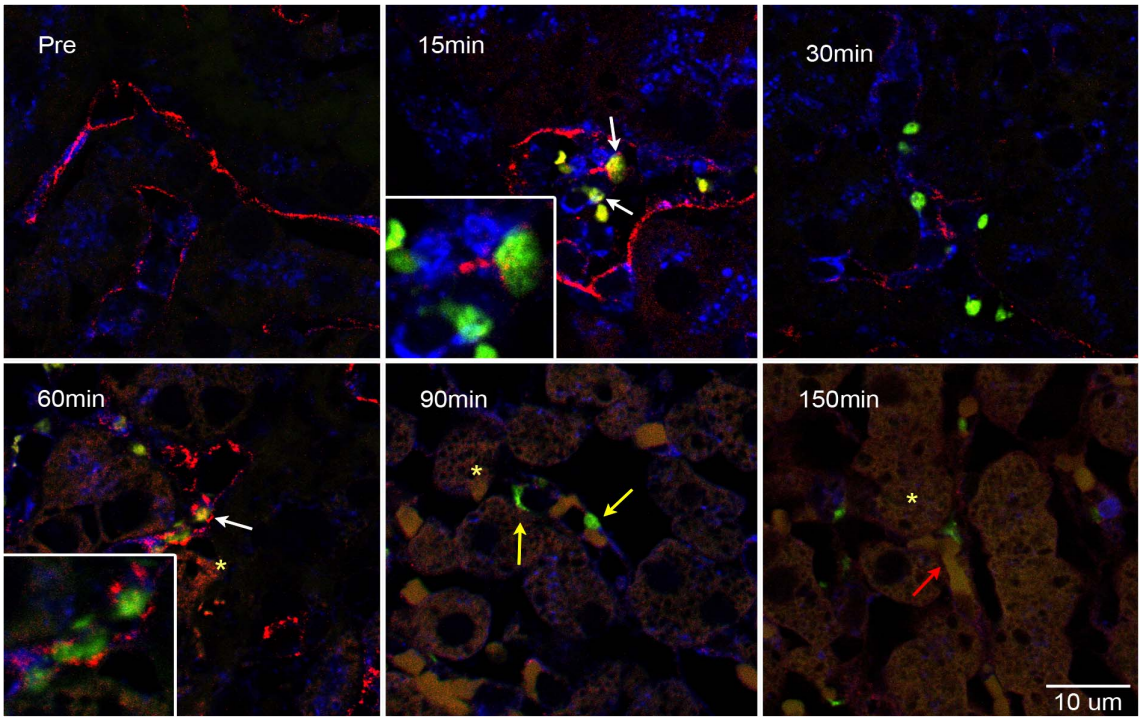


Figure 1-8. Chilled platelet clearance model adapted from Hoffmeister 2011 (59). Platelets with increased exposure of terminal N-acetylglucosamine are recognized by $\alpha M\beta_2$ integrin (CD11b/CD18, complement receptor 3, macrophage antigen complex 1). Platelets with increased terminal galactose are recognized by Hepatic lectin 1 (HL1) and Hepatic lectin 2 (HL2) also known as Asialoglycoprotein receptors 1 and 2. Platelets with sialic acids that cap exposure of terminal N-acetylglucosamine and galactose continue to circulate.

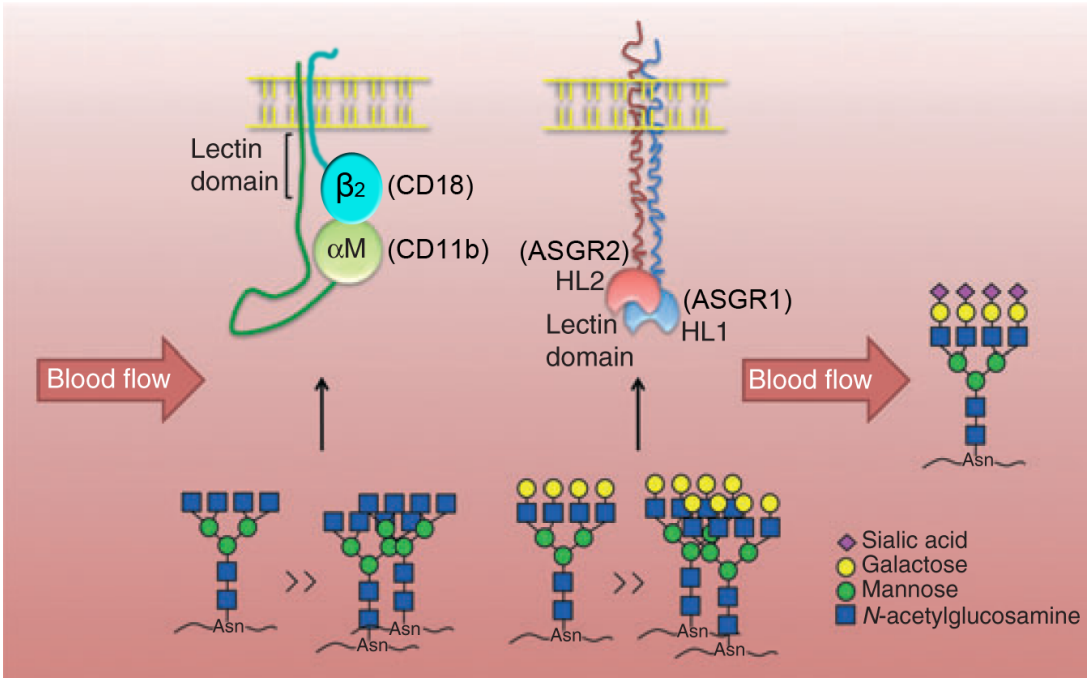


Figure 1-9. Antibody-mediated rejection model. Human antibodies recognize graft resulting in complement activation and damage to graft endothelial cells. Contraction and damage to endothelial cells expose the extracellular matrix resulting in platelet activation and thrombosis leading to downstream ischemia and eventual graft loss.

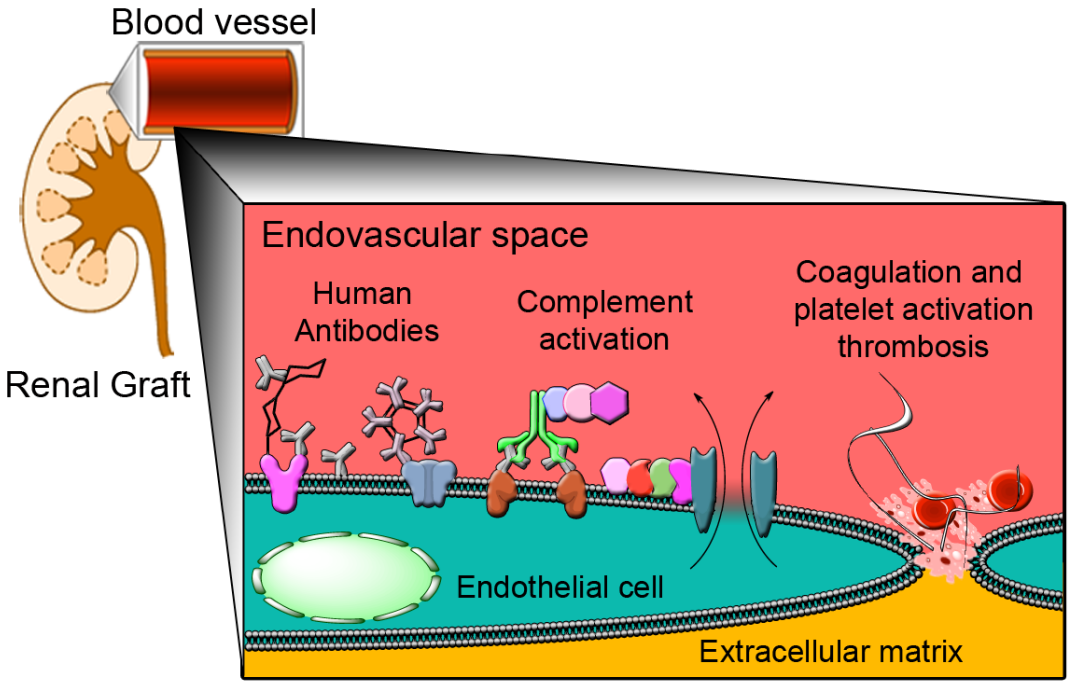
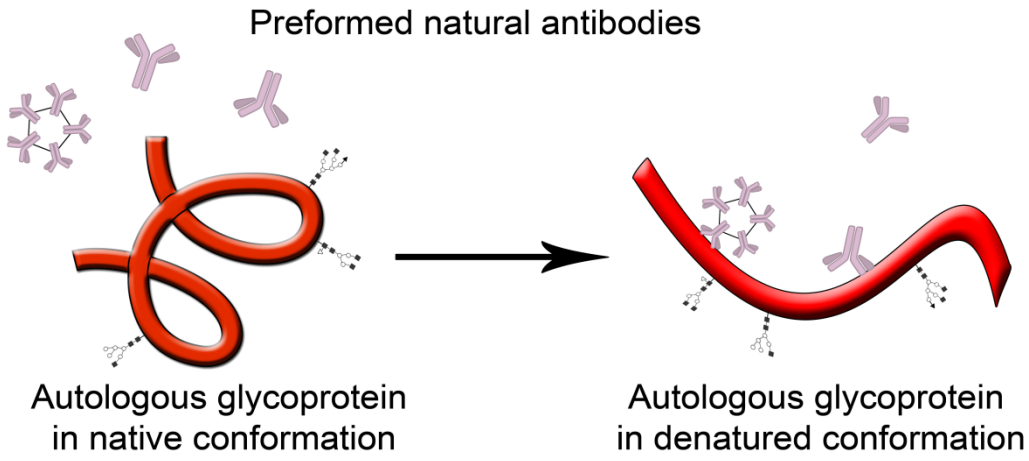


Figure 1-10. (A) Diagram representation of the exposure of normally hidden physiologic antigenic epitopes resulting from denaturation of a glycoprotein. (B) Normal human serum antibody recognition of native conformation and heat denatured human albumin. Native conformation albumin travels farther on the gel due secondary protein structure. Denatured human albumin is recognized by both normal serum IgM and IgG.

A.



B.

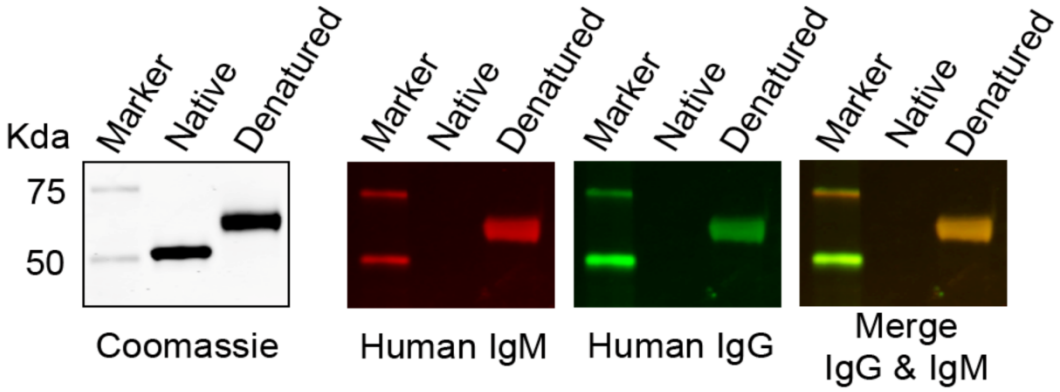


Figure 1-11. Diagram for known carbohydrate antigens between domestic pigs, α1,3 galactosyltransferase knockout (GTKO) pigs and humans. Domestic pigs express galactose α1,3 galactose (αGal) and N-glycolylneuraminic acid (Neu5Gc) containing carbohydrates known as Hanganutziu-Deicher antigens (HD). GTKO pigs lack the αGal oligosaccharide on glycoproteins but continue to express Neu5Gc. Humans express N-acetylneuraminic acid (Neu5Ac) and lack αGal and Neu5Gc expression.

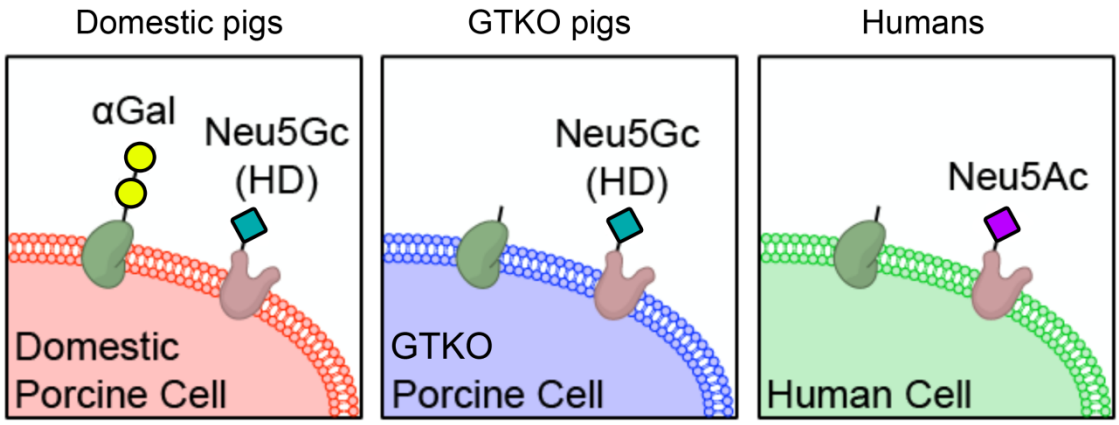


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Figure 1-3

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Chapter 2

Primary porcine Kupffer cell phagocytosis of human platelets involves the CD18 receptor

Transplantation

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ABSTRACT

Background: Hepatic failure has been treated successfully with clinical extracorporeal perfusions of porcine livers. However, dog to pig and pig to baboon liver xenotransplant models have resulted in severe bleeding secondary to liver xenograft-induced thrombocytopenia. Kupffer cells (KC) are abundant phagocytic cells in the liver. Kupffer cells express the CD11b/CD18 receptor, which has been implicated in chilled platelet binding and phagocytosis through interaction with platelet surface proteins and carbohydrates. We sought to identify the role of KC CD18 in liver xenograft induced thrombocytopenia.

Methods: Primary pig KC were characterized by flow cytometry, immunoblot and quantitative PCR. Pig KC were used in inhibition assays with fluorescently labeled human platelets. The CD18 receptor was targeted for siRNA knockdown.

Results: Domestic and α1,3-galactosyltransferase double knockout porcine KC cultures were ~92% positive for CD18 as detected by qPCR and flow cytometry. Use of CD18 blocking antibodies resulted in reduction of human platelet binding and phagocytosis. Additionally, asialofetuin, not fetuin, inhibited platelet phagocytosis suggesting the involvement of an oligosaccharide binding site. Furthermore, reduced CD18 expression by siRNA, resulted in decreased human platelet binding.

Conclusions: Our data suggest primary pig KC bind and phagocytose human platelets with involvement of CD18. Further understanding and modification of CD18 expression in pigs may result in a liver xenograft with reduced

thrombocytopenic effects, which could be used as a bridge to allogeneic liver transplantation.

INTRODUCTION

The major barrier to liver xenotransplantation is thrombocytopenia that occurs minutes after graft reperfusion (1, 2). Non-human primates transplanted with a α1,3-galactosyltransferase knockout (GTKO) porcine liver xenograft survive for 4-7 days despite the loss of platelets (1). In an ex vivo pig liver perfusion model, human platelets were removed by sinusoidal endothelial cells and Kupffer cells (KC) (3).

KC have the capacity to phagocytose large numbers of cells during xenogeneic interactions. Pig KC exposed to normal human blood during a normothermic ex vivo pig liver perfusion phagocytosed nearly all the erythrocytes from three units of human blood in 72 hours (4). Xenogeneic uptake of human erythrocytes is caused, in part by a disparity between pig and human carbohydrates expressed on the erythrocyte surface (5). KC have also been implicated in phagocytosis of cold activated platelets through the heterodimer CD11b/CD18 through the recognition of exposed β-glucans, specifically β-N-acetyl D glucosamine (β-GlcNac) (6-8). CD18 forms heterodimers with CD11a, b, c, d which comprise the family of cell surface receptors, β_2 integrins, which are expressed on macrophages and are involved in binding bacterial cell wall proteins, facilitating cellular adhesion, cell-cell interactions and phagocytosis (9-11). The necessity of CD18 and the formation of heterodimers with CD11a, b, c, d in binding and phagocytosis of platelets is unclear. However, specific point mutations in CD18 abolished ligand recognition by the receptors CD11a/CD18 and Mac-1 implicating the importance of CD18 in receptor-ligand interaction (12).

Mac-1 differs from other β_2 integrins in that CD11b has a β-glucan binding domain capable of binding β-GlcNac (13). In addition, Mac-1 has been shown to bind foreign fungal oligosaccharides containing β-GlcNac, also found on autologous-branched chains on the extracellular matrix and glycoproteins on mammalian cells (14).

The production of the GTKO pig has made it possible to consider using a pig liver for temporary life support as a bridge to allotransplantation. The loss of platelets in the immediate reperfusion period represents a barrier that must be overcome before liver xenotransplantation can be applied clinically. The mechanism for platelet loss in the liver xenotransplant setting is currently unknown. Porcine liver KC and liver sinusoidal endothelial cells are involved in xenotransplantation-induced thrombocytopenia (3). In this manuscript, evidence is presented which shows that the CD18 cell surface receptor on domestic and double α-galactosyltransferase knockout/human decay accelerating factor transgenic (GTKO/hDAF) porcine Kupffer cells bind and induce phagocytosis of human platelets in vitro.

RESULTS

Kupffer cells isolated from porcine liver sinusoids express macrophage markers

Primary porcine domestic and GTKO/hDAF KC were isolated to study their role in xenogeneic thrombocytopenia. Day-three live cultured primary porcine GTKO/hDAF KC under 10x magnification or fixed under 40x magnification and Giemsa stained had a characteristic slightly indented macrophage nuclear morphology (Figure 2-1A). There were no histological differences observed between domestic and GTKO/hDAF porcine KC. Primary KC from domestic and GTKO/hDAF pigs were positive for macrophage markers CD11b, CD14, CD18 and CD45 by flow cytometric analysis with gate set on propidium iodide-negative cells (Figure 2-1B). The live cell population had uniform histograms with average values of 92.5% (domestic) and 91.3% (GTKO/hDAF) KC purity based on macrophage markers. The anti-CD18 monoclonal blocking antibody with cross reactivity with porcine CD18 was used for flow cytometric analysis (15). Domestic and GTKO/hDAF porcine KC have a statistically significant higher relative gene expression of CD11b and CD18 compared to RNA profiles of domestic and GTKO/hDAF porcine whole liver using unpaired Student's t-test (Figure 2-1C). Isolated and cultured primary domestic and GTKO/hDAF fibroblasts were used as negative control for Mac-1 expression ($n=3$, $p<0.05$) (Figure 2-1C). These results suggest that KC are the predominant source of Mac-1 in the liver. Measurements of CD11b and CD18 mRNA levels between domestic and GTKO/hDAF KC, pulmonary alveolar macrophages,

splenic macrophages and peripheral blood monocytes were not statistically different using one-way ANOVA and Neuman-Keuls multiple comparison tests indicating similarity in gene expression for Mac-1 (data not shown).

Pig Kupffer cells bind and phagocytose human platelets in vitro

To assess KC ability to bind and phagocytose platelets, in vitro assays and confocal microscopy were used. Domestic and GTKO/hDAF porcine KC rapidly bound and phagocytosed more human platelets compared to autologous platelets ($n=2\text{-}5$, $p<0.05$) (Figure 2-2A) demonstrating xenogeneic platelet uptake in vitro. The highest rate of platelet uptake occurred in the first 15 minutes after incubation with platelets. Unlabeled platelets and KC had low levels of auto-fluorescence, which contributed minimally to the detectable fluorescence of CFSE-labeled human platelets.

Confocal microscopy of domestic and GTKO/hDAF KC in culture demonstrated the ability of KC to bind and phagocytose platelets and expression of CD18 and CD11b receptors on porcine Kupffer cells. Confocal analysis revealed that CFSE-labeled platelets (green) co-localized with CD11b (red) rich areas in the cell shown in the merged confocal image (orange) (Figure 2-2B, yellow arrows). Porcine CD18 (red) also co-localized (orange) with human platelets (green) at various stages of degradation in what are likely phagosomes (Figure 2-2C, yellow arrows).

Inhibition of human platelet binding and phagocytosis by domestic and GTKO/hDAF pig Kupffer cells

Experiments to prevent KC from phagocytosing human platelets were undertaken utilizing two methods of inhibition. Fetuin, a glycoprotein composed of sialic acid terminated β-glucans had no inhibitory effects on KC platelet uptake at concentrations tested (Figure 2-3A). However, asialofetuin (fetuin with terminal sialic acids removed exposing more β-glucans including β-GlcNac oligomers) had significant levels of platelets blocked from binding and phagocytosis in a dose-dependent manner (2-3A). Inhibition of human platelet phagocytosis was shown as a ratio of asialofetuin to fetuin control treatment at each concentration. Domestic KC were inhibited from binding human platelets with asialofetuin by 35% (5mg/mL), 72% (10mg/mL) and 75% (20mg/mL) (n=2-7) (Figure 2-3A). Kupffer cells isolated from GTKO/hDAF pigs were inhibited with asialofetuin by 47% (5mg/mL), 60% (10mg/mL), 76% (20mg/mL) (n=2-3) (Figure 2-3A). Inhibition percentages between concentrations for both domestic and GTKO/hDAF pig KC reached statistical significance using one-way ANOVA (n=2-7, $p<0.05$) with Newman Keuls post hoc test when 5 mg/mL was used. No statistical difference was observed between the 10mg/mL and 20mg/mL treatments.

The mouse anti-CD18 blocking antibody used to identify CD18 expressing KC in 2-1B, and 2-2C was used to inhibit human platelet binding and phagocytosis in a dose-dependent manner represented as anti-CD18 blocking antibody normalized to IgG_{1k} isotype control treatment at each dose. Human

platelets were inhibited from phagocytosis by domestic pig KC by 43% (2.5ug/mL), 70% (5ug/mL) and 73% (10ug/mL) (n=2-7) compared to no treatment (Figure 2-3B). Kupffer cells from GTKO/hDAF pigs were inhibited by 18% (2.5ug/mL), 31% (5ug/mL) and 55% (10ug/mL) (n=2-3) (Figure 2-3B). Percent inhibition between no treatment and antibody treatment for both domestic and GTKO/hDAF porcine KC were statistically significant using one-way ANOVA and Neuman-Keuls multiple comparison tests (n=2-3, $p<0.05$).

siRNA inhibition of platelet binding

To study the CD18 receptor more directly, porcine CD18 antisense RNA oligos were designed, incubated with ferrous beads and used for transfection of primary domestic and GTKO/hDAF KC. The results were statistically significant with a 27% and 26% decrease in CD18 transcript levels, respectively for domestic (n=3, $p<0.05$) and GTKO/hDAF (n=3, $p<0.05$) KC when compared to siGenome negative control, non-specific antisense off-target oligos using an unpaired Student's t-test (Figure 2-4A). CD18 protein expression from replicate domestic and GTKO/hDAF KC cultures showed a 24% and 11% decrease in CD18 receptor expression, respectively (n=3, $p<0.05$) (Figure 2-4A). Ferrous bead-siRNA treated KC were used in the human platelet binding and phagocytosis assay to determine the functional effect of reduced CD18 expression. Human platelet binding and phagocytosis was reduced by 31.5% (n=3, $p<0.05$) and 13.5% (n=3) for domestic and GTKO/hDAF KC, respectively (Figure 2-4C). Although platelet binding and phagocytosis for CD18 siRNA treated GTKO/hDAF was not statistically significant, there was a trend towards

decreased platelet binding similar to domestic KC. No statistical difference was found between siGenome and untreated group for mRNA transcript levels, receptor expression and platelet binding and phagocytosis (data not shown).

MATERIALS AND METHODS

Production of GTKO/hDAF pigs

Double α1,3galactosyltransferase knockout / human decay accelerating factor cells were acquired from the National Swine Resource and Research Center (NSRRC:0009) at the University of Missouri and cultured in Dulbecco's Modified Eagle Medium with 15% FBS and allowed to grow to confluence and then harvested (24). A single GTKO/hDAF cell was injected into matured enucleated in vitro oocytes (Minitub of America, Mount Horeb, WI and/or ART Madison WI, USA). Electrical pulses induced fusion and activation (25), and resulting embryos were transferred to a recipient on the first day of estrous.

Pig liver procurement

Domestic (primarily Landrace breed) and GTKO/hDAF (Miniature background) pigs, weighing 22-45kg, were anesthetized, intubated, prepped with chlorohexidine and draped in sterile fashion. The liver was dissected, excised, flushed with 2 liters of histidine-tryptophan-ketoglutarate preservation solution (Essential Pharmaceuticals. LLC, Newtown, PA) and stored on ice until time of cell isolation. Cadaveric domestic pig livers were also harvested from the abattoir in similar fashion within 2 minutes of exsanguinations with the exception of performing a liver flush with heparin-containing preservation solution.

Isolation of Kupffer cells

Procured chilled porcine livers were flushed with 0.0125ug/mL collagenase type IV (Sigma, St. Louis, MO) diluted in Hanks' balanced salt solution (HBSS) (Sigma-Aldrich, St. Louis, MO). Cells were pelleted by

centrifugation at 400 x g at room temperature, erythrocytes lysed using ammonium chloride solution, washed with phosphate buffered saline (PBS), then washed with PBS containing 1mM EDTA. Cells were cultured in polystyrene plates in the incubator in RPMI-1640 medium supplemented with 10% fetal bovine serum (v/v), 10mM HEPES, penicillin-streptomycin and amphotericin B. Cultures were washed after one hour incubation with generous amounts of PBS then HBSS to remove non-adherent cells. Cultures were washed daily with HBSS until assayed on day 3.

Platelet isolation and preparation

Fresh human or porcine whole blood was collected in anticoagulant citrate dextrose (ACD) and centrifuged for 3 minutes. Platelet-rich plasma was collected and centrifuged at 5000 x g for 5 minutes. Platelets were re-suspended and washed with PBS. Platelets used for in vitro KC assays were incubated and labeled with carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA) for 30 minutes then washed with PBS. Platelets were stored at room temperature and used within a few hours.

Flow cytometry

KC were released from culture plates on day 3 using 12mM lidocaine in PBS at 37°C (26). Cells were blocked with PBS containing 0.5% BSA, 0.02% sodium azide and stained with anti-CD11b (American Type Culture Collection, Manassas, VA. HB-249), anti-CD14 (R&D Systems, Minneapolis, MN.), anti-CD18 (Developmental Studies Hybridoma Bank, Iowa City, IA, H52) and anti-CD45 (ABD Serotec, Raleigh, NC.) for one hour followed by two washes with

buffer. Mouse isotypes IgG_{1k} and IgG_{2b} (eBioscience, San Diego, CA.) were used as negative control. Propidium iodide staining was used to exclude dead cells. Goat anti-mouse IgG PE (Jackson ImmunoResearch Laboratories, West Grove, PA.) was used as secondary. All flow cytometry data was collected and analyzed on the Accuri C6 flow cytometer and CFlow software (Accuri, Ann Arbor, MI).

Histological Analysis of Kupffer cells

Live cultured and Giemsa stained KC microscopy was completed using Olympus CX41, CKX41 microscopes. Confocal microscopy was performed using a Olympus IX81/FV1000 microscope. For confocal analysis, KC were incubated with CFSE-labeled platelets for 1 hour at 37 °C, fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton-X 100, blocked with 1% BSA in PBS. Cells were labeled with anti-CD18 or anti-CD11b, followed by donkey anti-mouse IgG DyLight 649 (Jackson ImmunoResearch Laboratories, West Grove, PA) and mounted with Prolong Gold with DAPI (Invitrogen, Carlsbad, CA). Slides were imaged using settings from the negative controls. A dashed white line was used to mark the outer membranes of each cell by increasing background fluorescence. Scans were run in sequential mode to prevent cross over between channels.

In vitro Kupffer cell-platelet binding, phagocytosis and inhibition assay

Primary KC, 1 x 10^6 per well, were cultured for 3 days at 37 ºC (5% CO_2) in opaque black wall-clear bottom cell culture-treated 96-well plates. Human and porcine platelets were isolated and labeled simultaneously and plated in empty

wells in eight dilutions as platelet fluorescence standard. Labeled platelets (4 x 10^6) were added to wells containing KC. Plates were centrifuged at 2000 x g to synchronize platelet settling. Replicate wells for each time point were washed with PBS three times then fixed with 4% paraformaldehyde in PBS for 20 minutes. After obtaining all time points, fluorescence was measured using a SpectraMax M2e plate reader (Molecular Devices Corp. Sunnyvale, CA). Assays comparing human and porcine platelets were completed on the same plate. Differences in fluorescence between human and porcine platelets were accounted for by a factor determined by the ratio between human and porcine platelet fluorescence standards. Adjusted fluorescent values were averaged and the highest value for each experiment was set to 100%. This normalization enabled comparison of data collected from different days by eliminating the technical variability. Wells containing KC without labeled platelets were used to determine and subtract auto-fluorescence. Inhibition assays were performed in similar manner with wells treated with anti-CD18 antibody, mouse IgG1k isotype control, or asialofetuin and fetuin (Sigma-Aldrich, St. Louis, MO) for 30 minutes prior to the addition of platelets.

siRNA knockdown of CD18

KC cultured for one day were washed with HBSS and fresh medium added. During optimization siGenome transfected, mock transfected with Purple NIMT beads (Genovis, Sweden) and non-transfected KC showed no significant differences in protein expression. Purple NIMT beads with CD18 or siGenome control siRNA (Dharmacon, Chicago, IL) were incubated for 30 minutes at room

temperature. Following incubation, the bead mixture was added to KC cultures at 60 pmol/mL final and 15uL 1 x NIMT beads/mL final. The target sequence for CD18 siRNA was sense strand: CCAAGUUUGCUGAGCGCUAAA and anti-sense strand UAGCGCUCAGCAAACUUGGAA. On day 3 of culture, 48 hours after addition of the siRNA bead complex, cells were incubated with platelets for the uptake assay or RNA was harvested for qPCR analysis. Knockdown was analyzed by qPCR using CD18 probes (Applied Biosystems: CD11b AssayID-Ss03374593_m1, CD18 AssayID-Ss03392626_u1) described above and whole liver control in a comparative CT experiment on a STEP-ONE PLUS (Applied Biosystems, Carlsbad, CA) real time PCR system or by In-Cell Western™ Assay Kit (LI-COR Biosciences Lincoln, Nebraska) using the H52 anti-CD18 blocking antibody without cell permeabilization.

Statistical analysis

GraphPad Prism software was used for graphical and statistical analyses using paired and unpaired Student's t-tests, one-way ANOVA and Newman-Keuls multiple comparison tests.

DISCUSSION

Liver xenotransplantation-induced thrombocytopenia prevents the use of porcine liver xenografts as a bridge to allotransplantation (1, 2). Both pig KC and liver sinusoidal endothelial cells have been shown to phagocytose human platelets in ex vivo pig liver perfusion (3). Understanding the mechanisms by which these cells recognize and phagocytose human platelets would generate targets for genetic modifications of donor pigs to prevent xenogeneic thrombocytopenia. Mouse Mac-1 antigen binds cold-stored platelets that have exposed β-GlcNac bearing oligosaccharides (7). The ability of the Mac-1 antigen to bind ligands is dependent on the I-domain, recognizing amino-acid sequences, and the lectin-binding domain, recognizing β-glucans (14, 16, 17). The cytoplasmic tail of CD18 interacts with the cytoskeleton suggesting direct involvement with receptor trafficking or phagocytosis (18). Involvement of the Mac-1 receptor for both non-opsonic and opsonic phagocytosis has been demonstrated (19). Analysis of leukocyte adhesion deficiency syndrome, a disease caused by the lack of CD18, indicates that CD18 is critical for maturation and expression of CD11a, b, c and the ability of Mac-1 antigen to recognize a ligand (12, 20). Studies completed on CD18-deficient mice showed a decreased ability to bind and phagocytose β-glucans (21). Therefore, we studied the role of porcine KC CD18 receptor in liver xenograft-induced thrombocytopenia.

We confirmed that our isolated cells had morphological characteristics and cell surface markers consistent with KC, including the ability of CD18 blocking antibody (clone H52) to recognize porcine CD18 (22). The H52 anti-CD18

antibody was used for flow cytometry, confocal microscopy and inhibition of CD18 function in platelet phagocytosis. Platelet uptake assays as well as confocal microscopy verified that isolated KC bound and phagocytosed human platelets in vitro. Co-localization of CD18 and CD11b receptors with platelets suggest that these receptors are involved with human platelet phagocytosis. CD18 inhibition assays resulted in a moderate reduction of platelet phagocytosis compared to the isotype control indicating that the blocking antibody does not completely abolish binding functions of porcine CD18 or that other receptors may be involved. It is also possible that intracellular stores of CD18 were mobilized to the cell surface when KC were treated with blocking antibody (23). To evaluate the involvement of platelet carbohydrates, inhibition assays with the glycoproteins asialofetuin and fetuin were undertaken. Asialofetuin treatment of KC resulted in substantial reduction in platelet binding and phagocytosis in comparison to fetuin, indicating the involvement of a lectin-binding domain such as in Mac-1. Asialofetuin has exposed β-GlcNac oligomers, however, many other oligosaccharides are exposed, implicating possible involvement of other lectin receptors.

To further define CD18 receptor involvement and to demonstrate that decreased CD18 gene expression attenuates platelet uptake, primary porcine KC CD18 knockdown was studied using siRNA. Knocking down gene expression using siRNA is difficult in primary cells especially terminally differentiated KC. The use of primary KC for siRNA was challenging due to variability in their maturation or differentiation state, primary cell resistance to chemical transfection

reagents and anionic nature of siRNA. There have been several methods used recently to target phagocytic cells using iron-oxide nanoparticles. Although we employed this technique, knockdown of primary KC CD18 mRNA expression proved difficult. CD18 protein expression may have been hampered by the presence of Mac-1 stores that exceed the protein's turnover time relative to CD18 mRNA production (23). Nonetheless, there was a corresponding decrease in CD18 receptor and platelet phagocytic functions supporting the hypothesis that a decrease in CD18 would reduce platelet binding and phagocytosis. The reduction of platelet phagocytosis by siRNA knockdown supports the CD18 antibody blocking and substrate inhibition results.

Our results show that CD18 is involved in liver xenotransplantation-induced thrombocytopenia suggesting that reduction or elimination of CD18 expression in pigs may yield a xenograft with reduced thrombocytopenic effects. The inhibition of platelet uptake by blocking CD18 was pronounced, but incomplete, which emphasizes the need for further investigation of CD18/CD11b and other receptors. The present study focuses on Kupffer cells but our prior studies demonstrated that liver sinusoidal endothelial cells also bind and phagocytose human platelets (3). Together, these studies suggest that receptors expressed by KC and liver sinusoidal endothelial cells should continue to be investigated.

Figure 2-1. Histological, flow cytometric and quantitative mRNA analyses of day 3 cultured domestic and GTKO/hDAF porcine KC. (A) Representative light micrograph [n=10] of isolated KC (10x) on left and Giemsa stained light micrograph (40x) [n=10] on right. (B) Flow cytometric histograms for macrophage markers (red) CD11b, CD14, CD18, CD45 [n=3] of domestic and GTKO/hDAF KC with isotype control (black). (C) Relative mRNA comparisons of CD11b and CD18 between isolated domestic and GTKO/hDAF KC, liver and fibroblasts (negative control) with values normalized to liver. *Indicate statistical significance ($p<0.05$, n=3).

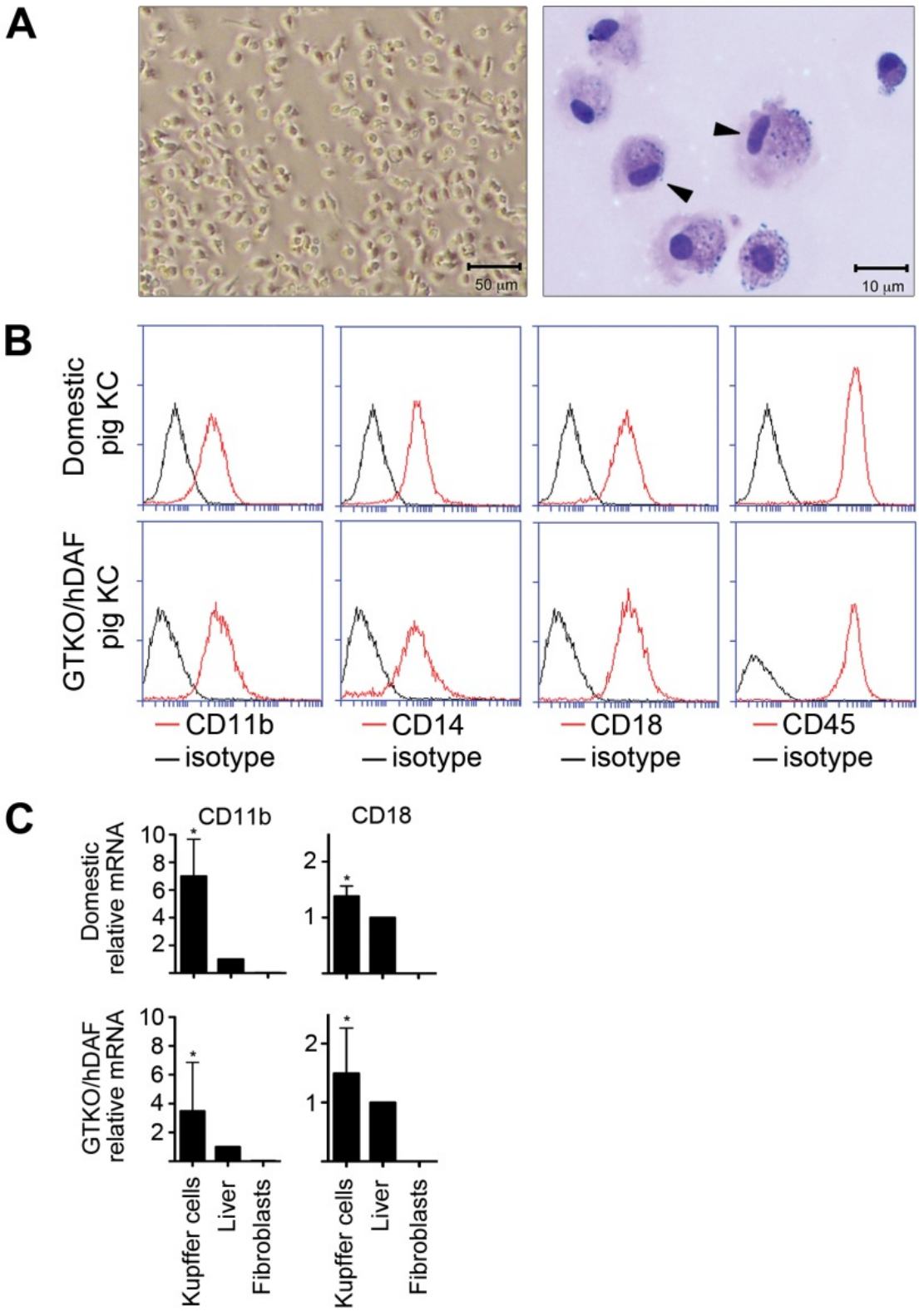

A
50 μm
10 μm
B
Domestic pig KC
GTKO/hDAF pig KC
CD11b
isotype
CD14
isotype
CD18
isotype
CD45
isotype
C
CD11b
CD18
Domestic relative mRNA
GTKO/hDAF relative mRNA
10
8
6
4
2
1
*
Kupffer cells
Liver
Fibroblasts

Figure 2-2. In vitro platelet binding and phagocytosis and confocal microscopy. (A) Plot of fluorescence representing percent human platelets bound and phagocytosed by domestic and GTKO/hDAF porcine KC at various time points shown with +/- 1 standard deviation bars. Domestic and GTKO/hDAF KC phagocytosis of human platelets was statistically significant compared to phagocytosis of autologous domestic platelets ($n=2\text{-}5$, $p<0.05$) and GTKO/hDAF platelets ($n=2\text{-}5$, $p<0.05$). (B) Confocal micrographs of domestic and GTKO/hDAF KC binding and phagocytosing human platelets with staining for CD11b. (C) Confocal micrograph of domestic and GTKO/hDAF KC binding and phagocytosing human platelets with staining for CD18. Outer membrane of cells was defined with a dashed white line. Images are representative of three biological replicates.

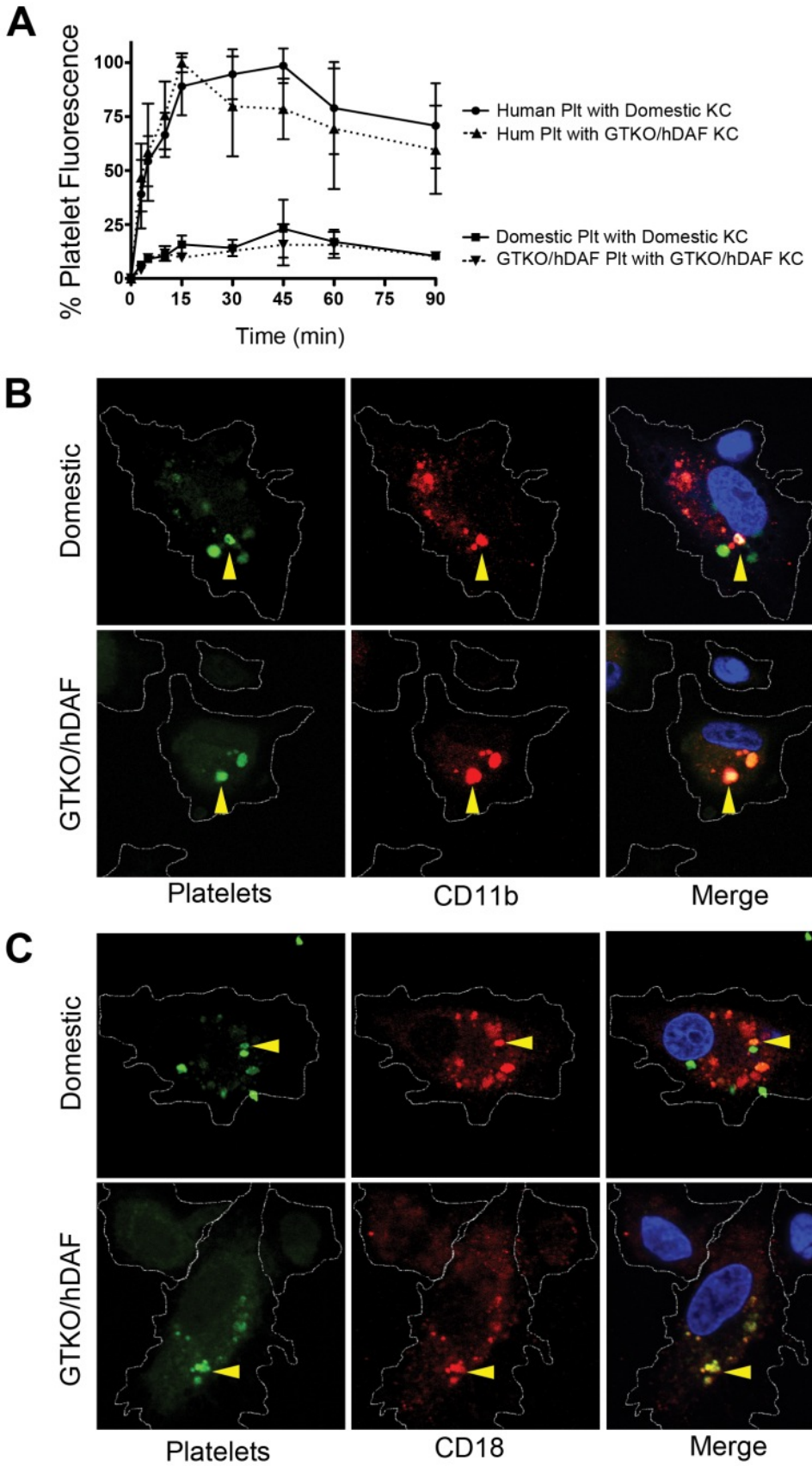
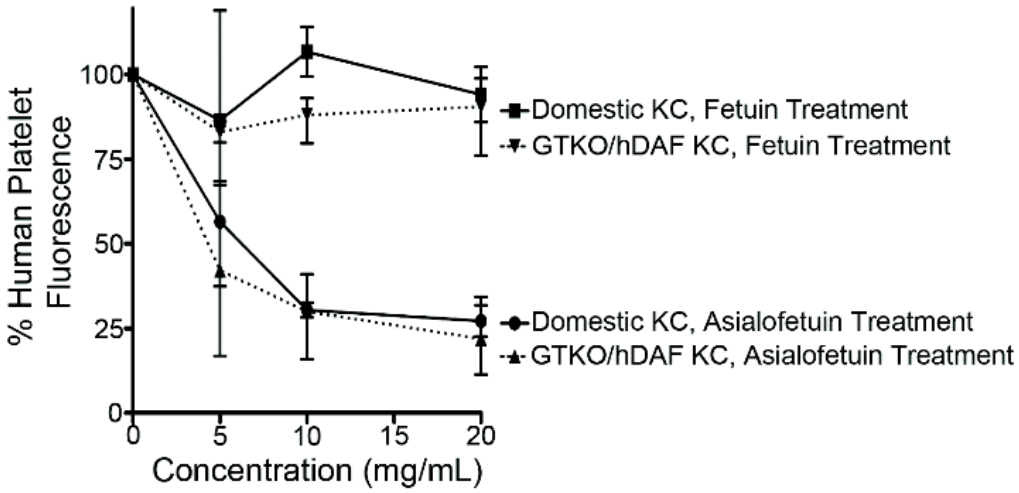

A
% Platelet Fluorescence
100
75
50
25
0
0
15
30
45
60
75
90
Time (min)
Human Plt with Domestic KC
Hum Plt with GTKO/hDAF KC
Domestic Plt with Domestic KC
GTKO/hDAF Plt with GTKO/hDAF KC
B
Domestic
GTKO/hDAF
Platelets
CD11b
Merge
C
Domestic
GTKO/hDAF
Platelets
CD18
Merge

Figure 2-3. Inhibition of human platelet phagocytosis by domestic and GTKO/hDAF porcine KC by asialofetuin and anti-CD18 blocking antibody. (A) Graphical representation of inhibition of human platelet phagocytosis by fetuin and asialofetuin treatments normalized to a non-treated control. Error bars represent +/- 1 standard deviation plotted against 5, 10 and 20 mg/mL concentrations were statistically significant for both domestic ($n=3$, $p<0.05$) and GTKO/hDAF ($n=3$, $p<0.05$) compared to no treatment and fetuin treated KC. (B) Anti-CD18 blocking of human platelet phagocytosis normalized to an isotype control with +/- 1 standard deviation. *Indicates statistical significance compared to no treatment ($n=3$, $p<0.05$). **Indicates statistical significance compared to all other values ($n=3$, $p<0.05$).

A


% Human Platelet Fluorescence
100
75
50
25
0
0
5
10
15
20
Concentration (mg/mL)
Domestic KC, Fetuin Treatment
GTKO/hDAF KC, Fetuin Treatment
Domestic KC, Asialofetuin Treatment
GTKO/hDAF KC, Asialofetuin Treatment

B

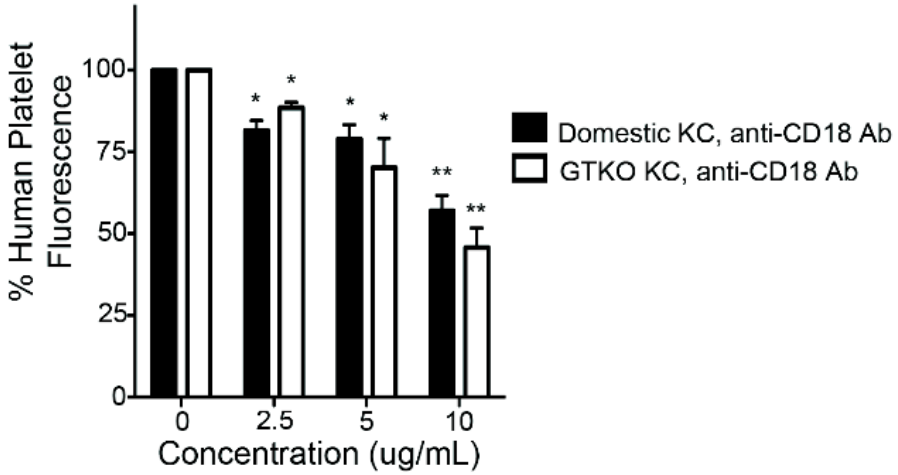
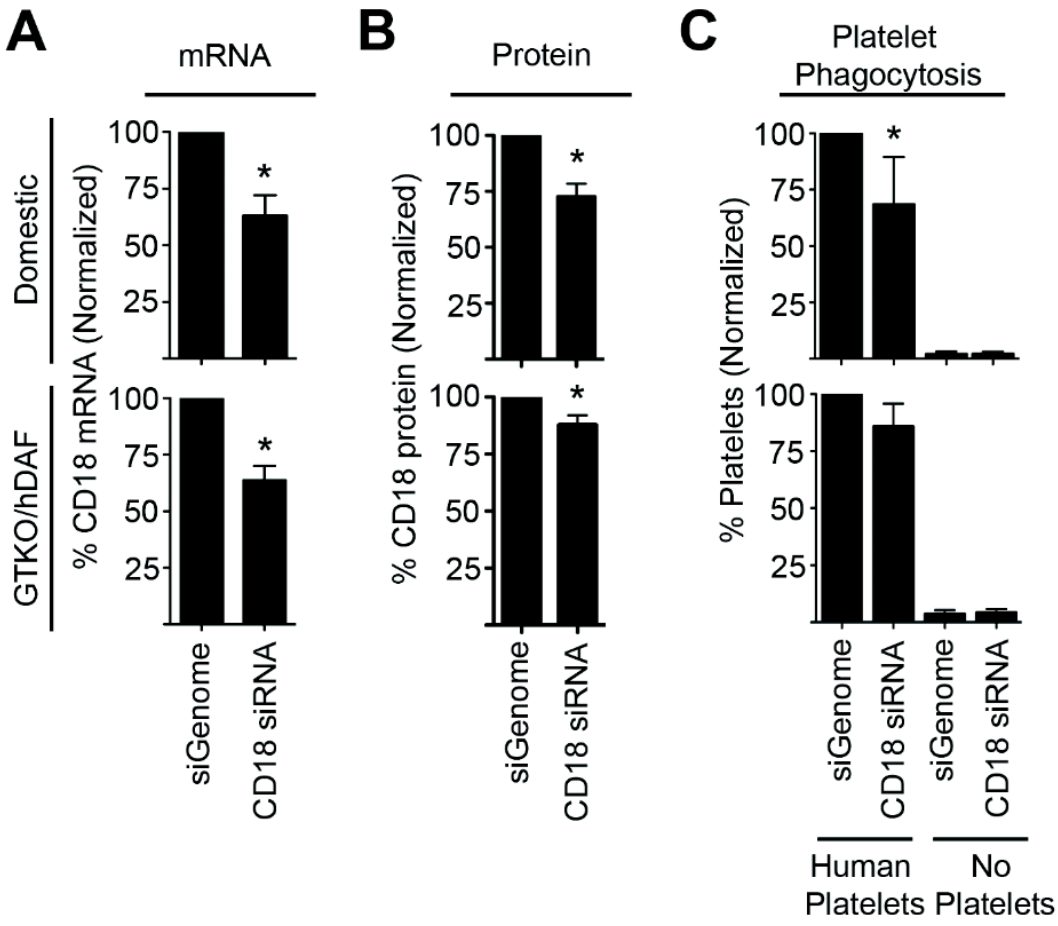

% Human Platelet Fluorescence
100
75
50
25
0
*
*
*
*
**
**
0
2.5
5
10
Concentration (ug/mL)
Domestic KC, anti-CD18 Ab
GTKO KC, anti-CD18 Ab

Figure 2-4. CD18 siRNA knockdown of CD18 mRNA, protein and phagocytosis of domestic and GTKO/hDAF porcine KC. (A) Domestic and GTKO/hDAF porcine KC CD18 mRNA (%) measured by quantitative PCR. (B) Domestic and GTKO/hDAF porcine KC surface CD18 expression (%) measured by In-Cell Western™. (C) Domestic and GTKO/hDAF porcine KC human platelet phagocytosis (%) with no platelet KC autofluorescence control measured by in vitro platelet assay. All error bars represent +/- 1 standard deviation. *Indicates statistical significance (n=3, $p<0.05$).



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Chapter 3

Differences in human and porcine platelet oligosaccharides may influence phagocytosis by liver sinusoidal cells in vitro.

Xenotransplantation

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Project design, writing and editing of the manuscript, platelet isolation, generation of figure 3-1, data analysis.

Richard A. Sidner
Assistance with platelet isolation, cell culture, manuscript editing, data anaylsis and statistics.

A. Joseph Tector
Manuscript editing, mentorship, guidance and funding.

Christopher Burlak
Manuscript editing, mentorship and guidance.

ABSTRACT

Background: Acute thrombocytopenia was revealed as a limiting factor to porcine liver xenotransplantation from in vitro and in vivo studies using porcine liver in human and baboon transplant models. The asialoglycoprotein receptor 1 (ASGR1) on liver sinusoidal endothelial cells (LSEC) and Macrophage Antigen Complex-1 (Mac-1) on Kupffer cells (KC) mediate platelet phagocytosis and have carbohydrate-binding sites that recognize galactose and N-acetyl glucosamine in the beta conformation. Analysis of these receptor carbohydrate-binding domains and surface carbohydrates on human and porcine platelets may shed light on the mechanism of xenotransplantation-induced thrombocytopenia.

Methods: An amino acid sequence comparison of human and porcine ASGR1 lectin binding domains was performed. Using fluorescent labeled-lectins, human platelets, domestic and α1,3 galactosyltransferase knockout/human decay accelerating factor (GTKO/hDAF) porcine platelets were characterized by flow cytometry and lectin blot analyses. After desialylation, human and porcine platelets were examined by flow cytometry to determine if sialic acid capping of galactose and N-acetyl glucosamine oligosaccharides in the beta conformation was a factor. Further, desialylated human platelets were studied on primary porcine liver sinusoidal cells with regard to binding and phagocytosis.

Results: Human platelets have four times more exposed galactose β1-4 N-acetyl glucosamine (Galβ) and N-acetyl glucosamine β1-4 N-acetyl glucosamine (βGlcNAc) than fresh porcine platelets. Gal$\beta$and βGlcNAc moieties on porcine

platelets were not masked by sialic acid. Removal of sialic acid from human platelets increased binding and phagocytosis by LSEC and KC.

Conclusions: Differences between human and porcine ASGR1 and Mac-1, in combination with a significantly higher number of galactose and N-acetyl glucosamine-containing oligosaccharides on human platelets contribute, in part, to platelet loss seen in porcine liver xenotransplantation.

INTRODUCTION

Acute thrombocytopenia observed during liver xenotransplantation is mediated partially by liver sinusoidal cells. Both the asialoglycoprotein receptor-1 (ASGR1) and Macrophage Antigen Complex-1 (Mac-1) participate in this process as knockdown of these proteins using siRNA resulted in a decrease in human platelet phagocytosis by porcine liver sinusoidal cells in vitro (1, 2). ASGR1, a component of the Ashwell receptor expressed on liver sinusoidal endothelial cells (LSEC), has a carbohydrate-binding site that recognizes exposed galactose β1-4 N-acetyl glucosamine (Galβ) (1). Kupffer cells express Mac-1, which contains a carbohydrate-binding site for N-acetyl glucosamine β1-4 N-acetyl glucosamine (βGlcNAc) oligosaccharides (3-5). Human platelet binding to porcine liver sinusoidal cells partially depends on the availability of carbohydrate binding sites based upon competition experiments using the glycoproteins fetuin and asialofetuin (1, 2).

The liver clears platelets from the circulation in normal and disease states including senescent platelets, cold-stored transfused platelets and during *Streptococcus pneumoniae* induced-sepsis (6-8). Loss of sialic acid from the surface of platelets during *S. pneumoniae* infection by the bacterium's neuraminidase induced Ashwell receptor-mediated platelet phagocytosis in mice (6). Mac-1 and ASGR1 mediated platelet phagocytosis also occurs with cold-stored platelets used for donation because there is an increase in both concentration and density of βGlcNAc and Galβ (7, 9). In both cases, the sialic acid was suggested to shield galactose and N-acetyl glucosamine

oligosaccharides, protecting platelets from uptake (6, 8). The same receptors are involved in xenogeneic platelet uptake, cold-stored platelet uptake and platelet uptake during *S. pneumoniae* induced-sepsis, hence the mechanism may be similar and analysis of these carbohydrates on human and porcine platelets may elucidate the means of xenotransplantation-induced thrombocytopenia.

Orthologs of ASGR1 from various mammals including humans, mice, and rats have high sequence identity (10). All natural mammalian forms of ASGR1 studied have a high binding affinity for terminal β-linked galactose or N-acetylgalactosamine (11-13). Some endogenous ligands for rat ASGR1 were found to be glycoprotein hormones with terminating Siaα2,6GalNAcβ1,4GlcNAc oligosaccaharides and not β-linked galactose or N-acetylgalactosamine (14). A comparison of ASGR1 from different species examining specificity for glycoproteins with terminal Siaα2,6GalNAcβ1,4GlcNAc revealed that even with very few differences in amino acid (AA) sequence there can be large changes in carbohydrate specificity (10).

Mac-1 orthologs have subunits exhibiting high similarity as well as subunits exhibiting significant differences in binding affinities (15, 16). Antibodies against cd11b, the α subunit of Mac-1, from various species were not found to react across species (15). In contrast, antibodies against cd18, the β subunit of Mac-1 cross-react among species (15). This indicates that the differences among species most likely occur in cd11b, the subunit containing the predicted carbohydrate-binding motif. Rat and human cd11b have significant differences in

affinity for the glycoprotein, recombinant neutrophil inhibitory factor (16). Variation in the CD11b sequence among species contributes to differences in ligand affinity.

In this study, the porcine ASGR1 sequence was identified and the carbohydrate-binding site was predicted based on the known crystal structure of human ASGR1. The predicted human and porcine CD11b carbohydrate binding motifs were compared. Variations in human and porcine ASGR1 and Mac-1 in combination with significantly higher endogenous levels of galactose and N-acetyl glucosamine-containing oligosaccharides may explain in part the phenomenon of liver xenotransplantation-induced thrombocytopenia.

MATERIALS AND METHODS

Structural and sequence analysis of ASGR1, sequence analysis of CD11b

The porcine ASGR1 protein sequence was translated using the Swiss Institute of Bioinformatics expert protein analysis system (SIB EXPASy) from the sequence AK233544.1 in the NCBI database. Porcine and human ASGR1 sequences were aligned and compared. The structural images were created using Pymol Molecular Graphics System (DeLano, W.L., World Wide Web.2002). Human ASGR1 binding domain (protein data bank: 1DV8) was superimposed with GlcNAc (protein data bank:1CBJ). Porcine ASGR1 binding domain was predicted by altering R262 to G262 in Pymol.

The *Sus scrofa* integrin alpha-M AA sequence (GenBank: AEE80503.1) was aligned with the *homo sapiens* integrin alpha-M isoform 1 precurser (GenBank: AAA59544.1) AA sequence using (Basic Local Alignment Search Tool- Protein) BlastP (NCBI), the protein sequence alignment algorithm. A BlastP comparison was also done with only the predicted carbohydrate-binding region and with the rest of the sequence without the predicted carbohydrate-binding domain (17-20). Positive matches include non-identical conserved substitutions using the BLOSUM26 scoring matrix (NCBI).

Platelet preparation

Human and porcine platelets were isolated from fresh whole blood containing anticoagulant citrate phosphate dextrose by centrifugation of whole blood at 2000 x g for 3 minutes, and then centrifugation of platelet enriched plasma at 5000 x g for 5 minutes. Platelets were resuspended in phosphate

buffered saline (PBS); some were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA) and counted using a hemocytometer.

Immunoblot/lectin blot analysis

Platelets were lysed in Laemmli sample buffer with Focus protease arrest (G-bioscience, Maryland Heights, MO) and heat denatured at 95°C. Samples were analyzed using SDS-PAGE and immunoblot or lectin blot. Membranes were blocked with 50% Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) in HBSS for 1 hour followed by incubation with anti-GAPDH (Millipore, Billerica, MA), anti-beta actin (Rockland Immunochemicals for Research Gilbertsville, PA) according to manufacturer recommended specifications. Membranes also were probed with erythrina cristagalli (ECA)-biotin, succinylated wheat germ agglutinin (sWGA)-biotin, and Datura strumonium (DSA)-biotin (EY laboratories, San Mateo, CA). Following primary antibody or lectin binding, membranes were probed with the appropriate secondary antibody or streptavidin conjugated to either IRDye 800CW or IRDye 680CW (LI-COR Biosciences, Lincoln, NE). Blots were scanned with a LI-COR near-infra-red Odyssey scanner (LI-COR Biosciences, Lincoln, NE).

Flow cytometry

Human, domestic, or GTKO/hDAF porcine platelets were washed then stained using lectins from EY Laboratories, (San Mateo, CA) as follows: ECA Lectin to determine quantity of galβ residues; sWGA lectin, to determine βGlcNAc oligosaccharides, or DSA lectin to determine βGlcNAc and Galβ residues. Anti-CD22/Siglec 2-FC (Invitrogen, Carlsbad, CA) was used to

measure exposed α2,6-linked N-glycolylneuraminic acid (Neu5Gc) (1mg/mL each). All platelets were washed and stained in 50% HBSS (Sigma, St. Louis MO) and 50% PBS with 0.02% sodium azide, 0.5% BSA. Secondary antibodies and avidin conjugates were used according to manufacturer's recommendations.

Sialidase treatment and quantification

Human, domestic porcine, and GTKO/hDAF platelets (5 x 10^8/mL PBS) were treated with 1/8 U Sialidase A for 1 hour at room temperature. Following treatment, platelets were collected by centrifugation. Platelets were labeled with CFSE for binding and phagocytoisis assays or stained for flow cytometric analysis.

Porcine liver procurement

GTKO/hDAF pigs were obtained from the National Swine Resource and Research Center (NSRRC) or produced from cells obtained from NSRRC (NSRRC 0009; www.nsrrc.missouri.edu). Livers from domestic or genetically modified pigs were removed as described by (1, 2). Briefly, the liver was removed and perfused through the portal vein with 1-2L cold histidine-tryptophan-ketoglutarate (HTK) solution (Essential Pharmaceuticals, LLC. Newtown, PA). Some livers were procured from the abattoir and flushed with HTK containing heparin (2000U/L) within 2 minutes of exsanguination. All livers were stored at 4°C until placed in the perfusion circuit. Cold-ischemia time was less than 3 hours.

Isolation of Liver Sinusoidal Endothelial Cells and Kupffer Cells

Cold porcine livers were placed in a chilled container (4°C) and flushed with 0.025% of collagenase type IV from *Clostridium histolyticum* (Sigma, St. Louis, MO) at 37°C. LSEC and KC were isolated by centrifugation and selective adherence as previously described (1, 2).

In vitro platelet binding assay

Primary KC or LSEC were cultured for 3 or 4 days, respectively, at 37 ºC and 5% CO_2 in black walled-clear bottomed, cell culture-treated, 96-well plates (100,000 cells/well). Four million CFSE-labeled platelets were added to wells containing primary KC or LSEC and medium. The assay was performed as previously described (1, 2). In short, following addition of platelets, plates were centrifuged at 2000 x g for 3 minutes. Wells with endothelial cells or Kupffer cells without CFSE-labeled platelets were used to determine levels of auto fluorescence. In LSEC and KC experiments the normalized fold change following sialidase treatment compared to no treatment was plotted.

Statistical Analysis

Data was analyzed using Graphpad Prism 5 (La Jolla, CA). Student's t-test, ANOVA, and Tukey Post-Hoc multiple comparison tests were performed as indicated in figure legends.

RESULTS

Sequence analysis, structural modeling of the ASGR1 binding site, and sequence analysis of CD11b

The sequence for ASGR1 in the porcine genome database (AK233544.1) was identified using the human sequence as a reference (assn: M10058). After translation of the nucleotide sequence, the AA sequences were aligned. The Galβ binding site identified in human ASGR1 (21) was 3 AA different from porcine ASGR1 (Figure 3-1A). The predicted porcine ASGR1 structure was compared to the published human ASGR1 structure (pdb: 1DV8) (Figure 3-1B).

The sequence identity between porcine and human CD11b was 79% and the positive matches were 87% when compared using NCBI Blastp. The proposed human and porcine CD11b carbohydrate-binding motifs (17, 19, 22) were aligned and compared (Figure 3-1C). There are multiple differences in AA with a significant number being non-conserved residues. The carbohydrate-binding motif of porcine CD11b had 70% sequence identity and 83% positive matches when compared with that of human CD11b. This is lower in sequence identity than the rest of CD11b excluding the carbohydrate-binding motif; identity 80%, positive matches 88%.

Concentrations of Galβ and βGlcNAc on platelets

Domestic and GTKO/hDAF porcine platelets had similar amounts of exposed Galβ and βGlcNAc as measured by flow cytometry using the lectins ECA (Figure 3-2A), sWGA (Figure 3-2B) and DSA (Figure 3-2C). Galβ on human platelets was 68.8% and 64.9% higher than on the domestic and

GTKO/hDAF porcine platelets respectively (n=3, $p<0.05$) (Figure 3-2A). βGlcNAc on human platelets was 74.6% greater than the βGlcNAc expression on domestic porcine platelets (n=3, $p<0.05$) and 77.2% higher compared to GTKO/hDAF porcine platelets (n=3, $p<0.05$) (Figure 3-2B). Total Galβ and βGlcNAc on human platelets was 73.2% greater than the expression on domestic porcine platelets (n=3, $p<0.05$) and 85.1% higher compared to GTKO/hDAF porcine platelets (n=3, $p<0.05$)(Figure 3-2C). The fluorescence intensities of ECA lectin, sWGA lectin and DSA lectin did not differ significantly between domestic and GTKO/hDAF porcine platelets using one-way ANOVA and the Tukey post Hoc test, however both differed significantly from human (Figure 3-2). Membranes with porcine and human platelet lysates were probed with ECA, sWGA, DSA lectins and revealed that numerous proteins are glycosylated with terminal Galβ or βGlcNAc oligosaccharides (Figure 3-2).

The role of sialic acid on Galβ and βGlcNAc availability

Human and porcine platelets were treated with sialidase A to remove terminal α2,3 and α2,6 Neu5Ac and Neu5Gc exposing Galβ and βGlcNAc epitopes. Human platelets treated with sialidase had 2.0 (n=6, $p<0.05$) fold more exposed Galβ epitopes compared with mock treated platelets (Figure 3-3A) and 5.1 (n=3, $p<0.05$) and 6.4 (n=3, $p<0.05$) fold more exposed Galβ epitopes compared to treated-domestic and GTKO/hDAF porcine platelets, respectively (Figure 3-3A). Human platelets treated with Sialidase A had a 2.1 (n=3, $p<0.05$) fold increase in exposed βGlcNAc epitopes compared with mock treated platelets (Figure 3-3B). Following treatment with Sialidase A, human platelets had 6.26

(n=3, $p<0.05$) and 5.4 (n=3, $p<0.05$) fold more sWGA lectin binding for domestic and GTKO/hDAF porcine platelets (Figure 3-3B). Total Galβ and βGlcNAc on human platelets increased by 2.8 (n=3, $p<0.05$) fold following Sialidase A treatment (Figure 3-3C). Human platelets had 4.7 (n=3, $p<0.05$) and 7.9 (n=3, $p<0.05$) fold more total Galβ and βGlcNAc compared to domestic and GTKO/hDAF porcine platelets, respectively, after treatment with sialidase (Figure 3-3C). In fact, the sialidase treatment of the pig platelets produced only a slight increase in reactivity of the various lectins tested. None of the lectins tested differed significantly between domestic and GTKO/hDAF porcine platelets for binding (Figure 3-3).

LSEC and KC phagocytosis of desialylated human platelets

To investigate the functional effects of increased exposure of Galβ and βGlcNAc oligosaccharides on sialidase-treated human platelets, in vitro assays were completed with domestic and GTKO/hDAF LSEC and KC. Desialylated human platelets incubated with domestic porcine LSEC or GTKO/hDAF porcine LSEC had a statistically significant 2.4 (n=5, $p<0.05$) and 2.2 (n=5, $p<0.05$) -fold increase in phagocytosis, respectively, when compared to mock-treated platelets (Figure 3-4A). Sialidase-treated human platelets incubated with domestic porcine KC and GTKO/hDAF porcine KC had a statistically significant 2.7 (n=6, $p<0.05$) and 1.9 (n=5, $p<0.05$) fold increase in platelet phagocytosis respectively suggesting the importance of platelet surface carbohydrates (Figure 3-4B). Data was analyzed using Student's t-test.

DISCUSSION

The basis of xenogeneic phagocytosis of human platelets via lectin receptors including ASGR1 and Mac-1 expressed on liver sinusoidal cells has not been well characterized. Most human platelets bind and are taken up by LSEC and KC within 15-30 minutes following addition, in vitro and ex vivo. There are multiple factors that may exist at the root of xenogeneic platelet phagocytosis. First, differences in receptor sequence and structure between pig and human may lead to differences in the binding capabilities and specificity of ligand. Another hypothesis is that there are differences in ligand concentration and density between human and porcine platelets. The data presented here indicates that it is most likely a combination of differences in receptor and ligand between human and pig.

ASGR1 and Mac-1 have been studied extensively with regards to binding specificity of Galβ and βGlcNAc (3, 23-31). It was found that livers from closely related mammals have distinct capacities to bind Galβ and Siaα2,6GalNAc (10). In particular, porcine liver extract showed very little binding capacity for Siaα2,6GalNAc (10). Recombinant mouse and human ASGR1 showed very little binding to Siaα2,6GalNAc, in contrast to rat ASGR1 differing in 12 AA shows a dramatic difference in ASGR1 binding capacity (10). Studies using mutant chimeras of rat and mouse ASGR1 indicate that small changes in AA sequence can reflect changes in affinity of ligand (10). Even though there are only 3 A.A. differences between porcine and human ASGR1, they are within the binding site and most likely yield differences in ligand binding affinity. When comparing the

predicted Mac-1 carbohydrate-binding domain of human and pig, there are many differences in the AA sequence between pig and human. With over half of the differences being non-similar AA, it is likely that porcine and human Mac-1 will differ in specificity and avidity for binding Galβ and βGlcNAc.

The xenogeneic uptake of human platelets by the porcine liver may in part be explained by differences in receptor affinity, but it may also be explained in part by differences in ligand. The known ligand for ASGR1 is terminal Galβ, while Mac-1 binds terminal Galβ and βGlcNAc oligosaccharides. These oligosaccharides are on both proteins and lipids covering the surface of platelets (32-34). Galβ and βGlcNAc, as seen by our data of platelet lysates on membranes probed with lectins, are on multiple proteins in both human and porcine platelets. There are reported differences between bovine and human platelets with respect to carbohydrate composition (32). Miyagawa et al. reported that there are differences between human and GTKO porcine endothelial cells and fibroblasts (35). In particular human endotheial cells showed stronger reactivity with ECA than the porcine endothelial cells (36). The porcine and human platelets exhibit differences in both Galβ and βGlcNAc amounts on the extracellular plasma membrane, as seen by the flow cytometric analysis. This difference in ligand concentration likely contributes to the xenogeneic uptake of platelets.

The removal of aged platelets from the circulation takes place following the loss of terminal sialic acid on the branched chain N and O linked oligosaccharides comprising platelet glycoproteins and glycolipids (8, 36).

However, there is convincing evidence that normal platelet uptake is more likely due to expression of markers of apoptosis and not sialic acid content (37, 38). A function of the ASGR1on KC and hepatocytes is to mediate removal of platelets that have been desialylated by bacterial neuraminidase, which exposes underlying galactose containing oligosaccharides during sepsis (6, 39, 40). These desialylated platelets were rapidly removed from circulation by the liver. In vitro removal of sialic acid from rabbit platelets caused an increase uptake in vivo following injection (41). It seems plausible that porcine primary LSEC and KC recognize human platelets as mice recognize bacteria-induced desialated platelets (6). By desialylating human and porcine platelets it was found that the release of sialic acid did not equalize the Galβ or the βGlcNAc differences between humans and pigs. Rather human platelet Galβ and βGlcNAc oligosaccharide exposure was increased while maintaining dramatically higher levels compared to the pig platelet, which translated to an increase in phagocytosis by primary porcine LSEC and KC. This parallels work done by Greenberg *et al* that found compared to other mammalian species there was little difference following desialation of porcine platelets with regards to aggregation indicating that there is little sialic acid capping on the pig platelet (41). Previous work proposed that Galβ epitopes on desialylated platelets were targets for ASGR1 in the liver (6, 8, 39, 40). When the enzyme that adds sialic acid to the galactose, St3gal4, was knocked out there was systemic dysregulation of thrombosis resulting in prolonged bleeding (8). The non-sialylated platelets from these mice were removed from circulation resulting in 66% of platelets removed

in 2.8 minutes (8). The xenogeneic uptake of human platelets by porcine LSEC and KC is not likely a result of differences in sialic acid capping, but a result of differences in the basal level of exposed Galβ and βGlcNAc between human and porcine platelets.

CONCLUSIONS

Variation in ASGR1 and Mac-1 sequences between species within the carbohydrate binding domains are present. Humans have more Galβ and βGlcNAc epitopes available compared to porince platelets regardless of sialylation state. Desialylation of human platelets increases available Galβ and βGlcNAc epitopes, thus increasing human platelet uptake by porcine sinusoidal cells.

ACKNOWLEDGEMENTS

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Figure 3-1. Comparison of Human and Porcine ASGR1 and CD11b. (A) A comparison of human and porcine ASGR1 saccharide binding sites revealed three amino acid (AA) differences (yellow boxes). An arginine in the human sequence is a glycine in the porcine sequence, possibly altering the availability of the binding pocket (box). (B) The human (protein data bank: 1DV8) and pig ASGR1 binding sites were constructed using the Pymol Molecular Graphics System with an N-acetylglucosamine (protein data bank: 1BCJ) in the binding site. A black arrow indicates the human arginine 262 that is glycine 262 in pigs. (C) A comparison of human and porcine CD11b predicted carbohydrate-binding domain. AA differences highlighted (yellow), dissimilar, non-conserved differences boxed.

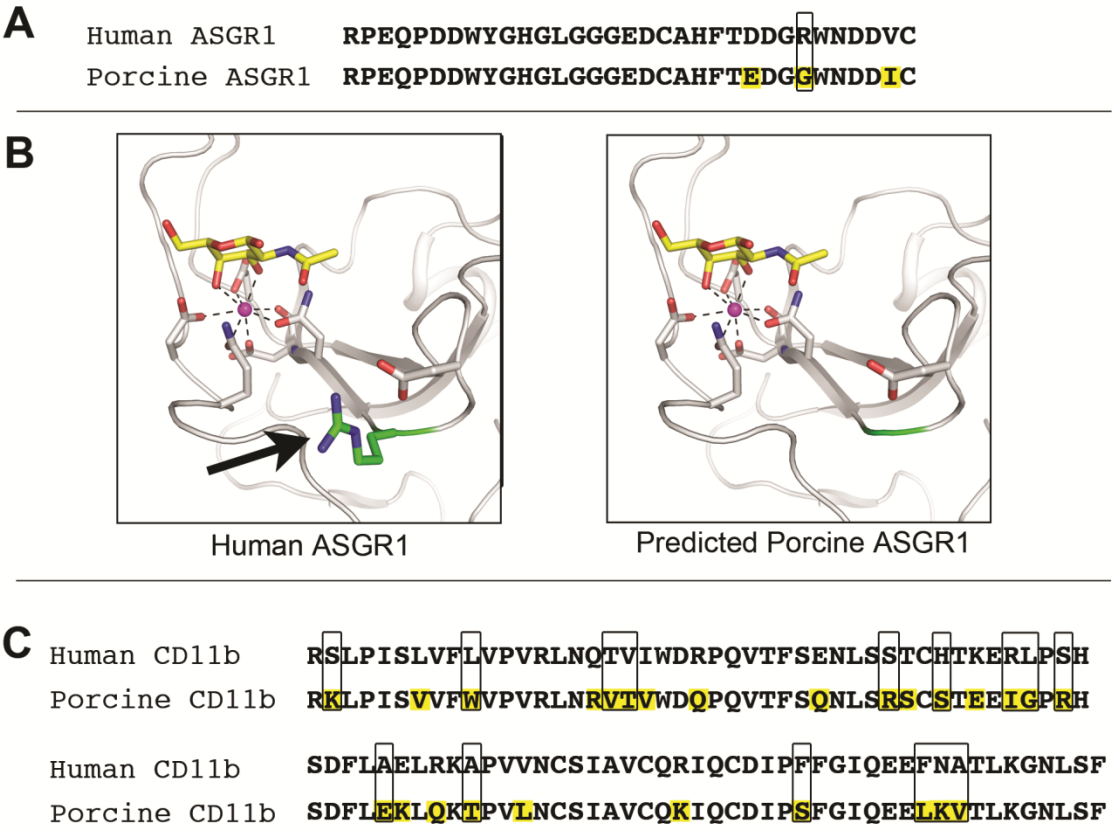


Figure 3-2. Differences in Galb and bGlcNAc between human and porcine platelets. (A) Flow cytometric analysis of Galb levels on human, domestic, and a1,3galactosyltransferase knockout (GTKO)/human decay accelerating factor (hDAF) porcine platelets measured by erythrina cristagalli (ECA) lectin binding represented by the mean fluorescent index. A representative histogram is shown. Immunoblot/Lectin blot analysis of human, domestic, and GTKO/hDAF porcine platelet lysates for Galb using ECA and GAPDH (indicated by arrow). (*) (n = 3, ANOVA/Tukey's test compared with Human $P < 0.05$). (B) Flow cytometric analysis of bGlcNAc levels on human, domestic, and GTKO/hDAF porcine platelets measured by succinylated wheat germ agglutinin (sWGA) lectin binding represented by the mean fluorescent index. A representative histogram is shown. Immunoblot/Lectin blot analysis of human, domestic, and GTKO/hDAF porcine platelet lysates for bGlc-NAc using sWGA and b actin indicated by arrow. (*) (n = 3, ANOVA/Tukey's test compared with Human $P < 0.05$). (C)
Flow cytometric analysis of bGal and bGlcNAc levels on human, domestic, and GTKO/hDAF porcine platelets measured by Datura stramonium (DSA) lectin binding represented by the mean fluorescent index. A representative histogram is shown. Immunoblot/Lectin blot analysis of human, domestic, and GTKO/hDAF porcine platelet lysates for bGal and bGlcNAc usingDSAand b actin indicated by arrow. (*) (n = 3,ANOVA/Tukey's test compared with Human $P < 0.05$).

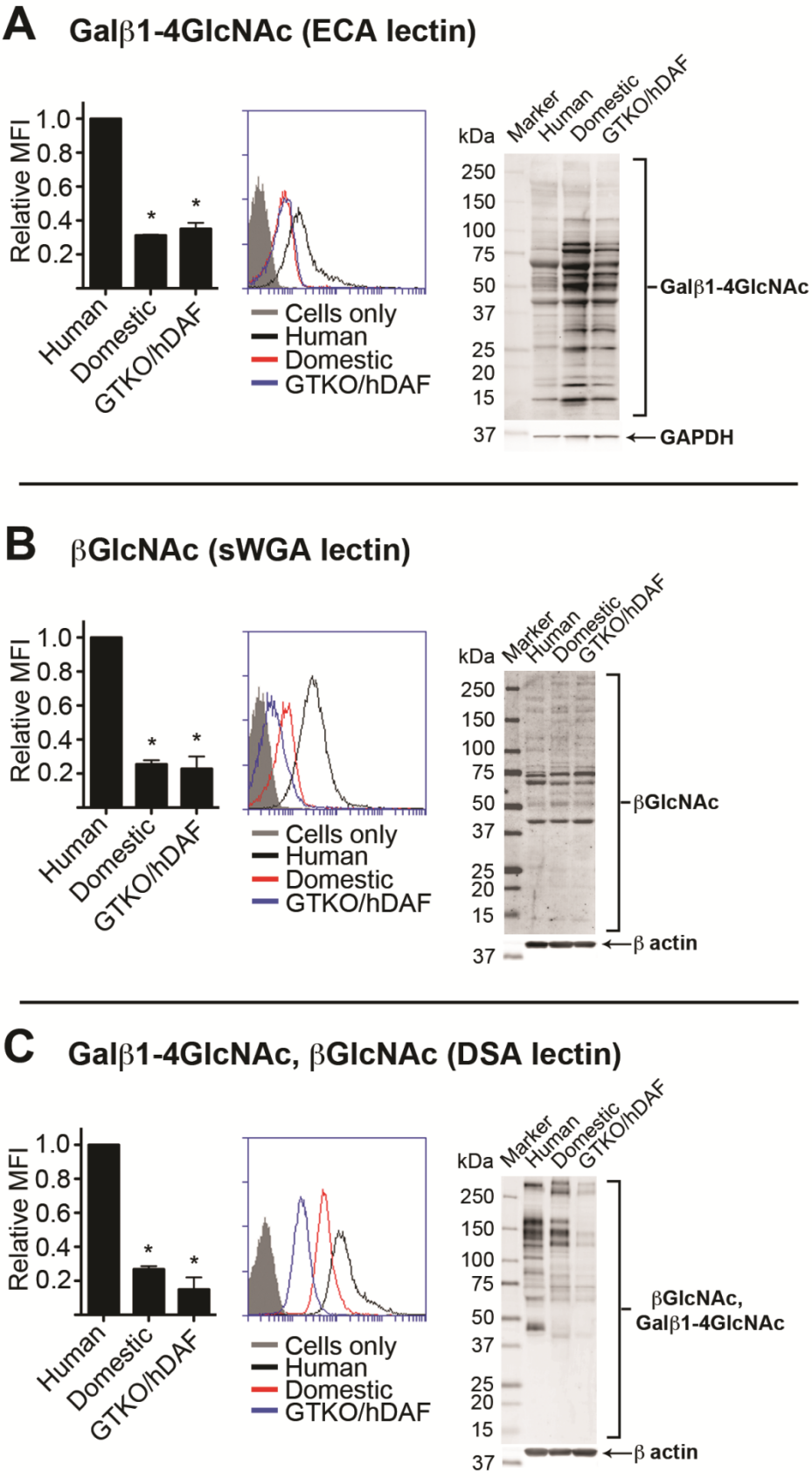

A
Galβ1-4GlcNAc (ECA lectin)
Relative MFI
1.0
0.8
0.6
0.4
0.2
*
*
Human
Domestic
GTKO/hDAF
Cells only
Human
Domestic
GTKO/hDAF
kDa
Marker
Human
Domestic
GTKO/hDAF
250
150
100
75
50
37
25
20
15
37
Galβ1-4GlcNAc
GAPDH
B
βGlcNAc (sWGA lectin)
Relative MFI
1.0
0.8
0.6
0.4
0.2
*
*
Human
Domestic
GTKO/hDAF
Cells only
Human
Domestic
GTKO/hDAF
kDa
Marker
Human
Domestic
GTKO/hDAF
250
150
100
75
50
37
25
20
15
37
βGlcNAc
β actin
C
Galβ1-4GlcNAc, βGlcNAc (DSA lectin)
Relative MFI
1.0
0.8
0.6
0.4
0.2
*
*
Human
Domestic
GTKO/hDAF
Cells only
Human
Domestic
GTKO/hDAF
kDa
Marker
Human
Domestic
GTKO/hDAF
250
150
100
75
50
37
25
20
15
37
βGlcNAc,
Galβ1-4GlcNAc
β actin

Figure 3-3. Differences in total Galb and bGlcNAc levels between human and porcine platelets are not a result of differences in relative sialylation. (A) Flow cytometric analysis of Galb levels on human, domestic, and α1,3galactosyltransferase knockout (GTKO)/human decay accelerating factor (hDAF) porcine platelets following sialidase treatment or mock treatment using erythrina cristagalli lectin binding represented as the mean fluorescent index. A representative histogram from three experiments is shown (n = 3, ANOVA/Tukey's test $P < 0.05$, *Compared with human mock-treated, **Compared with human sialidase-treated). (B) Flow cytometric analysis of bGlcNAc levels on human, domestic, and GTKO/hDAF porcine platelets following sialidase treatment or mock treatment using sWGA lectin binding represented as the mean fluorescent index. A representative histogram from three experiments is shown. (n = 3, ANOVA/Tukey's test $P < 0.05$, *Compared with human mock-treated, **Compared with human sialidase-treated). (C) Flow cytometric analysis of human, domestic pig, and GTKO/hDAF porcine platelets for bGal and bGlcNAc acid using Datura strumonium following sialidase or mock treatment. Representative histogram from three experiments is shown. (n = 3, ANOVA/Tukey's test $P < 0.05$, *Compared with human mock-treated, **Compared with human sialidase-treated).

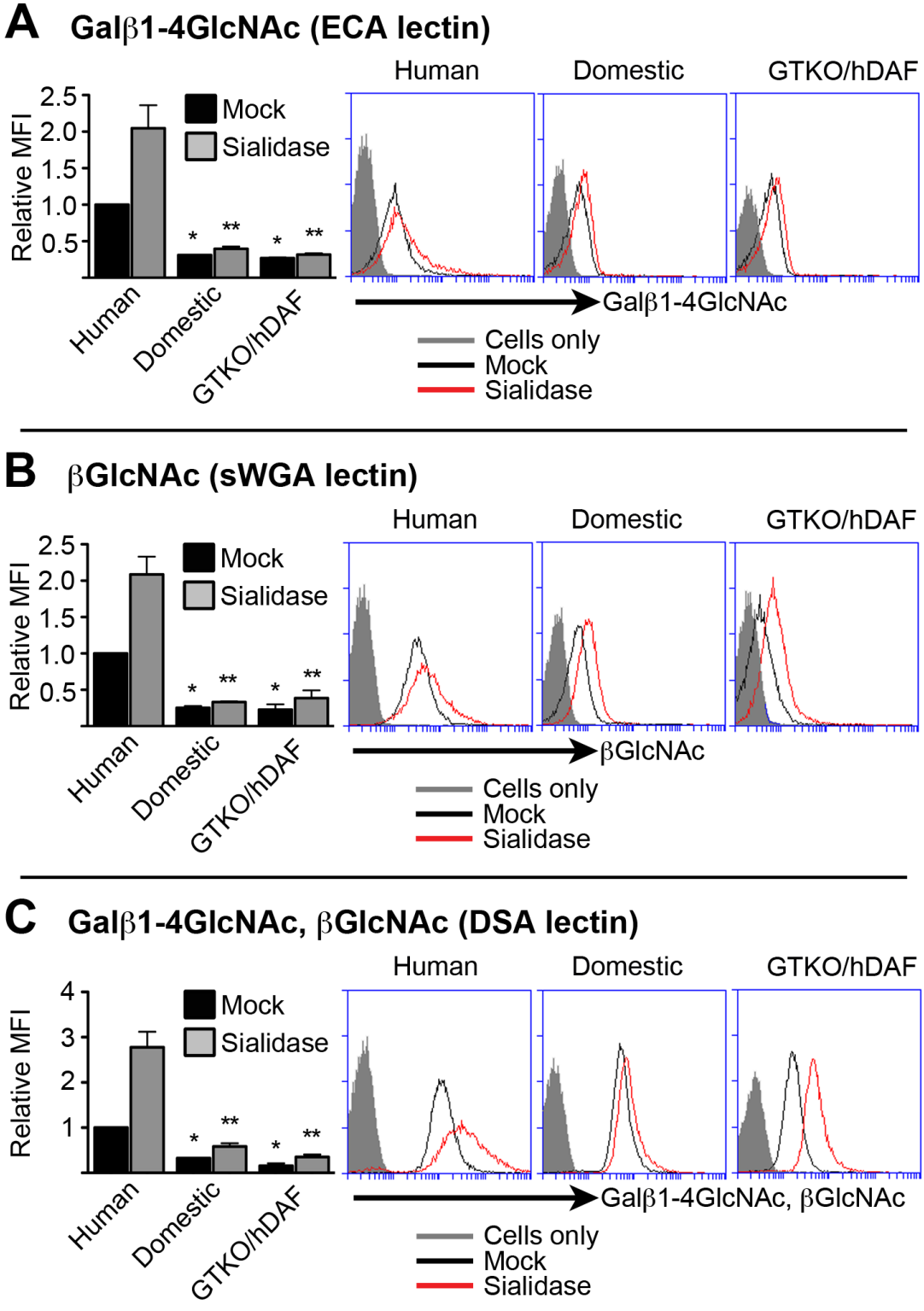
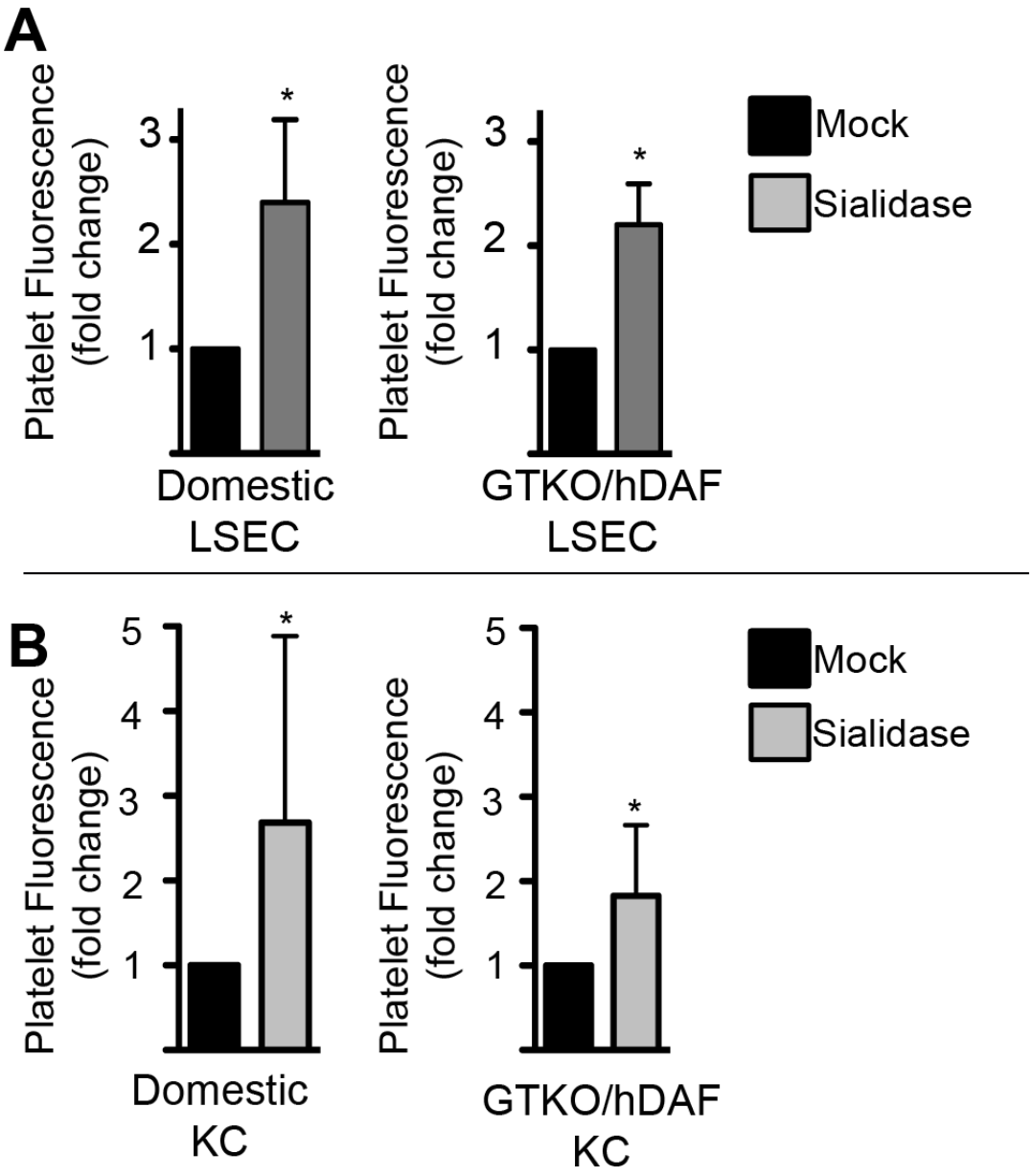

A
Galβ1-4GlcNAc (ECA lectin)
Human
Domestic
GTKO/hDAF
Relative MFI
2.5
2.0
1.5
1.0
0.5
Mock
Sialidase
*
**
*
**
Human
Domestic
GTKO/hDAF
Galβ1-4GlcNAc
Cells only
Mock
Sialidase
B
βGlcNAc (sWGA lectin)
Human
Domestic
GTKO/hDAF
Relative MFI
2.5
2.0
1.5
1.0
0.5
Mock
Sialidase
*
**
*
**
Human
Domestic
GTKO/hDAF
βGlcNAc
Cells only
Mock
Sialidase
C
Galβ1-4GlcNAc, βGlcNAc (DSA lectin)
Human
Domestic
GTKO/hDAF
Relative MFI
4
3
2
1
Mock
Sialidase
*
**
*
**
Human
Domestic
GTKO/hDAF
Galβ1-4GlcNAc, βGlcNAc
Cells only
Mock
Sialidase

Figure 3-4. Removal of Sialic acid from human platelets increases capture by Domestic and a1,3galactosyltransferase knockout (GTKO)/human decay accelerating factor (hDAF) porcine liver sinusoidal endothelial cells (LSEC) and Kupffer cells (KC). Human platelets either mock-treated or treated with sialidase for 1 h, labeled with carboxyfluorescein succinimidyl ester and incubated with domestic and GTKO/hDAF porcine (A) LSEC or (B) KC for 15 or 30 min respectively. Captured platelets were measured with a Spectramax plate reader for fluorescence (n = 5). Data analyzed using Student's t-test at $P < 0.01$.



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Chapter 4

Fibronectin from Alpha-Gal Knockout Pigs is a Xenoantigen

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ABSTRACT

Background: Antibody-mediated rejection continues to be an obstacle for xenotransplantation despite development of α1,3-galactosyltransferase knockout (GTKO) pigs. Fibronectin (Fn) from GTKO pigs was identified as a xenoantigen in baboons. N-glycolylneuraminic acid (Neu5Gc), similar to galactose α1,3-galactose, is an antigenic carbohydrate found in pigs. We evaluated human antibody reactivity and performed initial antigenic epitope characterization of Fn from GTKO pigs.

Materials and Methods: GTKO pig aortic endothelial cells (AEC) were isolated and assessed for antibody-mediated complement dependent cytotoxicity (CDC). Human and GTKO pig Fns were purified and analyzed using immunoblots. GTKO pig and human AEC absorbed human sera were assessed for CDC and anti-GTKO pig Fn antibodies. GTKO pig proteins were assessed for Neu5Gc. Immunoaffinity-purified human IgG anti-GTKO pig fibronectin (hIgG-GTKOp Fn) using a GTKO pig Fn column were evaluated for cross-reactivity with other proteins.

Results: GTKO pig AEC had greater human antibody binding, complement deposition and CDC compared to allogeneic human AEC. Human sera absorbed with GTKO pig AEC resulted in diminished anti-GTKO pig Fn Ab. Neu5Gc was identified on GTKO pig Fn and other proteins. The hIgG-GTKOp Fn cross-reacted with multiple GTKO pig proteins and was enriched with anti-Neu5Gc antibody.

Conclusions: Removal of antigenic epitopes from GTKO pig AEC would improve xenograft compatibility. GTKO pig Fn has antigenic epitopes, one identified as Neu5Gc, which may be responsible for pathology and cross-reactivity of hIgG-GTKOp Fn. Genetic knockout of Neu5Gc appears necessary to address significance and identification of non-Neu5Gc GTKO pig Fn antigenic epitopes.

Keywords: xenotransplantation, xenoantigen, fibronectin, antibody-mediated rejection, N-glycolylneuraminic acid, Neu5Gc

INTRODUCTION

Antibody-mediated rejection (AMR) is a barrier demonstrated in pig to non-human primate transplant models despite creation of α1,3-galactosyltransferase gene knockout (GTKO) pigs (1-9). Removal of other antigens appears necessary to prevent AMR.

Fibronectin (Fn), a glycoprotein produced by endothelial cells, was identified as a xenoantigen in GTKO pig to baboon cardiac transplant studies (1). Glycoproteins are of special interest, because in addition to antigenic peptides, antigenic glycosylations, for example galactose α1,3-galactose (αGal) carbohydrate antigen, may be shared by multiple pig glycoproteins. Similarly, N-glycolylneuraminic acid (Neu5Gc), a foreign sialic acid equivalent of N-acetylneuraminic acid (Neu5Ac) in humans, is found on pig glycoproteins and was identified as an antigen demonstrated by the presence of cytotoxic anti-Neu5Gc antibodies in normal human sera (10-13). The significance of the Neu5Gc antigen for xenotransplantation continues to be controversial (14).

Discovery of new antigenic epitopes responsible for AMR is necessary to identify new genetic targets for modification. Given that knockout of the Fn gene was lethal in mice (15) and Fn is involved in many cellular activities including cell growth, adhesion, migration, differentiation, coagulation, pathogen opsonization, wound healing, embryogenesis (16, 17), characterization of pig Fn epitopes is necessary. We characterized the reactivity of preformed human antibodies towards endothelial cells and Fn purified from GTKO human CD55 transgenic

pigs (GTKOp) to evaluate its potential role in AMR and to identify potential antigenic epitopes for modification.

MATERIALS AND METHODS

Source of Pigs and Procurement of Tissue

GTKOp were produced through somatic-cell nuclear transfer using cells from the National Swine Resource and Research Center (NSRRC:0009) at the University of Missouri. After pigs were anesthetized, the abdominal and thoracic cavities were entered followed by cannulation of the aorta and histidine-tryptophan-ketoglutarate solution (Essential Pharmaceuticals. LLC, Newtown, PA) flush. Liver and kidney frozen sections were obtained followed by harvest of the aorta. Samples were taken from 3 different pigs.

Aortic Endothelial Cell Isolation

The GTKOp aortic thoracic and abdominal branches were tied off. Aortas were filled with 0.0125ug/mL collagenase type IV (Sigma-Aldrich, St. Louis, MO) diluted in Hank's balanced salt solution (HBSS) and incubated at 37°C for 30min. The collagenase and endothelial cell mix was transferred into a 50mL conical tube and centrifuged at 400xG for 10min. Cells were re-suspended in Medium 200 with Low Serum Growth Factor Supplement (Invitrogen, Carlsbad, CA) and plated.

Flow Cytometry

Isolated GTKOp cells and human AEC (Invitrogen) were trypsinized, washed and stained in phosphate buffer saline (PBS) with 0.5% bovine serum albumin (BSA) and 0.02% sodium azide using mouse anti-CD31 IgG_1 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-swine leukocyte antigen (SLA) Class 1 IgG_1 (ABD Serotec, Raleigh, NC), mouse anti- human leukocyte antigen

(HLA) Class 1 ABC IgG_{1k} (BD, Franklin Lakes, NJ), mouse IgG_1 isotype (ABD Serotec) and mouse IgG_{1k} isotype (eBioscience, San Diego, CA) and donkey anti-mouse IgG Dylight®649 (Jackson ImmunoResearch Laboratories, West Grove, PA) was used for detection. Heat inactivated human sera were incubated with GTKOp and human AEC at various dilutions ranging from 0 to 50% for 1.5 hours, followed by washes with PBS. Donkey anti-human IgM Dylight®649 and IgG Dylight®649 (Jackson ImmunoResearch) were used for detection. Flow cytometry was performed and analyzed on the Accuri™ C6 flow cytometer (Accuri, Ann Arbor, MI). Flow histograms were used to characterize isolated AEC. Mean fluorescence index values were recorded for human antibody recognition of AEC.

Confocal Microscopy

Frozen tissue sections and cell monolayers were fixed with 4% paraformaldehyde. Tissues were permeabilized but not cell monolayers. GTKOp kidney sections were stained with goat anti-CD31 IgG (R&D Systems, Minneapolis, MN), mouse anti-beta-actin IgG (Sigma-Aldrich) and sheep anti-fibronectin IgG (ABCAM, Cambridge, MA) and detected using bovine anti-goat IgG Dylight®488 (Jackson ImmunoResearch), donkey anti-sheep IgG Alexa Fluor®555 (Invitrogen) and anti-mouse IgG Dylight®649. GTKOp liver sections were stained with the same antibodies as above except for mouse anti-human CD18 IgG_1 (Developmental Studies Hybridoma Bank, Iowa City, IA) instead of beta-actin. Sheep, goat IgG isotype (SouthernBiotech, Birmingham, AL) and mouse IgG isotype (Santa Cruz) were used as controls. GTKOp and human

AEC were stained with mouse anti-CD31 and sheep anti-fibronectin primary antibody (Ab) and anti-sheep Alexa Fluor®555, anti-mouse IgG Dylight®649 secondary Ab. Live GTKOp AEC and human AEC were incubated with heat inactivated human serum at various dilutions or number of cell absorptions in HBSS for 1.5hr and washed with warm HBSS three times. Slides were fixed and stained with donkey anti-human IgM Dylight®549 and IgG Dylight®649 (Jackson ImmunoResearch) or incubated with baby rabbit complement (Cedarlane, Burlington, NC), fixed and stained with sheep anti-human C4 IgG (ABD Serotec) followed by anti-sheep Alexa Fluor®555. Nuclei were stained with DAPI. Neu5Gc images were obtained using the Sialix®Gc-Free kit (Sialix, Vista, CA) in conjunction with donkey anti-chicken IgY Dylight®649 (Jackson ImmunoResearch). Quantification images were obtained separately in triplicate using the photon count acquisition setting. Images were taken using an Olympus IX81/FV1000 microscope 60x/1.40 oil objective and analyzed on FluoView software. Image sets were captured using consistent laser and detector settings and processed with consistent brightness and contrast settings in Adobe Photoshop®CS4.

Purification of Fibronectin and Human Antibodies

Human and GTKOp Fns were affinity purified from plasma using Gelatin Sepharose™4B (GE Biosciences, Pittsburg, PA) column. Trace contaminants were removed using two serial Sephacryl™S-400 size exclusion columns 25mm (diameter) x 500mm (height) (GE Biosciences). Fns were desalted in Tris buffered saline (TBS) using 100kDa centrifugal concentrators. GTKOp Fn was

conjugated with CNBr-activated Sepharose™4B at 5mg/mL (protein/mL of beads) for a 5mL total column bed volume. Total human IgG was purified from a blood group O donor with a HiTrap™Protein G column (GE Biosciences). Human anti-GTKOp Fn IgG were immunoaffinity-purified by application of 100mg of 5mg/mL total human IgG to the column, washed with TBS, eluted with TBS 4M urea and desalted to yield 70μg of IgG estimated using the Nanodrop®1000 (Thermoscientific, Wilmington, DE). All liquid chromatography was performed on a BioLogic Duoflow system (Bio-Rad, Hercules, CA).

Protein Preparation and Immunoblot Analysis

Fibronectins were normalized based on OD280 measurements taken on SpectraMax®M2e (Molecular Devices, Sunnyvale, CA). GTKOp Fn and sera and human Fn, sera and albumin were diluted into TBS 100mM ethylenediaminetetraacetic acid (EDTA) 10% glycerol pH 7.5 buffer. Ground frozen GTKOp liver and kidney and *Escherichia coli* DH10B™strain (Invitrogen) were solubilized and lysed in TBS 1% Igepal CA-630 (NP-40 substitute) 10% glycerol pH 8 on ice for 30min followed by three freeze-thaw cycles, centrifugation at 4°C and collection of supernatant. Electrophoresis was accomplished using the Criterion™ system and 4-10% TGX™ precast gels (Bio-Rad), Tris Glycine buffer with or without 0.1% SDS corresponding to denaturing or native conditions. Fns were separated under native conditions whereas GTKOp tissue lysates and sera were analyzed under denaturing conditions along with *E. coli* positive and human albumin negative controls. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes or stained with Bio-

Safe Coomassie (Bio-Rad). Membranes were blocked with Odyssey Blocking Buffer (LI-COR, Lincoln, NE) then incubated with human sera at 10%, total human IgG at 1mg/mL, hIgG-GTKOp Fn at 10ug/mL or sheep anti-fibronectin at 1ug/mL. After washes with PBS, blots were probed with goat anti-human IgG IRDye®800CW or IgM IRDye®800 (Rockland Immunochemicals, Gilbertsville, PA) or anti-sheep IgG Dylight®649 (Jackson ImmunoResearch). Neu5Gc immunoblots were completed with Sialix®Gc-Free kit (Sialix) and donkey anti-chicken IgY IRDye®800CW (LI-COR). Blots were scanned on the Odyssey infrared imaging system (LI-COR).

Antibody-mediated Complement Dependent Cytotoxicity Assay

Absorbed human sera were prepared by dilution to 50% in HBSS followed by seven serial applications to confluent monolayers of GTKOp and human AEC in two-chamber coverglass slides. Antibody absorption was analyzed by confocal microscopy as described above. Sera dilutions ranging from 0-50% were incubated in triplicate with confluent GTKOp or human AEC for 2hr, washed with warm HBSS then incubated with 12.5% baby rabbit complement for 1hr. WST-1 Cell Proliferation Assay System (Takara, Mountain View, CA) was used to assess cell viability and growth.

Enzyme linked immunosorbent assay (ELISA) and Mass Spectometry

Neu5Gc/Neu5Ac-polyacrylic acids (500ng/mL) and goat anti-human IgG/IgM H+L chain $F(ab')_2$ (Jackson ImmunoResearch) (10ug/mL) were coated onto plates using carbonate bicarbonate pH 9.5 buffer. GTKOp Fn was coated at 20ug/mL in PBS. Recombinant human albumin (Sigma-Aldrich) (1%) in PBS

was used for blocking and antibody/sera dilutions. Serum ranging from 0-25% were incubated in duplicate with coated plates for 4hrs, then bound antibody was detected using goat anti-human IgM $Fc_{5\mu}$ or IgG Fc_{γ} $F(ab')_2$ HRP (Jackson ImmunoResearch) and o-Phenylenediamine dihydrochloride with acid stop. Standard curves were generated from purified human IgM and IgG (Sigma-Aldrich). Absorbance at 490nm was measured and data analyzed using SpectraMaxM2e and SoftMaxPro®5 software (Molecular Devices). HRP secondary antibody only measurements were subtracted as background. Antibody measurements for Neu5Ac were subtracted as background from Neu5Gc. Measurements of diluted serum incubated in blocked wells followed by secondary Ab was recorded as non-specific background binding. Proteins were identified using the AB SCIEX TOF/TOF™5800 System as previously described (18).

Statistical Analysis

GraphPad Prism 5 (Prism, La Jolla, CA) was used for generation of graphs and statistical analyses. Flow cytometric, confocal and absorbance measurements used for Figures 4-1C, 4-1F, 4-3B and 4-3C were normalized to the highest value per serum used on GTKOp and human AEC set. Band intensities in 4-2C were normalized relative to the human Fn band for each serum. ELISA measurements for Figure 4-3C were normalized using unaltered human serum set at 100%. Linear regression and binding saturation non-linear fit analyses were used for IgM and IgG respectively in Figure 4-1C. One-tailed paired Student's t-test was

used for Figures 4-1E, 4-2C, 4-3B, 4-3C and 4-3D. One-way analysis of variance with Tukey's post hoc test was used to analyze Figures 4-1F, 4-3D.

RESULTS

Preformed Human Antibody-Mediated Complement Dependent Cytotoxicity

Flow cytometric analysis demonstrated that isolated GTKOp aortic endothelial cells (AEC) were a uniform population of CD31 positive cells similar to commercially available human AEC (Figure 4-1A). The histogram for GTKOp AEC Swine Leukocyte Antigen class 1 (SLA1) was comparable to HLA1 in human AEC (Figure 4-1A). Human IgM and IgG bound live GTKOp AEC greater than human AEC after incubation with 25% human serum (Figure 4-1B). Human IgM and IgG partially colocalized (Figure 4-1B). Flow cytometric analysis using sera showed greater dose dependent binding of GTKOp AEC by human IgM ($n=3$, $p<0.001$) and IgG ($n=3$, $p<0.001$) compared to human AEC (Figure 4-1C). Live GTKOp AEC showed greater complement component 4 (C4) deposition than to human AEC after incubation with 5% human serum followed by complement (Figure 4-1D). C4 deposition on GTKOp was 8 fold higher than on human AEC, at MFI=122 and MFI=15.8 ($n=3$, $p=0.007$) respectively (Figure 4-1E). Antibody-mediated complement dependent cytotoxicity (CDC) assay showed an initial increase in relative growth and viability for human sera incubated with GTKOp AEC or human AEC, followed by a decrease in viability for GTKOp AEC (xenogeneic) but not human AEC (allogeneic) in a dose dependent manner ($n=3$, $p=0.007$) (Figure 4-1F). The difference between CDC curves for GTKOp AEC incubated with GTKOp serum (autologous) and human AEC with human serum (allogeneic) were not statistically significant (Figure 4-1F).

Expression and Localization of GTKOp Fibronectin

GTKOp kidney and liver express CD31 and Fn (Figure 4-2A). Beta-actin outlines the cellular structures of kidney (Figure 4-2A). CD18 staining in the liver demonstrates Kupffer cells on the surface of liver sinusoidal endothelium (Figure 4-2A). CD31, an endothelial cell marker, and Fn colocalized in GTKOp kidney and liver (Figure 4-2A). Non-permeabilized GTKOp AEC and human AEC had similar expression of CD31 and Fn on the cell surface (Figure 4-2A). One band for each purified Fn was separated on native Coomassie stained gels and was identified as Fn in the anti-Fn immunoblot (Figure 4-2B). GTKOp Fn had four-fold greater IgM (n=4, p=0.028) and two-fold greater IgG (n=4, p=0.004) relative band intensity compared to human Fn (Figure 4-2B). *E. coli* lysate but not human albumin was recognized by human IgM and IgG.

Evaluation of Absorbed Human Sera and Anti-GTKOp Fibronectin Antibodies

Human sera serially absorbed to GTKOp AEC or human AEC cell monolayers seven times showed diminishing IgM and IgG binding to GTKOp AEC compared to human AEC (Figure 4-3A). Human IgM (n=3, p=0.004) and IgG (n=3, p=0.012) bound GTKOp AEC more than human (Figure 4-3B). The seventh and final absorption demonstrated continued antibody binding to GTKOp AEC by 1.7 fold for IgM and 1.1 fold for IgG compared to human AEC (Figure 4-3B). GTKOp AEC absorbed human sera resulted in higher growth and viability (n=3, p=0.01) in CDC assays compared to serum absorbed against human AEC (Figure 4-3C). Human sera absorbed with GTKOp AEC compared to human

AEC resulted in decreased relative concentrations of GTKOp Fn IgM (83% to 62%) with background at 32% (n=3, $p<0.001$) and IgG (106% to 58%) with background at 42% (n=3, $p=0.005$) (Figure 4-3D).

Assessment of N-glycolylneuraminic Acid on GTKOp Fibronectin, Tissue and Cells

GTKOp liver, kidney, AEC were positive for Neu5Gc but was minimal for human AEC (Figure 4-4A). Presence of Neu5Gc on GTKOp Fn and mouse glycoprotein positive control and not human Fn or human albumin was determined by immunoblot analysis (Figure 4-4B). Neu5Gc was detected on multiple proteins in GTKOp serum, kidney and liver lysates, but not in human serum (Figure 4-4C).

Evaluation of Human IgG Antibodies Recognizing GTKOp Fibronectin

Immunoaffinity-purified human IgG on a GTKOp Fn column (hIgG-GTKOp Fn) recognized GTKOp Fn and to a lesser degree human Fn and *E. coli* (Figure 4-5A). Protein G purified total human IgG recognized multiple proteins from *E. coli*, GTKOp kidney and liver lysates while fewer proteins were recognized by hIgG-GTKOp Fn (Figure 4-5B). Fn was not seen by immunoblot (data not shown). One high molecular weight protein band (arrow) in the GTKOp kidney lysate was clearly visible in the Coomassie stained gel, total human IgG and hIgG-GTKOp Fn immunoblots (Figure 4-5B). The band was analyzed by mass spectrometry leading to identification of several proteins screened based on mammalian origin and Molecular Weight Search score (Figure 4-5C). Original sample concentrations for the human anti-GTKOp Fn IgG and total human IgG

were 4.125mg/mL and 10.3mg/mL respectively (Figure 4-5D). Anti-Neu5Gc antibodies were detected at 20.75 ng/mL and 8.05 ng/mL from the human anti-GTKOp Fn IgG and total human IgG respectively representing a 6-fold enrichment of anti-Neu5Gc IgG in the hIgG-GTKOp Fn (Figure 4-5D).

DISCUSSION

Preformed human antibodies cause greater antibody-mediated complement dependent cytotoxicity to wildtype than to GTKO pig endothelial cells (18, 19). This study demonstrated continued cytotoxicity for GTKOp AEC (xenogeneic) and not human AEC (allogeneic) antibody response. Presence of non-αGal antibodies detected through immunoaffinity methods and diminished capacity of human sera absorbed with GTKOp AEC to elicit CDC further demonstrates the need to remove xenoantigenic epitopes. Fibronectin from wildtype pigs was discovered as a xenoantigen with antigenicity attributed mostly to αGal (20). After generation of GTKO pigs, Fn was rediscovered as a xenoantigen in pig to baboon cardiac xenotransplantation (1). Here, we have characterized GTKOp Fn antigenicity using normal human sera.

The relevance of a xenoantigen largely depends on its abundance, location and the presence of pathologic antibodies (21). Data presented here demonstrate rich expression of Fn in the vascular space of GTKOp kidney, liver and on the surface of endothelial cells suggesting exposure of Fn to host antibodies immediately after reperfusion. Since recent studies suggest that autoantibodies to normal and damaged endothelial cells and proteins exist to play a role in homeostasis (22-25), one cannot attribute a pathologic significance for all antibodies that bind a protein. GTKOp and human Fn were both recognized by preformed human antibodies, IgM and IgG recognized native GTKOp Fn to a greater extent. Human sera absorbed to GTKOp AEC resulted in

reduced CDC and anti-GTKOp Fn antibodies. These findings implicate antigenic epitopes on GTKOp Fn as contributors to CDC.

Immunoaffinity-purified human anti-GTKOp Fn IgG was cross-reactive with multiple GTKOp kidney and liver proteins suggesting the existence of a shared antigenic epitope among multiple GTKOp proteins and/or multiple antigenic epitopes on GTKOp Fn. Neu5Gc was found on GTKOp Fn, kidney and liver lysates confirming the presence of a known carbohydrate antigen common to multiple GTKOp proteins. Human anti-GTKOp Fn IgG demonstrated affinity towards Neu5Gc linked to polyacrylic acid implicating cross reactivity of anti-Neu5Gc antibodies obtained using GTKOp Fn. Several studies have shown possible increases in Neu5Gc in α1,3-galactosyltransferase deficient pigs (26, 27) suggesting increased importance of Neu5Gc in GTKO pigs. Although enrichment for anti-Neu5Gc IgG in hIgG-GTKOp Fn implied involvement in the antigenicity of GTKOp Fn, anti-Neu5Gc IgG was measured to be a small portion of hIgG-GTKOp Fn. The Neu5Gc and hIgG-GTKOp Fn immunoblots for GTKOp kidney and liver lysates were dissimilar and GTKOp fibronectin was also identified as a xenoantigen in non-human primates expressing Neu5Gc (1) suggesting presence of antigenic non-Neu5Gc epitopes on GTKOp Fn.

Several proteins were identified in the GTKOp Fn cross-reactive band identified in the kidney lysate. Two out of five proteins identified were cell surface transmembrane glycoproteins, which were 4F2 cell-surface antigen heavy chain (CD98 heavy subunit, 58 kDa) and low-density lipoprotein receptor-related protein 2 precursor (megalin, 518 kDa). Megalin, a known antigenic

target responsible for subepithelial glomerular immune deposits for autoimmune Heymann nephritis (28, 29), has been studied as a sialoglycoprotein containing oligo/polysialic acid, N-linked and O-linked posttranslational glycosylations (30, 31). Although the status of Neu5Gc on GTKOp CD98 heavy subunit and megalin are currently unknown, the cross-reactive band in the GTKOp kidney lysate was positive for Neu5Gc suggesting at least partial responsibility for the observed cross-reactivity with GTKOp Fn. Cross-reactive proteins were evaluated using a protein preparation preventing solubilization of Fn in GTKOp kidney and liver lysates by use of a mild detergent and cold temperature conditions simplifying analysis.

Xenotransplantation provides a unique opportunity to genetically modify donor grafts to enhance compatibility to the host. Comparison of xenogeneic and allogeneic CDC indicates the need for antigen removal from GTKOp endothelial cells to reach allogeneic levels of immune compatibility. GTKOp Fn has antigenic epitopes, one identified as Neu5Gc, recognized by preformed human antibodies. Since Neu5Gc is a potentially significant xenoantigenic epitope for humans, any porcine glycoprotein expressing Neu5Gc could be an important xenoantigenic protein. Removal of Neu5Gc through knockout of cytidine monophosphate-n-acetylneuraminic acid hydroxylase appears warranted to further characterize the importance and identification of non-Neu5Gc GTKOp Fn antigenic epitopes.

CONCLUSIONS

Preformed human antibodies still recognize epitopes on porcine endothelial cells even in the absence of αGal. Natural human antibodies bind GTKOp Fn. Natural human antibodies absorbed against GTKOp AEC bind GTKOp less and yield reduced CDC suggesting GTKOp Fn has antigenic epitopes responsible for CDC. One epitope on GTKOp Fn is Neu5Gc. Natural human antibodies that bind GTKOp Fn epitopes also bind epitopes on other proteins of GTKOp kidney, GTKOp liver, and *E.coli*.

Figure 4-1. Preformed human antibody response to aortic endothelial cells (AEC) from α1,3-galactosyltransferase knockout human CD55 transgenic pigs (GTKOp). (A) CD31 (red), SLA1 (blue) and HLA1 (blue) for GTKOp (left) and human AEC (right). (B) Confocal images of GTKOp and human AEC with human IgM (green), IgG (red), nuclei (blue) and merge colocalization (yellow) after incubation with 25% normal human serum. (C) Flow cytometric quantification represented as relative preformed human IgM (n=3, error bars=SD, $p<0.01$) and IgG (n=3, error bars=SD, $p<0.01$) binding of GTKOp and human AEC. (D) Confocal images of complement component 4 (C4) deposition onto GTKOp and human AEC after incubation with 5% human serum follow by complement. (E) Confocal MFI quantification of C4 deposition onto GTKOp and human AEC (n=3, error bars=SD, $p<0.05$). (F) Antibody-mediated complement dependent cytotoxicity of GTKOp serum on GTKOp AEC, human sera on human AEC and human sera on GTKOp AEC (n=3, error bars=SD, $p<0.01$).

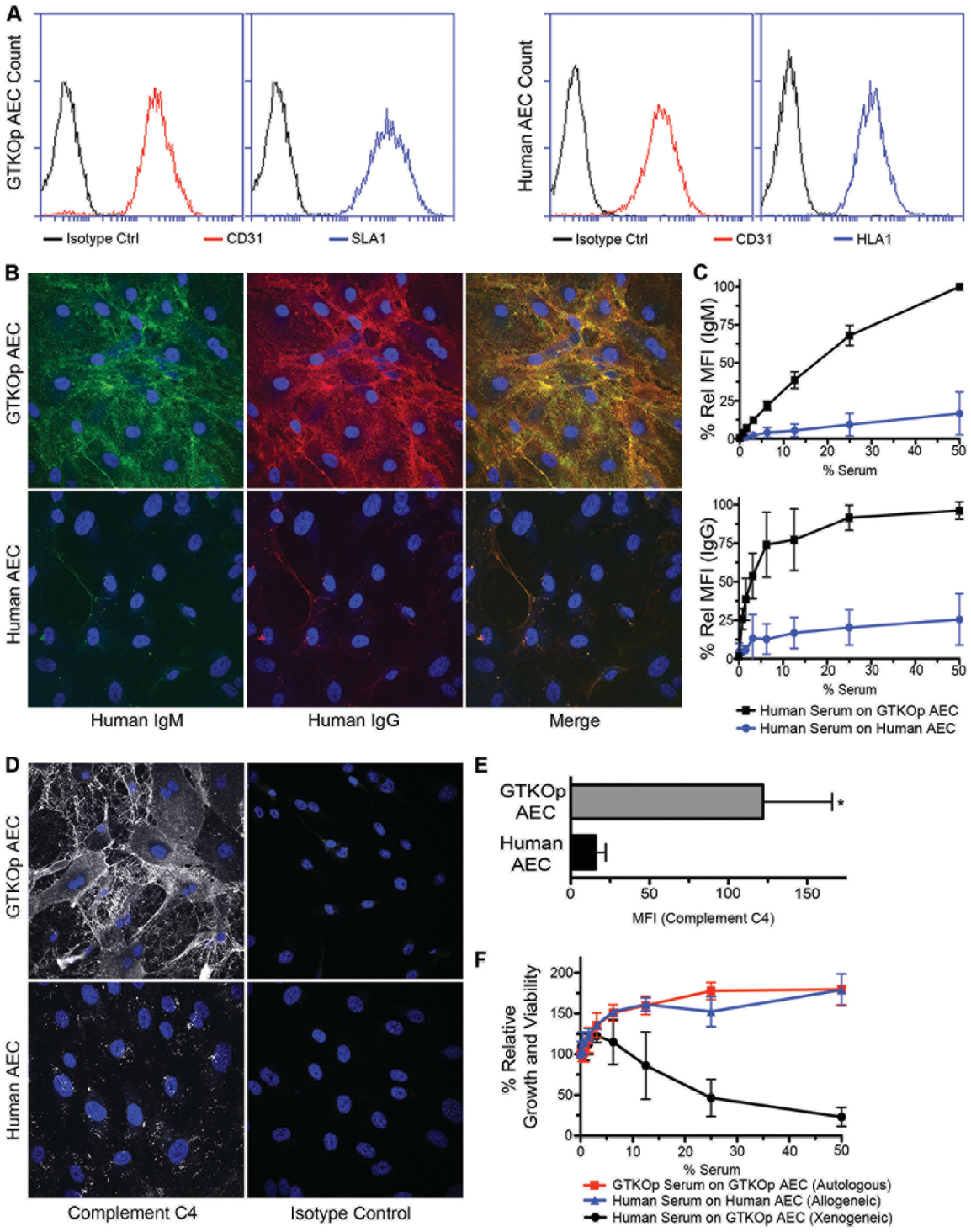

A
GTKOp AEC Count
Isotype Ctrl
CD31
SLA1
Human AEC Count
Isotype Ctrl
CD31
HLA1
B
GTKOp AEC
Human AEC
Human IgM
Human IgG
Merge
C
% Rel MFI (IgM)
% Serum
% Rel MFI (IgG)
% Serum
Human Serum on GTKOp AEC
Human Serum on Human AEC
D
GTKOp AEC
Human AEC
Complement C4
Isotype Control
E
GTKOp AEC
Human AEC
MFI (Complement C4)
F
% Relative Growth and Viability
% Serum
GTKOp Serum on GTKOp AEC (Autologous)
Human Serum on Human AEC (Allogeneic)
Human Serum on GTKOp AEC (Xenogeneic)

Figure 4-2. Evaluation of GTKOp fibronectin (Fn) expression and preformed antibody reactivity. (A) Confocal images of GTKOp kidney, liver, AEC and human AEC for CD31 (green), Fn (red), CD18 or beta-actin (magenta), AEC isotype controls, nuclei (blue) and merge. Colocalization (yellow) of CD31 and Fn in the kidney and liver is shown in the merge. (B) Coomassie stained gel, Fn, total human IgM and IgG immunoblots of *E. coli* lysate, human albumin, GTKOp and human Fn and (C) corresponding band density quantification and comparison of GTKOp and human Fn recognizing antibodies (n=4, error bars=SD, *$p<0.05$).

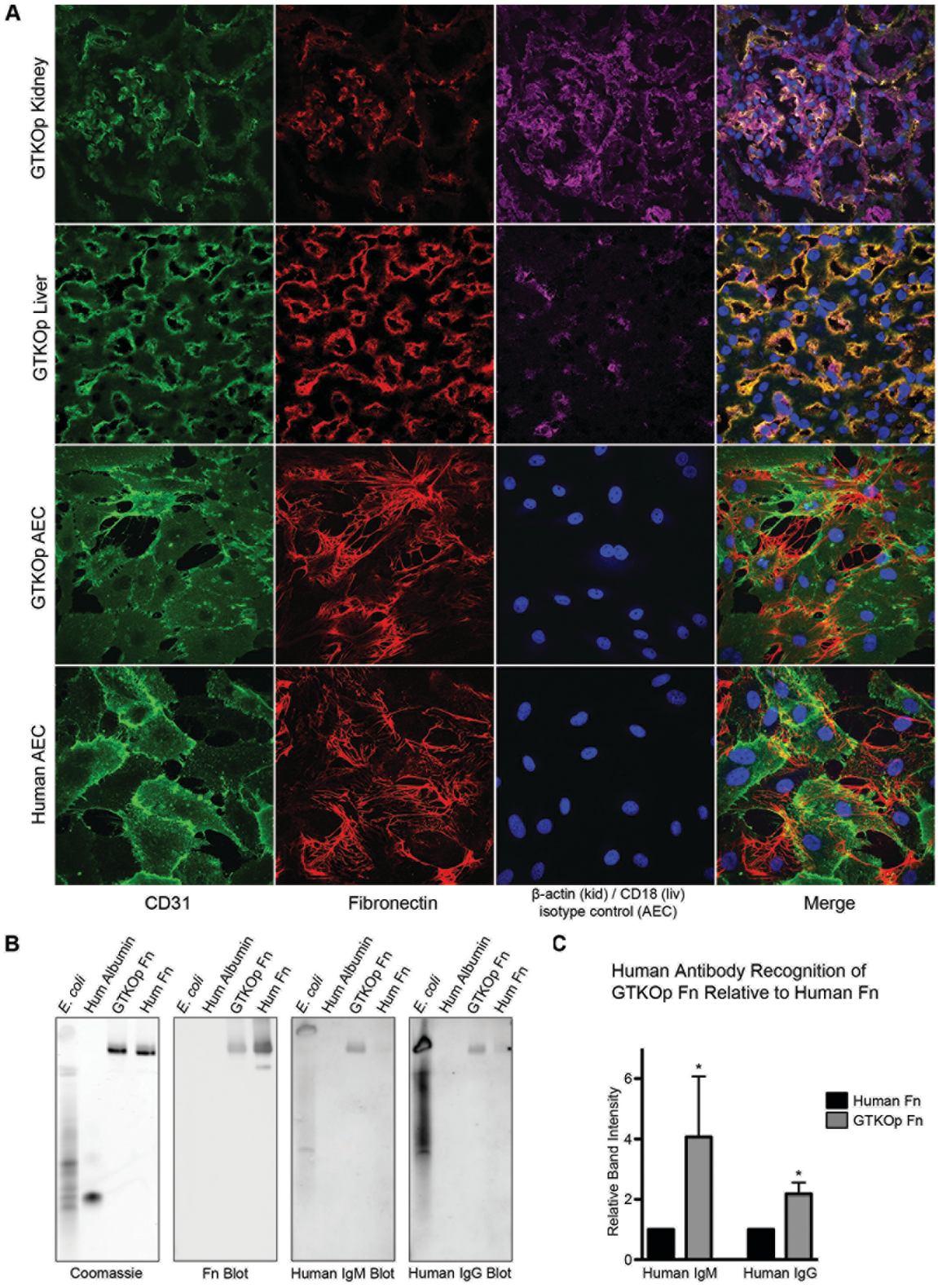

A
GTKOp Kidney
GTKOp Liver
GTKOp AEC
Human AEC
CD31
Fibronectin
β-actin (kid) / CD18 (liv)
isotype control (AEC)
Merge
B
E. coli
Hum Albumin
GTKOp Fn
Hum Fn
Coomassie
Fn Blot
Human IgM Blot
Human IgG Blot
C
Human Antibody Recognition of
GTKOp Fn Relative to Human Fn
Relative Band Intensity
Human Fn
GTKOp Fn
Human IgM
Human IgG

Figure 4-3. Comparison of human sera absorbed with GTKOp or human AEC monolayers. (A) Representative confocal images of serial 1hr absorptions to GTKOp or human AEC monolayers. (B) Confocal MFI quantification and comparison of human IgM (n=3, error bars=SD, $p<0.01$) and IgG (n=3, error bars=SD, $p<0.05$) binding of GTKOp and human AEC monolayers. (C) Antibody-mediated complement dependent cytotoxicity comparison of GTKOp and human AEC absorbed serum (n=3, error bars=SE, $p<0.05$). (D) Relative human anti-GTKOp Fn IgM and IgG concentration after absorption of human sera to GTKOp or human AEC (n=3, error bars=SD, $*p<0.001$).

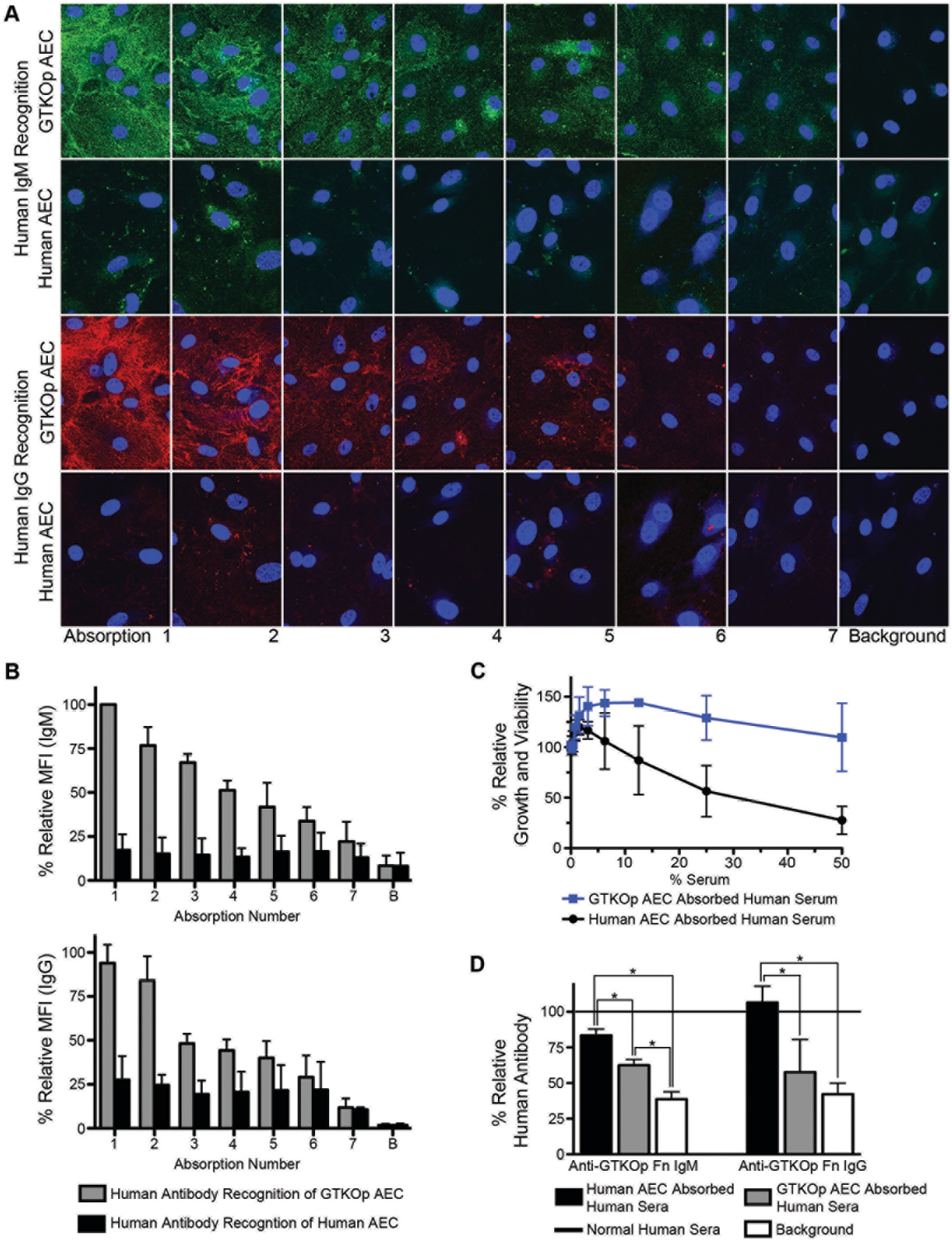

A
Human IgM Recognition
GTKOp AEC
Human AEC
Human IgG Recognition
GTKOp AEC
Human AEC
Absorption 1 2 3 4 5 6 7 Background
B
% Relative MFI (IgM)
Absorption Number
% Relative MFI (IgG)
Absorption Number
Human Antibody Recognition of GTKOp AEC
Human Antibody Recogntion of Human AEC
C
% Relative Growth and Viability
% Serum
GTKOp AEC Absorbed Human Serum
Human AEC Absorbed Human Serum
D
% Relative Human Antibody
Anti-GTKOp Fn IgM
Anti-GTKOp Fn IgG
Human AEC Absorbed Human Sera
GTKOp AEC Absorbed Human Sera
Normal Human Sera
Background

Figure 4-4. N-glycolylneuraminic acid (Neu5Gc) evaluation on GTKOp glycoproteins. (A) Confocal images of GTKOp liver, kidney, AEC and human AEC expression of Neu5Gc (cyan) with isotype controls. (B) Coomassie stained gel, Neu5Gc and Fn immunoblots for mouse glycoprotein positive control, human albumin, GTKOp and human Fn. (C) Coomassie stained gel and Neu5Gc blot for GTKOp serum, human serum, GTKOp kidney and liver lysates.

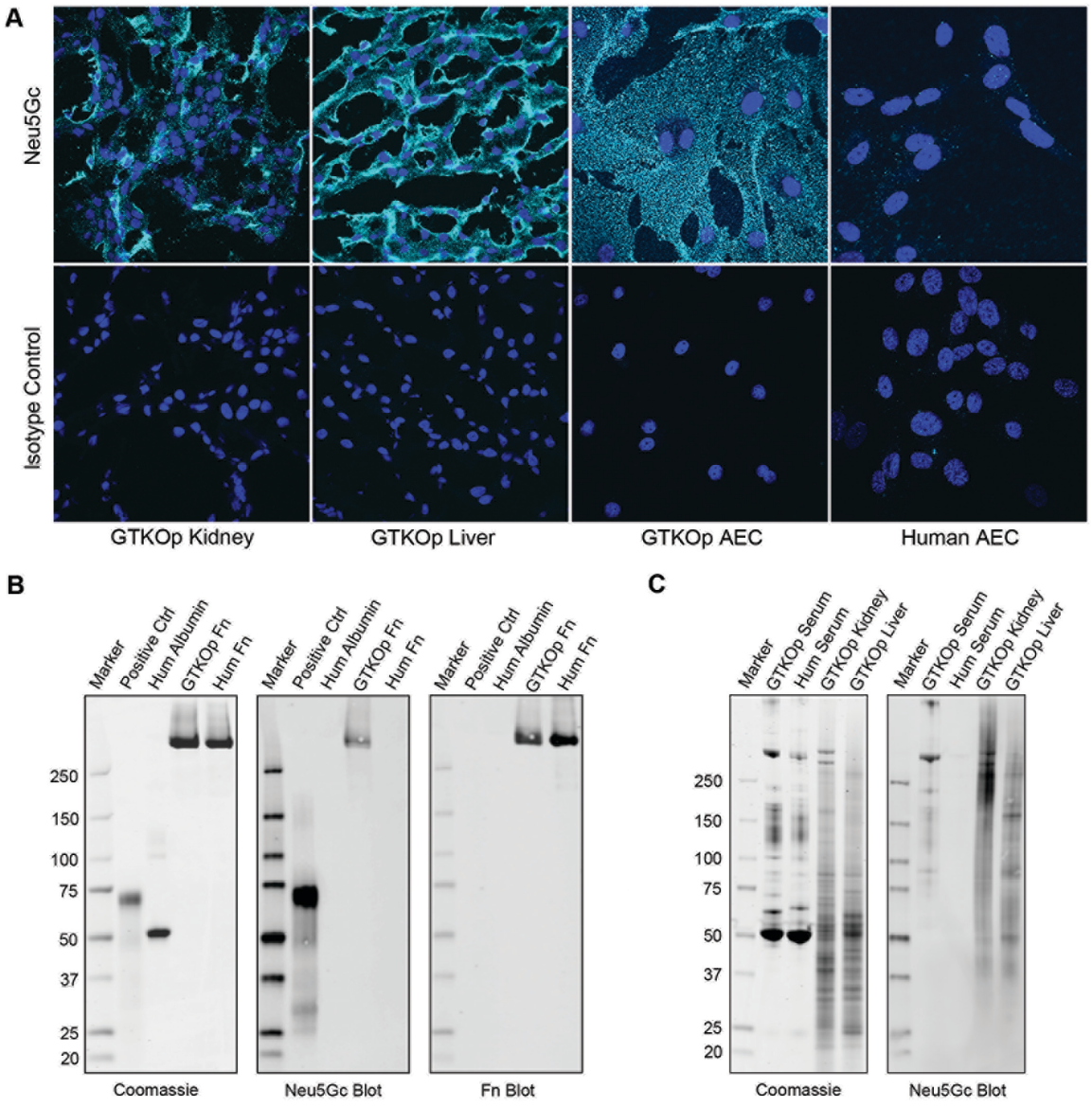
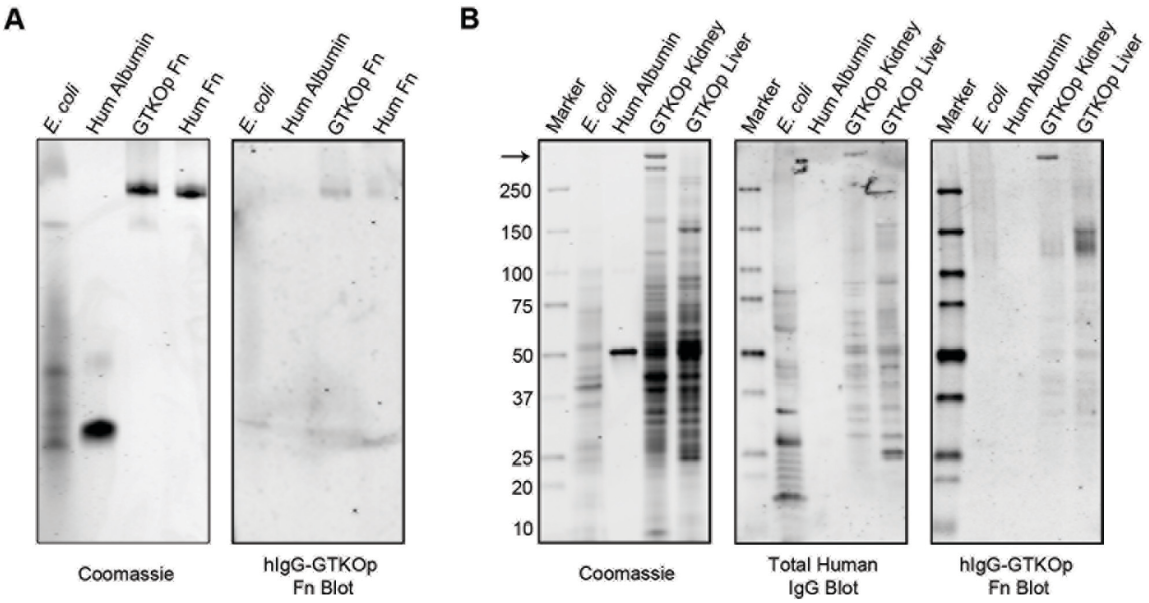
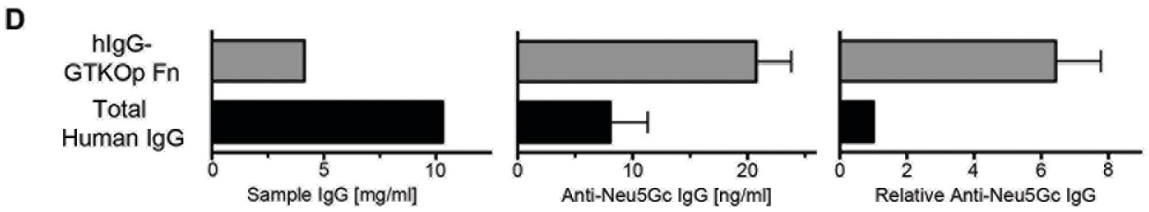


Figure 4-5. Cross-reactivity of immunoaffinity-purified human IgG on a GTKOp Fn column (hIgG-GTKOp Fn). (A) Coomassie stained gel and hIgG-GTKOp Fn immunoblot of *E. coli* lysate, human albumin, GTKOp and human Fn. (B) Coomassie stained gel, total human IgG and hIgG-GTKOp Fn immunoblots of *E. coli* lysate, human albumin, GTKOp kidney and liver lysates. One high molecular weight cross-reactive band was identified across the gel and immunoblot (black arrow). (C) List of proteins identified in the cross-reactive high molecular weight band. * Molecular Weight Search (MOWSE) Scores > 36 indicate identity or extensive homology ($p<0.05$). (D) Comparison of total human IgG and hIgG-GTKOp Fn for sample IgG (mg/mL), anti-Neu5Gc IgG (ng/mL) (n=1, error bars = SE) and a normalized relative anti-Neu5Gc IgG concentration (n=1, error bars = SE).



C

Protein Name	MW (kDa)	MS/MS MOWSE Score*
4F2 cell-surface antigen heavy chain (4F2)	58	45
Argininosuccinate lyase (ARLY)	50	42
Low-density lipoprotein receptor-related protein 2 precursor (LRP2)	519	62
Probable E3 ubiquitin protein ligase TOM1 (TOM1)	37	40
Uncharacterized protein C12orf26 homolog (CL026)	67	42



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Chapter 5: Discussion

Discussion Objectives

Our research has shed light on physiologic and immunologic barriers to clinical xenotransplantation more specifically xenotransplant-related thrombocytopenia and antibody-mediated rejection respectively. Although a distinction is made between physiologic and immunologic obstacles, xenotransplant-related thrombocytopenia and antibody-mediated rejection appear interdependent upon further inspection. Genetic modifications of pigs to improve antibody-mediated rejection may affect xenotransplant-related thrombocytopenia and vice versa resulting in many upcoming challenges in the field. This chapter will summarize our research, comment on additional findings and thoughts, and discuss future directions and the relationship between physiologic and immunologic incompatibilities related to clinical xenotransplantation.

Xenotransplant-Related Thrombocytopenia

Since no current therapies exist to treat life threatening acute on chronic liver failure, porcine liver xenotransplantation as a bridge to allotransplantation was proposed as a solution (1). Xenotransplant-related thrombocytopenia and resulting hemorrhage was determined to be a major barrier (2). Ex-vivo porcine liver perfusion experiments implicated liver sinusoidal endothelial cells (LSEC) and Kupffer cells (KC) to be involved with clearance of human platelets (3). Since cold storage of platelets cause rapid clearance after transfusion, associated mechanisms were evaluated for relevance in xenotransplant-related thrombocytopenia. Receptors implicated in cold stored platelet clearance included CD11b / CD18 (Mac-1, CR3, $\alpha_M\beta_2$) and asialoglycoprotein receptor 1 (ASGR1) (4, 5).

Porcine KC and human platelet interactions were studied demonstrating human platelet clearance by porcine KC in vitro (Chapter 2). CD18 receptor was targeted with antibodies and siRNAs and determined to be an important receptor for platelet clearance by porcine KC (Chapter 2) and recently the involvement of CD11b / CD18 receptor was identified in porcine liver sinusoidal endothelial cells (LSEC) (6). Platelet clearance by KC could not be completely blocked through inhibition of the CD18 receptor implying redundant mechanisms. Studies in porcine liver sinusoidal endothelial cells (LSEC) have determined asialoglycoprotein receptor 1 (ASGR1) to be involved with platelet clearance (7). Similar to CD18, inhibition of ASGR1 through antibody blocking and siRNAs led to reduction in human platelet uptake by porcine LSEC. KC have been shown to

express the ASGR1 receptor by others (8) representing another area of further investigation.

Platelet surface carbohydrates exposed from loss of sialic acids from platelet membrane glycoproteins have been implicated in the platelet clearance (4). The heterodimer CD11b / CD18 and ASGR1 are pattern recognition receptors. Mac-1 and ASGR1 recognize carbohydrates through carbohydrate binding domains, which are lectin-like (9) and C-type lectin domains (10) respectively. Mac-1 has a broad range of carbohydrate recognition including glucose derivatives, mannose and β-glucans, for example galactose β1-4 N-acetyl glucosamine (Galβ) and N-acetyl glucosamine β1-4 N-acetyl glucosamine (βGlcNAc) (9, 11). ASGR1 has affinity towards β-glucans with terminal galactose such as Galβ (10). Differences in human and pig carbohydrate binding domains for Mac-1 and ASGR1 were identified (chapter 3) which may alter affinities for carbohydrate substrates resulting in cross species incompatibility. Comparison of human and pig platelet surface β-glucans demonstrated higher amounts of Galβ and βGlcNAc on human platelet surface before and after removal of sialic acids (chapter 3). Receptor differences and larger amount of β-glucans on human platelets implicate possible aberrant recognition of human platelets by porcine Mac-1 and ASGR1 receptors. Knockout, diminished expression or humanizing the carbohydrate domains of porcine Mac-1 and ASGR1 receptors may result in attenuation of xenotransplant-related thrombocytopenia.

Many questions remain regarding the mechanisms of human platelet clearance by porcine sinusoidal cells summarized in Figure 5-1. Our lab has

demonstrated pig LSEC and KC are capable of human platelet phagocytosis in vitro, however other sinusoidal cells may be involved directly or indirectly with this process including hepatocytes and stellate cells. Further, incomplete inhibition of human platelet phagocytosis by pig KC through inhibition of the CD18 receptor indicates that our understanding of the mechanisms for pig KC phagocytosis of human platelets is not comprehensive.

Apart from the incomplete understanding of the mechanisms behind xenotransplant-related thrombocytopenia, many concerns exist for generating pigs with altered CD18 and ASGR1. The knockout of the CD18 receptor was successful in mice (12), however the leukocyte adhesion deficiency disease 1 in humans with complete lack of CD18 often results in patient death within one year (13) questioning viability of a CD18 knockout pig. Although changes to carbohydrate binding domains of porcine Mac-1 and ASGR1 may be a viable alternative, incompatibilities may result in the demise of such genetically modified pigs. In vitro experiments have demonstrated a reduction in human platelet uptake through inhibition of CD18 and ASGR1. Whether or not such modification will result in clinical attenuation of xenotransplant-induced thrombocytopenia remains unknown. Although generation of CD18 or ASGR1 modified pigs may provide insight to such questions, further in vitro evaluation appears necessary given incomplete attenuation of thrombocytopenia attained in our experiments.

Future Directions for Xenotransplant-Related Thrombocytopenia

Since phagocytosis is a complex highly regulated process with involvement of many receptors, lack of complete inhibition of human platelet phagocytosis through inhibition of CD18 on porcine KC (chapter 2) was not surprising. Phagocytosis may functionally be divided into two categories depending on the target engulfed. Invading pathogens and apoptotic cells (AC) must be engulfed to prevent infection and maintain cellular homeostasis respectively (14, 15). Mac-1 functions as part of the innate immune system (16) and as a result, human platelets may be recognized as foreign and cleared by porcine KC. ASGR1 is implicated in clearance of AC (15) and glycoproteins through recognition of carbohydrates following desialylation (17). Human platelets may appear apoptotic to porcine phagocytes leading to recognition and clearance through ASGR1 expressing cells. Since Mac-1 and ASGR1 are implicated in platelet and apoptotic cell clearance (18, 19), exploration of AC clearance mechanisms and its relation to xenotransplant-related thrombocytopenia appears warranted.

Apoptotic cell phagocytosis process has been divided into several phases, which includes recognition, tethering, signaling and phagocytosis corresponding to initial interaction, binding, extra and intra-cellular communications and engulfment respectively (19). CD11b / CD18 and ASGR1 belong to receptors responsible for signaling and tethering (19, 20). Many receptors have been studied regarding each phase of apoptotic cell clearance and among these receptors, signal-regulatory protein alpha (Sirp-α, Src homology 2 domain-

bearing protein tyrosine phosphatase substrate-1, SHPS-1) and CD31 (platelet-endothelial cell adhesion molecule-1, PECAM-1), appear noteworthy for further evaluation for their implications in CD18 regulation in addition to AC phagocytosis (Figure 5-2) (19, 21-23).

The interaction between macrophage SIRP-α and platelet CD47 was found to regulate platelet homeostasis through inhibition of platelet clearance (24). Phagocyte SIRP-α mediates the tethering stage of AC phagocytosis by interaction with AC CD47 (25) and inhibits phagocytosis through immunoreceptor tyrosine-based inhibition motif (ITIM) signaling by negatively regulating CD11b / CD18 expression (22). Xenotransplant studies have identified an incompatibility between SIRP-α on human macrophages and CD47 on porcine cells resulting in lack of downstream inhibitory signaling leading to clearance of porcine cells by human phagocytes (26, 27). The reverse, compatibility of SIRP-α on porcine macrophages and CD47 on human platelets signaling to our knowledge has not been established in literature. If porcine phagocyte SIRP-α and human platelet CD47 do not interact appropriately, inhibitory phagocytic signals would not be initiated possibly leading to human platelet phagocytosis justifying further evaluation (Figure 5-3).

CD31 is described as part of the recognition phase of AC phagocytosis and has been demonstrated to upregulate several integrins including CD18 (β_2 integrin) expression in monocytes upon cell surface treatment with anti-CD31 antibodies (21, 23, 28). Homophilic interaction between CD31 on macrophages and AC has been implicated in initiating downstream stages of AC clearance (18,

28-31). CD31 may disable phagocyte-AC detachment by inhibitory signals dependent on ITIM located in the cytoplasmic tail of CD31 (31). Oligomerization of the CD31 receptors appear necessary to activate integrins (21). Comparison of pig and human CD31 protein sequences using Basic Local Alignment Search Tool (BLAST) resulted in 71% maximum identity demonstrating substantial amino acid sequence differences. A physiologic incompatibility between pig and human CD31 expressed on porcine KC, LSEC and human platelets may result in abnormal signaling resulting in the inhibition of platelet release and CD31 oligomerization leading to integrin activation and human platelet phagocytosis.

Since porcine CD18 inhibition resulted in attenuation of human platelet clearance by porcine KC (Chapter 2), additional investigation of receptors such as SIRP-α and CD31 with ability to regulate CD18 appears to be the next logical step. Incompatibilities of inhibitory receptors, porcine SIRP-α / human CD47 and porcine CD31 / human CD31 may result in upregulation of CD18. ASGR1 involvement in human platelet clearance demonstrated for porcine LSEC suggests redundant mechanisms involved. Receptors involved with ASGR1 regulation could be other potential targets for evaluation in xenotransplant-related thrombocytopenia.

Antibody-Mediated Rejection and Xenoantigens

Host antibodies recognizing antigens on transplanted grafts result in complement fixation and graft dysfunction known as antibody-mediated rejection as demonstrated in xenotransplant models (32-41). When wild-type pig organs are transplanted into non-human primate models, graft rejection within minutes to hours occurs known as hyperacute rejection. Deletion of a major carbohydrate antigenic epitope, galactose α1,3-galactose (αGal), on wild-type pigs has significantly reduced the incidence of hyperacute rejection in pig to non-human primate xenotransplant models (34, 35, 37, 42). Generation of pigs lacking αGal through knockout of the α1,3-galactosyltransferase gene (GTKO) demonstrated ability to improve xenograft immune compability through genetic modifications of pigs. The ensuing acute or delayed rejection due to other antigens continue to be an obstacle to overcome, since antibodies towards donor graft antigens have been attributed for hyperacute, acute and more recently chronic rejection (43, 44). This highlights the importance of host antibody and graft antigen interaction in transplant rejection. Since human antibodies have been shown to recognize antigens and cause fixation of complement on pig cells lacking αGal (Chapter 3), antibodies reacting towards GTKO pig xenoantigens were further evaluated.

Porcine proteins, oligosaccharides, lipids, glycoproteins, glycolipids, DNA, RNA and other biological materials recognized by host antibodies are xenoantigens. Specific sites on porcine biological materials that are bound by human antibodies are xenoantigenic epitopes. For example, αGal was determined to be an antigenic epitope on porcine glycoproteins (45). Rejection

studies involving GTKO pig organs in non-human primates suggest evidence of acute vascular type of rejection (35, 46, 47). Human antibodies recognized GTKO porcine endothelial cells that line vasculature resulting in complement fixation and decreased cell viability (Chapter 4). Evaluation and identification of xenoantigenic epitopes on the endothelial cell surface appears most relevant to improving xenograft compatibility. Since many cell surface proteins are glycosylated and antigenic epitopes may be peptides or oligosaccharides, antigenic porcine glycoproteins expressed on the cell surface of the endothelium would be ideal for study. GTKO fibronectin (Fn) glycoprotein was chosen for evaluation, because of abundant expression on pig endothelial cells (Chapter 4) and induction of antibodies in pig to non-human primate cardiac xenotransplant studies (42).

Confirming the existence of preformed human antibody towards GTKO Fn was much more difficult than initially anticipated. Determining appropriate negative control conditions required alterations to many commonly used protocols in proteomic studies due to existence of preformed human antibodies towards denatured, unfolded or misfolded human Fn. This phenomenon could be explained by exposure of antigenic epitopes previously hidden when Fn unfolds allowing recognition by preformed human antibodies (48). Although reasons for the presence of these antibodies remains largely unknown, a possible functional explanation could be a clearance mechanism for misfolded or damaged Fn in plasma similar to damage associated molecular pattern hypothesis of preformed human antibody recognition of damaged cells (49).

Anecdotally, human antibody recognition of human endothelial cells was noted on several occasions when cells used were overgrown in cell culture dishes. Regardless, presence of such antibodies has lead to stringent protein preparation conditions. Purification of Fn for antigen evaluation required an affinity column and size exclusion column followed by urea refolding process to attain native conformation Fn. Denaturing agents such as strong detergents and reducing agents such as 2-Mercaptoethanol frequently used to increase resolution in electrophoresis was avoided to prevent erroneous results leading to confirmation of greater preformed human antibody recognition of GTKO Fn relative to human Fn.

Carbohydrate evaluation demonstrated the presence of the N-glycolylneuraminic acid (Neu5Gc), non-human sialic acid, on GTKO Fn (Chapter 4). Normal human serum antibodies toward Neu5Gc have been shown (50) and several studies have identified Neu5Gc as an antigenic determinant capable of initiating complement dependent cytotoxicity (51-53). When human IgG was purified against GTKO Fn, presence of anti-Neu5Gc IgG was detected confirming Neu5Gc as an antigenic epitope on GTKO Fn (Chapter 4). The amount of human anti-Neu5Gc IgG was relatively small compared to the total amount of human anti-GTKO Fn IgG purified on a GTKO Fn column suggesting the presence of other antigenic epitopes on GTKO Fn. Although several other potential antigenic proteins were identified using human anti-GTKO Fn IgG, possible cross reactivity of the anti-Neu5Gc antibodies to Neu5Gc on identified proteins complicates the end results. In order to more conclusively identify

presence of further antigenic epitopes of GTKO Fn, sialic acids were removed using Sialidase A. Unfortunately, removal of sialic acids from human Fn negative control resulted in positive human antibody recognition on western blotting. The findings were not surprising given that glycosylations have a role in protein folding resulting in aforementioned issues (54). Similar to the pre-GTKO pig era and studies evaluating non-αGal antigens, definitive evidence for non-Neu5Gc antigens was difficult to confirm experimentally using Neu5Gc positive pigs.

Future Directions for Antibody-mediated Rejection and Xenoantigens

Although our GTKO Fn studies suggest other antigenic epitopes present other than Neu5Gc, knockout of the Neu5Gc antigen in addition to αGal appear necessary to conclusively determine clinical relevance of Neu5Gc and identify non-Neu5Gc antigenic epitopes. Recently, generation of pigs lacking αGal and Neu5Gc was accomplished in our lab. Zinc finger nucleases were used to knockout α-1,3 galactosyltransferase gene responsible for the αGal antigen followed by knockout of cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) gene required for the conversion of N-acetylneuraminic acid (Neu5Ac) to Neu5Gc (Figure 5-4) (55). Somatic nuclear cell transfer was used to generate GTKO pigs then GTKO/CMAHKO pigs (Figure 5-5) (55). The phenotypes of these pigs were confirmed through flow cytometry and confocal analysis (Figure 5-6) (55). Evaluation of GTKO/CMAHKO peripheral blood mononuclear cells (PBMC) demonstrated decreased recognition by preformed human antibodies and reduced complement dependent cytotoxicity compared to GTKO PBMC (Figure 5-7) (55).

Initial evaluation comparing antibody binding and complement dependent cytotoxicity between human and baboon, chimpanzee, GTKO/CMAHKO pigs demonstrated similar levels of cytotoxicity (Figure 5-8). Concordant xenograft transplantation such as chimpanzee to human renal transplantion (Reetsma et al) has previously shown some promise in clinical studies even without modern immunosupression drugs (Chapter 1), suggesting possible clinical success with the comparable GTKO/CMAHKO pig renal xenografts. Although the generation

of GTKO/CMAHKO pigs appears to be a significant advancement in pig to human immune compatibility, there continues to be antibody recognition and cytotoxicity of GTKO/CMAHKO pig cells compared to human control. Further evaluation of xenoantigens in the absence of Neu5Gc is necessary to reach allotransplant level of immune compatibility. Continued evaluation of fibronectin from GTKO/CMAHKO pigs may allow identification of other significant antigenic epitopes for removal.

Concluding Remarks

Utilization of life saving organ transplantation and treatment of critical illnesses involving organ failure continue to be limited by the shortage of donor organs. Clinical xenotransplantation could alleviate the donor organ shortage. Research in xenotransplant-related thrombocytopenia and immune incompatibility has not only improved my understanding of the mechanisms behind these barriers, but has changed my overall thought process behind the barriers to xenotransplantation.

Physiologic and immunologic incompatibilities between humans and pigs appear to be intimately related scientific obstacles preventing successful clinical xenotransplantation. For instance, differences in human and porcine cell surface receptors CD11b / CD18 were implicated in causing xenogeneic platelet clearance. Since CD11b / CD18 is a known pattern recognition receptor involved with the innate immune system (16), receptor differences between humans and pigs could affect intrinsic immune compatibility. Genetic modifications such as knockout or humanization of porcine receptors to improve physiologic compatibility may dually lead to a reduction in xenoantigens diminishing antibody-mediated rejection.

Immune incompatibility may also affect physiologic incompatibility. The ability of antibodies to block the function of cell surface receptors has been commonly used in scientific experiments. For example, mouse anti-CD18 antibodies were used to block the function of the receptor in porcine Kupffer cells resulting in human platelet phagocytosis (Chapter 2). Similarly, human

antibodies that recognize porcine cell surface receptor antigens may alter receptor function resulting in physiologic dysfunction. Modifications to pigs that reduce antigenic epitopes may improve in vivo physiologic compatibility.

The creation of GTKO/CMAHKO pigs lead to humanization of porcine sialic acids, Neu5Gc to Neu5Ac, found on many cell surface receptors. Sialic acids have been implicated in numerous physiologic and immunologic functions that involve molecular and cellular interactions through the ability to block antigenic peptide epitopes, carbohydrate ligands and cell receptors and its capacity to act as a ligand especially for antibodies and mammalian lectins (56). The capability of sialic acids to block recognition of cells through masking of carbohydrate epitopes was demonstrated in chapter 3 when desialylation of platelets resulted in increased human platelet recognition and clearance by porcine cells. Variation in sialic acid masking of GTKO pig and human Fn could be partially responsible for human antibody recognition of GTKO pig Fn, yet another area needing evaluation. Blocking the function of receptors through sialylation has been demonstrated for the β_1 integrin receptor preventing cell adhesion and silencing of the LYVE-1 receptor in lymphatic endothelium (57). Siglecs, which bind sialic acids, have ability to alter cell-to-cell interactions (56). Comparison of Siglec-2 between mice and humans has revealed affinity differences for Neu5Gc and Neu5Ac highlighting potential physiologic and immunologic changes in pigs lacking Neu5Gc (58). Since sialic acid changes from Neu5Gc to Neu5Ac may affect receptor function, reevaluation of xenotransplant-related thrombocytopenia for GTKO/CMAHKO pigs is needed.

In conclusion, continued research is necessary for clinical xenotransplantation to become a reality given the complexities for bridging the physiologic and immunologic gaps between humans and pigs. Recent advances in genetic engineering have allowed quicker generation of genetically modified pigs for human compatibility analysis enhancing xenotransplant research efforts. Resolution of xenotransplant-related thrombocytopenia and antibody-mediated rejection barriers will require further research and likely further genetic alterations to pigs in order to attain clinical success.

Figure 5-1. Model of porcine liver sinusoidal endothelial cell (LSEC) and Kupffer cell (KC) clearance of human platelets. Porcine LSEC shown on the left expresses asialoglycoprotein receptor 1 (ASGR1) with affinity towards exposed galactose linked to N-acetylglucosamine (GlcNac). Porcine KC shown on the right expresses CD11b / CD18 with affinity mainly towards exposed GlcNac. Pig platelet on the left with fewer exposed galactose and GlcNac comparted to the human platelet on right. Pig platelets express N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Human platelets express Neu5Ac.

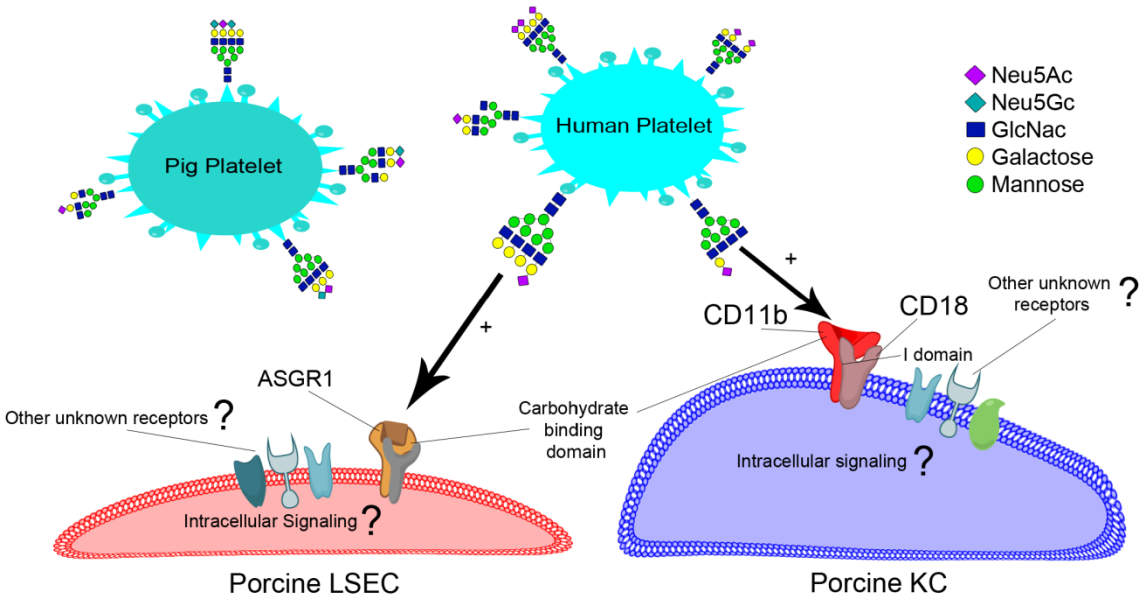

Figure 5-2. Receptors implicated in apoptotic cell clearance by macrophages adapted from Gregory 2004 (19). Receptor abbreviations: β_2 integrin, CD11b/CD18; ASGPR, asialoglycoprotein receptor (ASGR1/2); SHPS-1, Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1 (signal-regulatory protein-alpha, Sirp-α); dCD31, disabled CD31 (platelet-endothelial cell adhesion molecule-1, PECAM-1); 2GPI-R, 2-glycoprotein I receptor; ABCA1, ATP-binding cassette transporter 1; Altered CHO, altered carbohydrate; BAI-1, brain-specific angiogenesis inhibitor 1; CRT, calreticulin; MFG, milk-fat globule EGF-8; OxLIs, oxidized LDL-like site; OxPL, oxidized phospho- lipid; TBS, TSP-binding site; Tim 1,3,4, T cell Ig and mucin domain-containing molecule 1, 3, or 4. CD31 and Sirp-α have regulatory roles for CD18 when interacting with apoptotic cells.

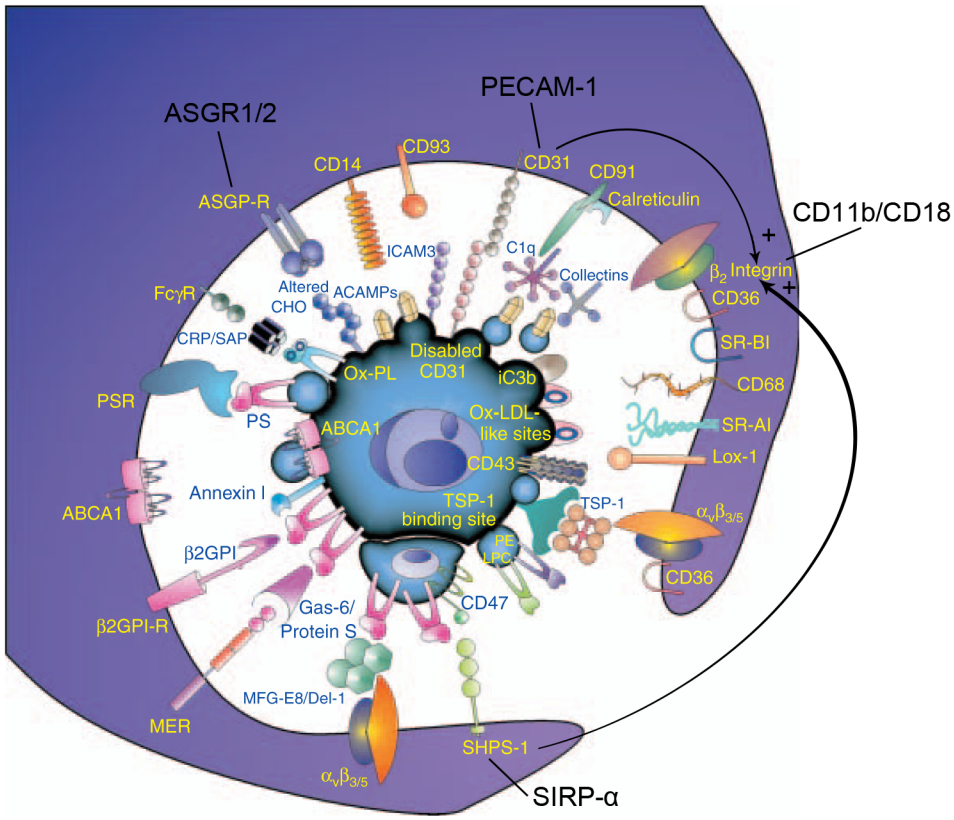

Figure 5-3. Diagram representation of porcine Sirp-α interaction with human CD47 from Leela Paris Ph.D 2013. Porcine CD47 (pCD47) interacts appropriately with porcine signal-regulatory protein-alpha (pSirp-α) resulting in phoshphorylation of the immunoreceptor tyrosine-based inhibitory motif recruiting SH2 tyrosine-specific protein phosphatase (SHP-2) inactivating the phagocytosis signal. Interaction between human CD47 (hCD47) and pSirp-α may not result in active SHP-2 resulting in continued phagocytosis signal.

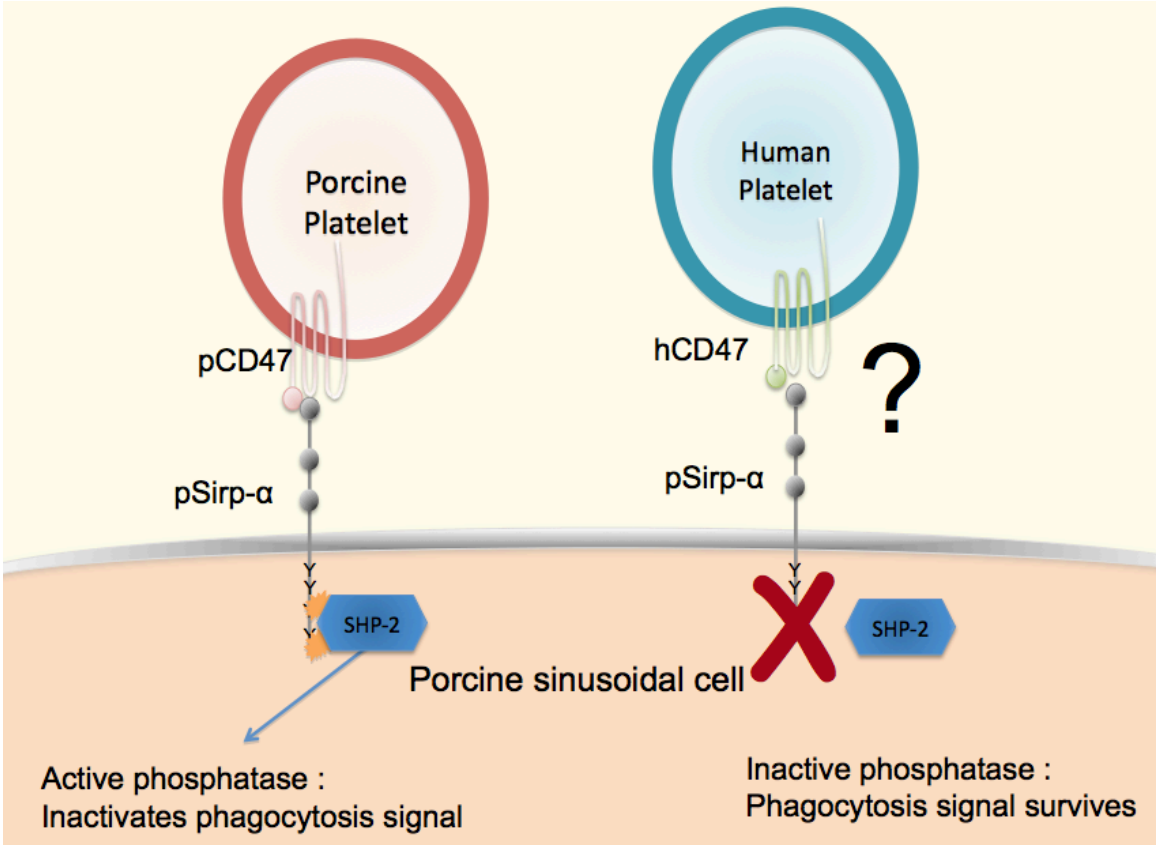

Figure 5-4. Diagram description of cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) function and result after knockout of the CMAH gene in α1,3 galactosyltransferase knockout (GTKO) pig background. GTKO pig cells express the CMAH enzyme necessary to catalyze the conversion of CMP-N-acetylneuraminic acid (CMP-Neu5Ac) to CMP-N-glycolylneuraminic acid (CMP-Neu5Gc). Knockout of CMAH results in inability to convert CMP-Neu5Ac to CMP-Neu5Gc resulting in cell surface expression of N-acetylneuraminic acid (Neu5Ac) and not N-glycolylneuraminic acid (Neu5Gc).

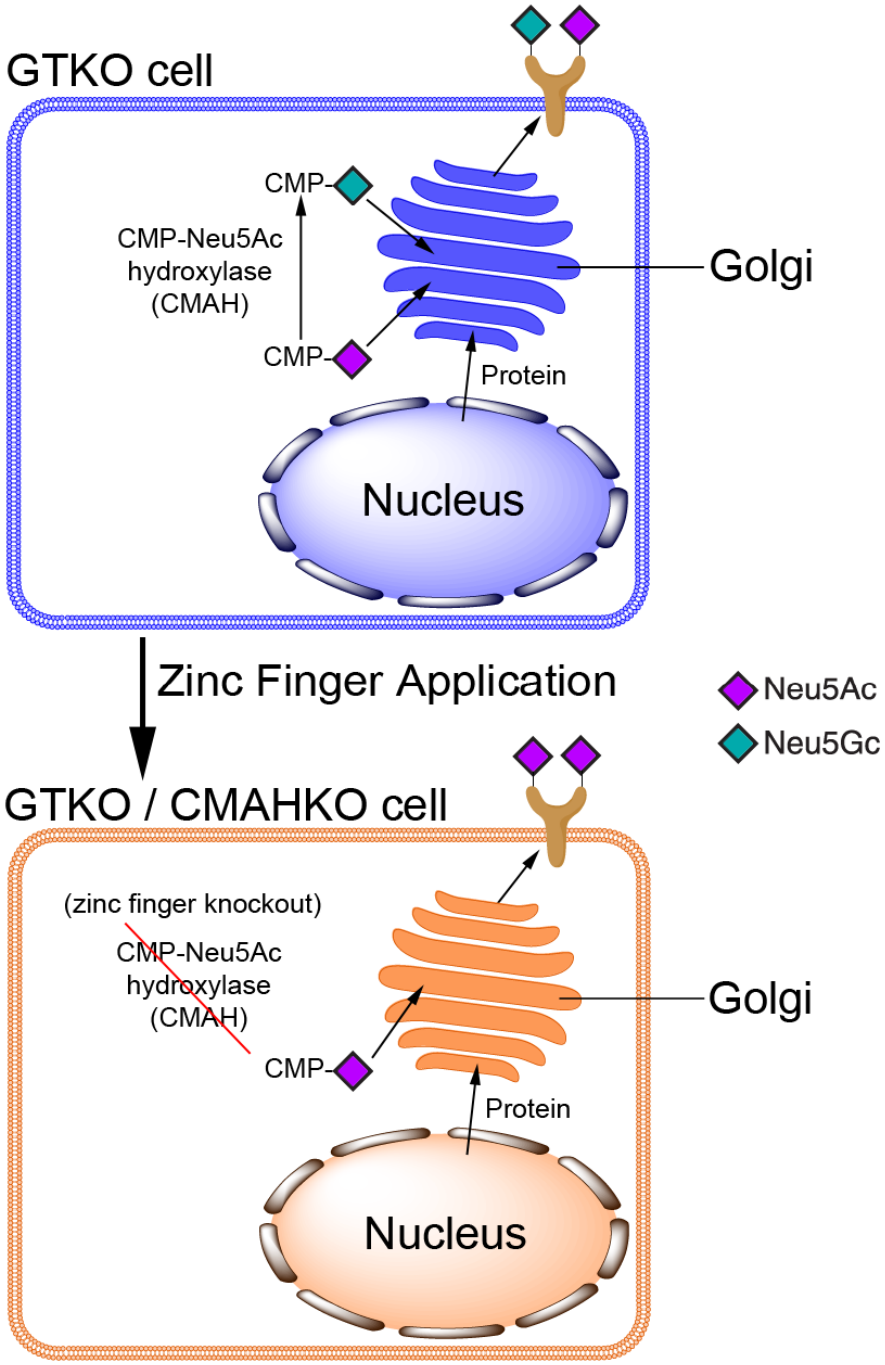

Figure 5-5. Photo of α1,3 galactosyltransferase and cytidine monophosphate-N-acetylneuraminic acid hydroxylase double knockout pigs at 1 week old.

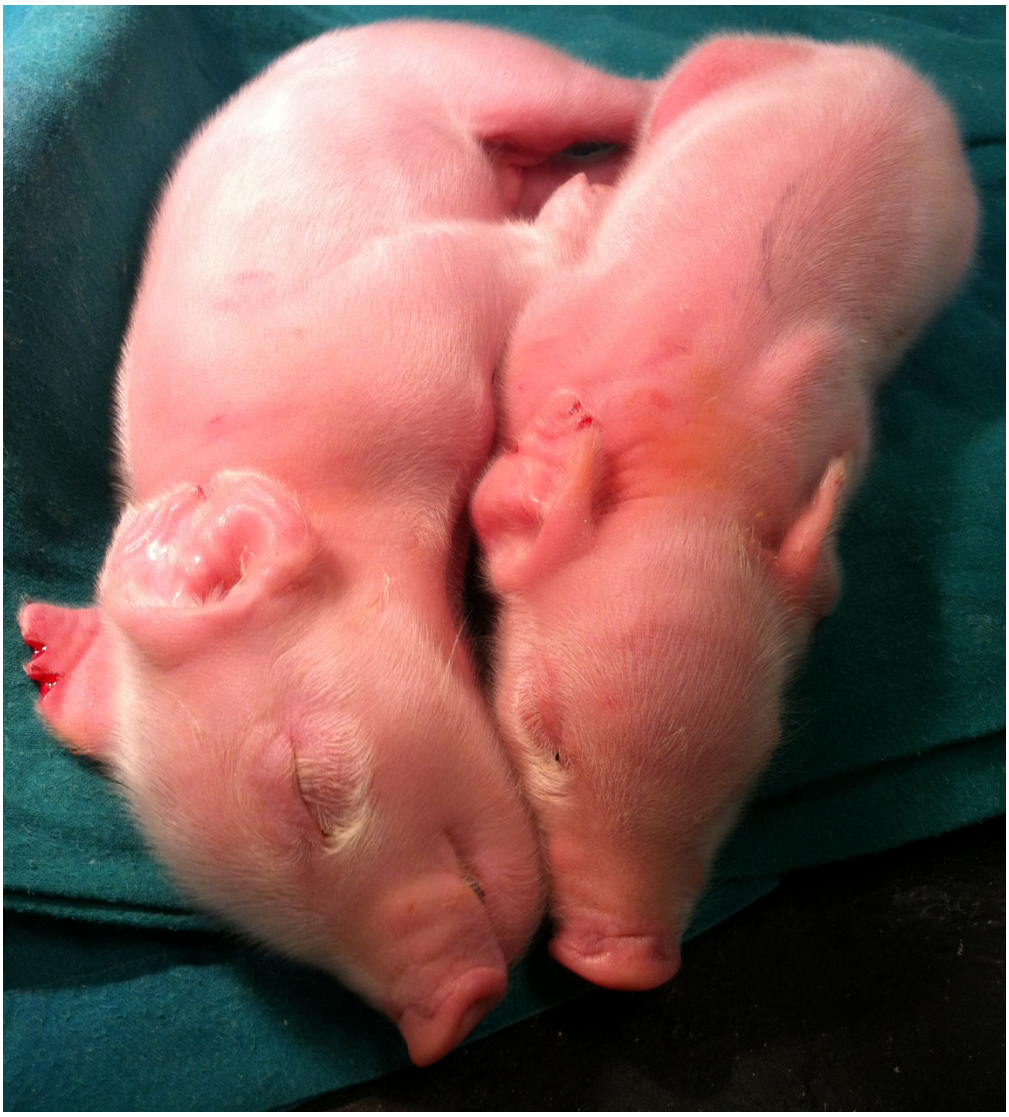

Figure 5-6. Confocal microscopy comparison of domestic, α1,3 galactosyl-transferase knockout (GTKO) and GTKO / cytidine monophosphate-N-acetylneuraminic acid hydroxylase double knockout (GTKO/CMAHKO) pigs modified from Lutz 2012 (55). Galactose α1,3 galactose epitope evaluated using Isolectin B4 staining. N-glycolylneuraminic acid was evaluated using chicken anti-N-glycolylneuraminic acid IgY.

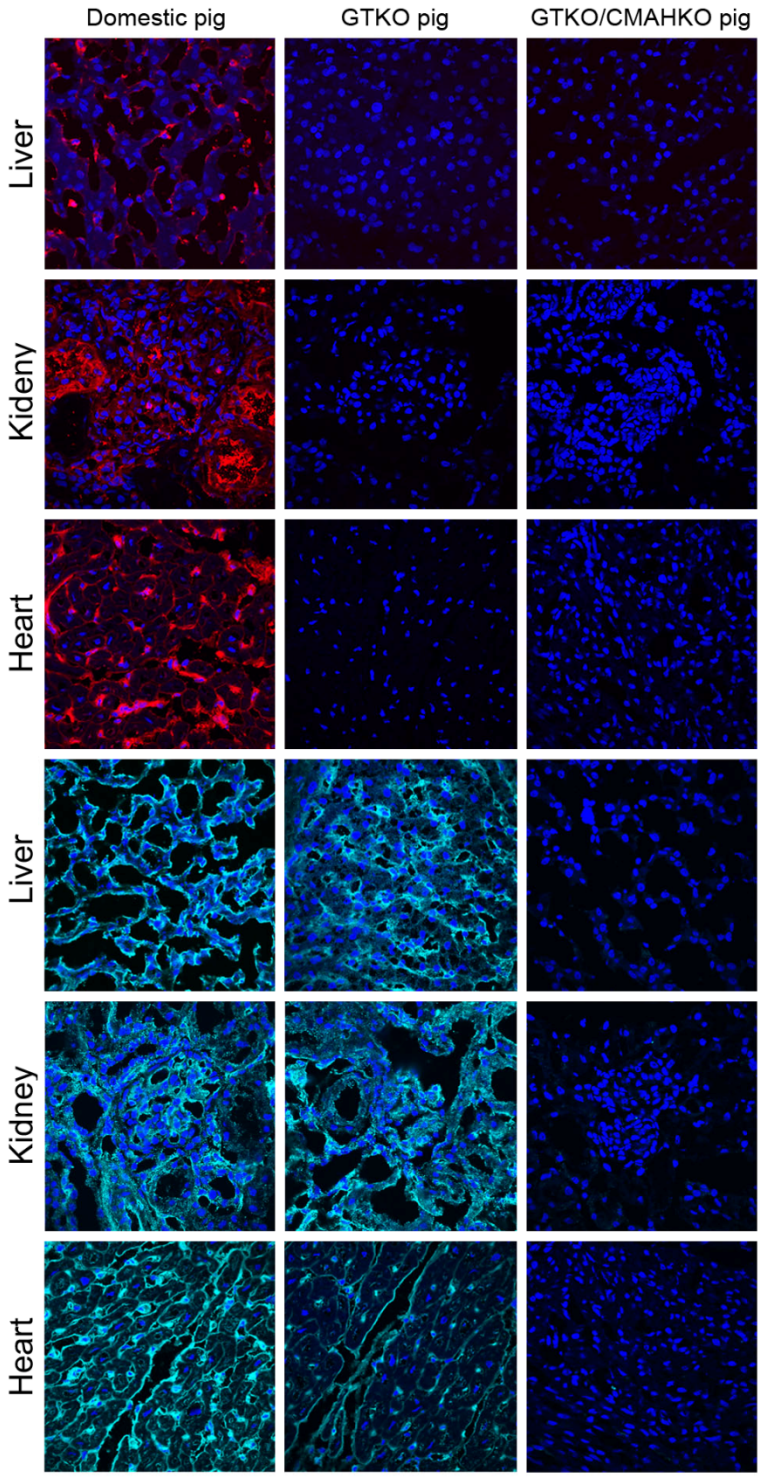

Figure 5-7. Human IgM, IgG antibody recognition and cytotoxicity analysis of peripheral blood mononuclear cells (PBMC) comparing α1,3 galactosyl-transferase knockout (GTKO) and GTKO / cytidine monophosphate-N-acetylneuraminic acid hydroxylase knockout (GTKO/CMAHKO) pigs Lutz 2012 (55). (A) Ten subjects were evaluated using flow cytometer with random representative histogram (left). Mean fluorescence intensities of IgM and IgG compared in the bar graphs (right). (B) Cytotoxicity compared by flow cytometer using fluorescein diacetate and propidium iodide staining. Random subject was chosen for graph of % cytotoxicity per concentration of serum (left) and bar graph comparison of cytotoxicity at 2% serum of ten subjects (right).

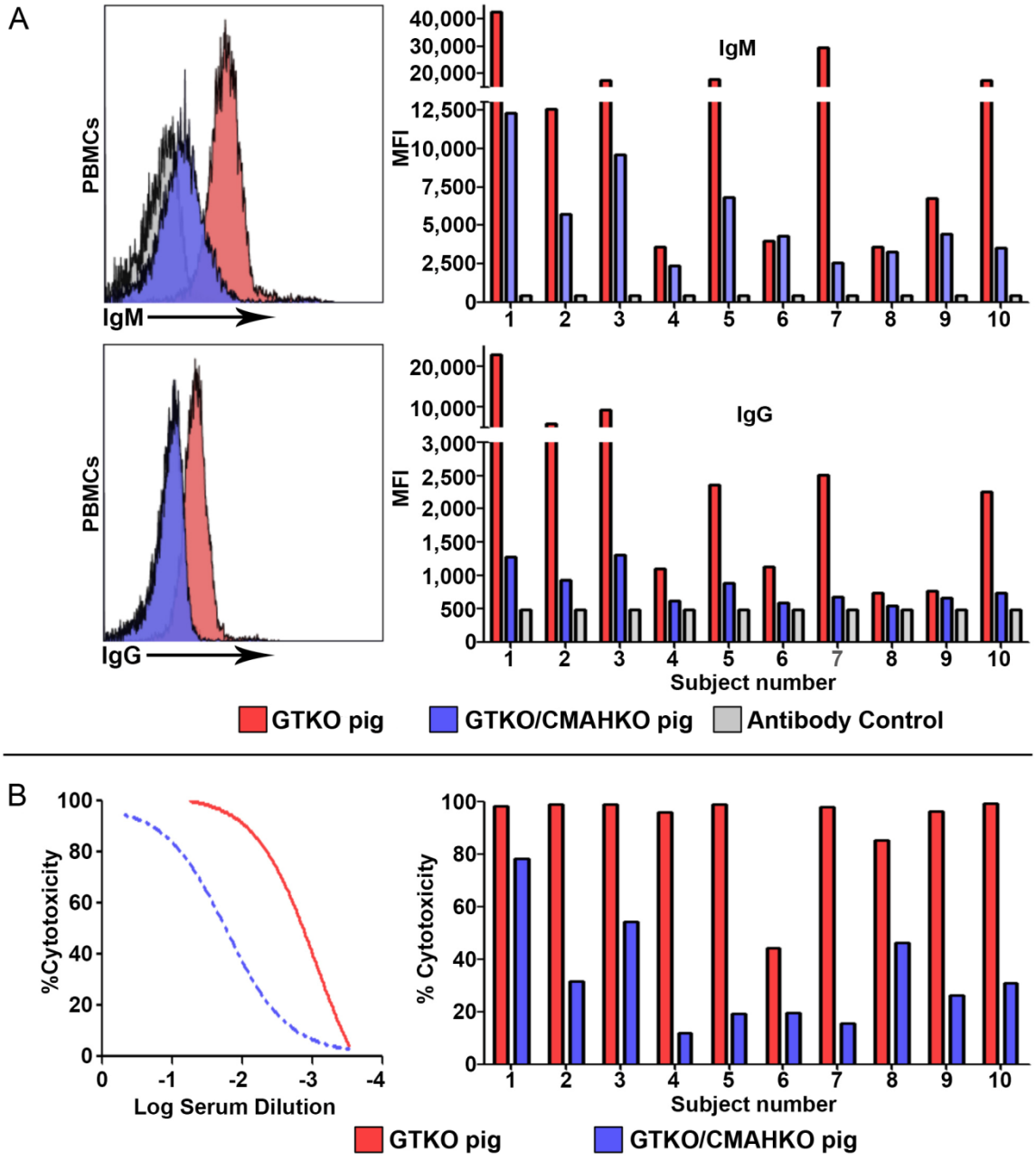

Figure 5-8. Bar graph comparison of human serum IgM recognition, IgG recognition and % cytotoxicy of α1,3 galactosyl-transferase knockout pig (GTKO), GTKO / cytidine monophosphate-N-acetylneuraminic acid hydroxylase knockout pig (GTKO/CMAHKO), baboon, chimpanzee and human peripheral blood mononuclear cells (PBMC) provided by Andrew Lutz MD 2013.

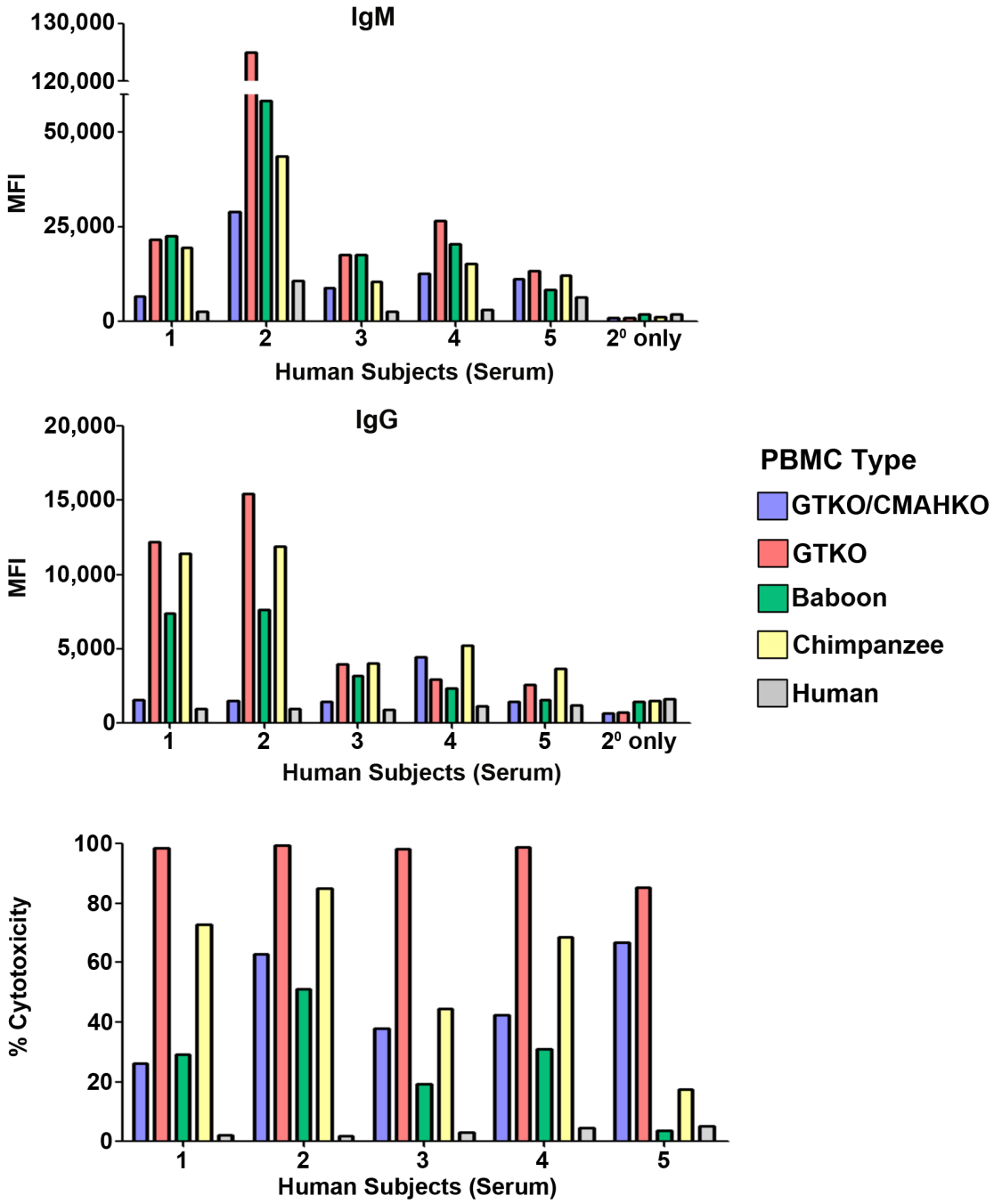

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Figure 5-2

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