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Peer-Reviewed Journal Articles

1. Kadam, Shilpa, **Beth French**, S-T Kim, Christy Morris-Berry, Andrew Zimmerman, Mary Blue, and Harvey Singer. "Altered Postnatal Cell Proliferation in Brains of Mouse Pups Prenatally Exposed to IgG from Mothers of Children with Autistic Disorder." *Journal of Experimental Neuroscience* (2013): 93-99. 13 Nov. 2013.
2. Harris DG, Quinn KJ, **French BM**, Schwartz E, Kang E, Dahi S, Phelps CJ, Ayares DL, Burdorf L, Azimzadeh AM, Pierson RN 3rd. "Meta-analysis of the independent and cumulative effects of multiple genetic modifications on pig lung xenograft performance during ex vivo perfusion with human blood." *Xenotransplantation* (2014): 2 Dec 2014
3. Laird, Christopher, Burdorf, Lars, **French, Beth**, Kubicki, Natalia, Cheng, Xiangfei, Braileanu, Gheorghe, Sun, Wenji, So, Edward, O'Neill, Natalie,

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 5. **Beth M. French**, Selin Sendil, Krishna Mohan Sepuru, Lars Burdorf, Emily Redding, Xiangfei Cheng, Christopher Laird, Krishna Rajarathnam, Richard N Pierson III, MD, Agnes M. Azimzadeh. “Interleukin-8 mediates neutrophil-endothelial interactions in pig-to-human xenogeneic models” *Xenotransplantation* (Under review)
 6. Arielle Cimeno, Wessam Hassanein, **Beth M. French**, Jessica M. Powell, Lars Burdorf, Olga Goloubeva, Xiangfei Cheng, Dawn M. Parsell, Jagdece Ramsoondar, Venkata Kuvvari, Mehmet C. Uluer, Todd Vaught, Emily Redding, Natalie O’Neill, Christopher Laird, Alena Hershfeld, Ivan Tatarov, Kathryn Thomas, David Ayares, Agnes M. Azimzadeh, Richard N. Pierson III, Rolf N. Barth, John C. LaMattina. “Neu5GcKO Reduces Erythrocyte Sequestration and Thromboxane Elaboration in an *ex vivo* Pig-to-Human Xenoperfusion Model” *Xenotransplantation*. (Under review)

Abstracts

1. **BM French**, MS; DG Harris, MD; P Benipal, BA; RN Pierson III, MD; AM Azimzadeh, PhD. “Role of endothelial sialic acid expression on xenogeneic neutrophil adhesion” Experimental Biology Conference, April 2014; Poster.
2. **BM French**, MS; DG Harris, MD; P Benipal, BA; Zhuo Gao, BA; RN Pierson III, MD; AM Azimzadeh, PhD. “Role of Sialic Acid Expression on Xenogeneic Neutrophil Adhesion and Protein C Activation” Gordon Conference/Seminar on Hemostasis, July 2014; Poster.
3. **BM French**, MS; DG Harris, MD; P Benipal, BA; RN Pierson III, MD; AM Azimzadeh, PhD. “Role of endothelial sialic acid expression on xenogeneic neutrophil adhesion” Society for Glycobiology Conference, Nov 2014; Poster.
4. **BM French**; P Benipal; DG Harris; X Cheng; N Ye; RN Pierson III; AM Azimzadeh. “Cellular sialylation and activation regulate xenogeneic neutrophil adhesion” Transplantation Science Symposium, Nov 2015; Poster.
5. **BM French**; P Benipal; DG Harris; X Cheng; N Ye; RN Pierson III; AM Azimzadeh. “Cellular sialylation and activation regulate xenogeneic neutrophil adhesion” International Xenotransplantation Association Conference, Nov 2015; Oral Presentation.
6. **BM French**; L Burdorf; S Dahi; N Kubicki; X Cheng; N Chen; David L. Ayares, PhD; RN Pierson III; AM Azimzadeh. “Role of human IL-8 in GalTKO.hCD46 xenogeneic lung inflammation.” Transplantation Science Symposium, Nov 2015; Poster.

7. **BM French**; L Burdorf; S Dahi; N Kubicki; X Cheng; N Chen; David L. Ayares, PhD; RN Pierson III; AM Azimzadeh. "Role of human IL-8 in GalTKO.hCD46 xenogeneic lung inflammation." International Xenotransplantation Association Conference, Nov 2015; Oral Presentation.
8. **Beth French**, MS; Donald G. Harris, MD; Prabhjot Benipal, BA; Zhuo Gao, MS; Xiangfei Cheng, MD, PhD; Christopher Laird, MD; Lars Burdorf, MD; David L. Ayares, PhD; Richard N. Pierson III, MD; Agnes M. Azimzadeh, PhD. "Activated Protein C Modulates the Thrombotic Phenotype and Vascular Permeability of Human Endothelial Protein C Receptor Transgenic Porcine Endothelium." Transplantation Science Symposium, Nov 2015; Poster.
9. **Beth French**, MS; Donald G. Harris, MD; Prabhjot Benipal, BA; Zhuo Gao, MS; Xiangfei Cheng, MD, PhD; Christopher Laird, MD; Lars Burdorf, MD; David L. Ayares, PhD; Richard N. Pierson III, MD; Agnes M. Azimzadeh, PhD. "Activated Protein C Modulates the Thrombotic Phenotype and Vascular Permeability of Human Endothelial Protein C Receptor Transgenic Porcine Endothelium." International Xenotransplantation Association Conference, Nov 2015; Oral Presentation.

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ABSTRACT

Title of Dissertation: Role of Sialic Acids in Xenotransplantation

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The sialic acid profile on the surface of cells can dynamically change upon cell activation and such change can dramatically affect various cellular functions. One pathway facilitating such change is through the loss of sialic acid by enzymatic cleavage caused by sialidases. In certain cell types, sialidases were found to mobilize from intracellular reservoirs to the cell surface after activation, however, the mechanism of the translocation is still not clear. This surface expressed sialidase activity causes desialylation of the cell surface either *in cis* or *trans* on other cell types in close vicinity. One of the mechanisms by which sialic acid cleavage has been shown to affect cell-cell binding is facilitated by the binding of galectins. When sialic acid is cleaved, underlying galactose molecules are exposed. Galactose is in turn the ligand for the lectin receptors galectins. Galectins are defined by sharing a structural homology in their carbohydrate-

recognition domains (CRDS). Galectins are expressed in different tissues and cells including endothelial cells (EC) , alveolar macrophages and PMN. It has been found that a change in the level of galectins can affect PMN recruitment.

A main limitation of pig lung xenograft injury is the fact that leukocyte (specifically PMN) and platelet sequestration occur in our *ex-vivo* human blood perfusion system within minutes, and the mechanisms driving these adhesive interactions are largely unknown. We believe that sialidase activity is increased during an *ex-vivo* lung xeno-perfusion and this causes an increase in desialylation of lung tissue and PMN. Here we characterize sialidase expression during an *ex-vivo* lung xeno perfusion and evaluate the role of cellular desialylation in PMN and endothelial cell adhesion under xeno settings, most specifically focusing on NEU1 and NEU3. We demonstrate that when sialic acid is cleaved from pig EC/human PMN this increases human PMN adhesion, and that PMN adhesion is further increased when PMN or EC are pre-activated. We show that the galectin binding is a mechanism behind this discovery/phenomenon. These findings provide the first mechanistic explanation of how galectin binding, consequent of sialic acid cleavage, affects human PMN adhesion during xenotransplantation.

Role of Sialic Acids in Xenotransplantation

by
Beth French

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
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Doctor of Philosophy
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List of Abbreviations

1,3 α Gal: 1,3 α -galactose

ASGR-1: Asialoglycoprotein receptor-1

B4GALNT2: β 1,4 N-acetyl1,3 α Galactosaminyltransferase

CD46/hCD46: Membrane cofactor protein

CD55/hCD55: Decay accelerating factor

CPRP: Complement pathway regulatory proteins

CRD: Carbohydrate-recognition domain

DANA: 2-deoxy-N-acetylneuraminic acid

DCs: Dendritic cells

EC: Endothelial Cells

ENA-78: Epithelial cell-derived PMN-activating peptide

EPCR/hEPCR: Endothelial cell protein C receptor

Gal: Galactose

Gal-1: Galectin-1

Gal-3: Galectin-3

Gal-8: Galectin 8

GalNAc: N-acetylgalactosamine

GalTKO: knocking out of α -1,3 galactosyltransferase

G-CSF: Granulocyte colony-stimulating factor

GlcNAc: N-acetylglucosamine

GRO-alpha: Growth-regulating oncogene

hAECs: Human aortic endothelial cells

HAR: Hyperacute rejection

HBSS: Hank's Balanced Salt Solution

ICAM: Intercellular adhesion molecule

ITIMs: Immunoreceptor tyrosine-based inhibitory motifs

JAM: Junctional adhesion molecule

Kdn: Ketodeoxynonulosonic acid
LacNAc: N-acetyllactosamine
LSEC: Liver sinusoidal endothelial cells
MADCAM-1: Mucosal vascular cell-adhesion molecule-1
NA: *Clostridium perfringens* neuraminidase
NETs: Neutrophil extracellular traps
NEU: Neuraminidase
Neu5Ac: N-acetylneuraminic acid
Neu5Gc: N-glycolylneuraminic acid
NHP: Non-human primate
NK: Natural killer cell
pAECs: Primary pig aortic endothelial cells
PECAM-1: Platelet endothelial-cell adhesion molecule-1
PMN: neutrophils
pMVECs: Primary microvascular pig lung endothelial cells
ROS: Reactive oxygen species
Sia: Sialic acids
STs: Sialyl-transferases
Siglecs: Sialic acid-binding immunoglobulin type lectins
TBM/hTBM: Thrombomodulin
TRPI/hTFPI: Tissue factor pathway inhibitor
VCAM-1: Vascular cell-adhesion molecule-1
WT: Wild type

Chapter 1: Introduction and Scope of Dissertation

Sialic Acid

Sialic Acid Structure

Sialic acids (Sia) are negatively charged monosaccharides typically expressed on the terminating branches of N-glycans, O-glycans, and glycosphingolipids (gangliosides) on the surface of virtually all cells in vertebrates. The Sia family contains more than 50 members, but two common "primary" Sia have been described: 5-acetamido-2-keto-3,5-dideoxy-D-glycero-D-galactononic acid (N-acetylneuraminic acid, or Neu5Ac) and 2-keto-3-deoxy-D-glycero-D-galactononic acid (ketodeoxynonosinic acid, or Kdn). All Sia contain a nine carbon backbone. With the exception of some bacterial Sia¹ all other types of Sia are metabolically derived from these two precursors. Neu5Ac is more common than Kdn in most vertebrate cell types and is expressed by all mammals. In all mammals except humans, Neu5Ac is converted into N-glycolylneuraminic acid (Neu5Gc) by conversion of CMP-Neu5Ac to CMP-Neu5Gc in the cytoplasm by the CMP-Neu5Ac hydroxylase, which is encoded by the CMAH gene. During evolution, the human CMAH gene became non-functional². Consequently, humans predominantly express Neu5Ac (and little to no Neu5Gc), whereas most other mammals predominantly express Neu5Gc. Since Neu5Gc is expressed by gut bacteria and not by 'self', it is not surprising that in humans (but not in non-human primates or other mammals) a substantial fraction of anti-carbohydrate antibodies that bind to cells from candidate xenograft organ donors are directed against the Neu5Gc antigen.

There are additional layers of diversity in the structure of Sia, related to their linkage to the underlying glycan chain. Sias are usually found at the non-reducing terminal position of glycoconjugate sugar chains, linked to galactose (Gal), N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc) residues. Sia bind to underlying residues via α -linkages between their second carbon (C-2) and either the third carbon (C-3) or sixth carbon (C-6) positions of Gal, GalNAc or GlcNAc. When Sia are linked (via C-2) to other Sia residues at the eighth carbon (C-8), they are classified as oligo- or poly-sialic acids.³ Additional chemical modifications of Sias such as hydroxylation and acetylation have been described which account for the enormous variety of glycan structures on cell surfaces, and contribute to the distinctive phenotype and physiologic diversity of different cell types.

Sialylation: a dynamic balance

The Sia family is made up of many structurally diverse sugars that are positioned to influence cell-cell and intermolecular interactions. The sialic acids exposed on the surface structures on endothelia and epithelia contribute to the cell surface glycocalyx, along with other membrane-bound polyanionic macromolecules.⁴ The 'net' cellular sialylation/desialylation state of a cell at any time depends on the combination of chemical synthesis of Sia-bearing proteins and lipids, the rate of transfer of Sia-expressing molecules to the cell surface, and the rate of loss of Sia residues. Loss of Sia from the cell surface may occur either by catabolism of its carrier protein or lipid, by active removal (which is believed to be primarily enzymatic) or by shedding of the carrier upon activation (such as with T cells, B cells, and neutrophils (PMN))⁵. In eukaryotic cells, free Sia (derived from biosynthesis or recycled/recovered from the lysosome) is activated into the nucleotide donor CMP-Sia, by CMP-Sia synthetases in the nucleus. The CMP-Sia products then return

to the cytoplasm, and are delivered into the lumen of Golgi compartments, where Sias are transferred onto newly synthesized glycoconjugates passing through Golgi compartments by the action of sialyltransferases (STs). Finally, during molecular turn-over and recycling of cell-surface molecules, glycoconjugates are desialylated in endosomal/lysosomal compartments and either return to the Golgi to undergo re-sialylation or are further metabolized into new glycoconjugates.

Some mammalian cells express sialidases on their plasma membrane (outer cell surface) conditionally after cell activation. Cell-surface sialidases (also termed neuraminidases when referring to Sia of bacterial or viral origin) are preformed and are mobilized from intercellular reservoirs such as endosomes during activation of certain cell types (e.g., PMN)⁶. The mechanism behind this is still not completely understood. Cell-surface sialidases have been implicated in the rapid cleaving of cell-surface Sias. Altogether, the net amount of sialylation on a cell's surface depends on the opposing activity of sialyltransferases, which promote sialylation, and sialidases, which strip Sia residues from the cell surface. This dynamic equilibrium of Sia was a driving factor behind the initial formulation of my hypothesis for my thesis research.

Sialyltransferases

Human sialyltransferases (STs) are a family of at least 20 different intracellular, Golgi membrane-bound glycosyltransferases.⁷ They transfer Sia onto newly synthesized glycoconjugates during their passage through the Golgi.³ STs are designated based on the acceptor structure on which they act, as well as on the sugar linkage they form. Specifically, STs are classified as ST3, ST6, or ST8 according to their formation of α -glycosidic bonds between the C2 of the Sia and the 3'-, 6'-, or 8'- hydroxyl group of the acceptor, respectively.

For example, a group of ST3s adds Sia with an α -2,3 linkage to galactose, resulting in Sia α 2,3 Gal, while ST6s add Sia with an α -2,6 linkage to galactose or N-acetylgalactosamine, resulting in Sia α 2,6 Gal or Sia α 2,6 GalNAc. Each of the three ST families has up to six subfamilies that differ based on their substrate specificity. For example, ST6Gal-II is an oligosaccharide-specific enzyme toward oligosaccharides that have a Gal β 1,4-GlcNAc sequence, whereas ST6Gal-I demonstrates broad substrate specificities. ST activity and expression varies in response to inflammation, which in turn is influenced by both physiological and pathological processes^{8,9,10}. It was indicated that in mouse livers during inflammation there was up-regulated expression of β -galactoside α 2,3-sialyltransferases (ST3Gal-I and ST3Gal-III) and β N-acetylgalactosaminide α 2,6 sialyltransferase (ST6GalNAc-VI) as well as β galactoside α 2,6-sialyltransferase (ST6Gal-I) mRNAs. It has also been found that the endothelial surface expresses ST activity.¹¹ Treatment of human EC with TNF α , IL-1, or LPS causes increased expression of the enzyme β -galactoside α -2,6-sialyltransferase. This coincided with increased expression of endothelial glycoproteins bearing N-linked glycans with α 2,6-linked Sia such as adhesion molecules E-selectin, ICAM-1, and VCAM-1. Thus, activation of EC during inflammatory and immunological processes may induce ST activity, which can participate in sialylation of other activation-dependent molecules. Most of the human and mouse sialyltransferase family members' sequences have been determined. In contrast, most pig sialyltransferase genes have not been fully characterized, but this is being looked into by other labs.¹²

Sialidases

By convention, eukaryotic neuraminidases are called sialidases, reserving the term neuraminidase for other organisms such as bacteria, viruses and fungi. Sialidases remove

the terminal Sia residues at the non-reducing end of glycoconjugates. There are four mammalian sialidase genes, which are classified as neuraminidase (NEU) 1-4. Neuraminidases are also expressed by human pathogens such as the influenza virus, where they contribute to viral cell entry mechanisms and are a target of human antiviral immunity in the context of natural infection and vaccine/treatment development.^{13,14,15}

Sialidases are differentially expressed in various cells and tissues/organs, and in subcellular spaces.³ NEU1 is localized primarily in lysosomes, where it is associated with Cathepsin A and β -galactosidase which act as chaperones for lysosomal compartmentalization and catalytic activation (optimum pH 4.4–4.6). NEU2 is found in the cytosol (pH 6.0–6.5). Although it has also been identified in the endosomal compartment, NEU3 is mainly associated with and possesses enzymatic activity on the plasma membrane (pH 4.5-4.7 and 6.0-6.5, depending on the changes occurring in the plasma membrane)^{16,17}. NEU4 is found in lysosomes, mitochondria or endoplasmic reticulum (pH 4.5-4.7).¹⁶ All summarized in Table 1.1.

Some believe that sialidases display different activities for various Sia linkages. NEU 1,2 and 4 preferentially catalyzes α 2,3 linkages, whereas NEU 3 also efficiently targets α 2,6 linkages.¹⁸ Each sialidase also target different substrates: NEU1, 2 and 3 target oligosaccharides; NEU2 and 4 glycoproteins; NEU2, 3 and 4 gangliosides; and NEU1 glycopeptides. In human tissues, NEU1 generally shows the strongest expression by PCR and Western blot, 10–20 times greater than those of NEU3 and NEU4, but is usually found on the cell surface only after cell activation. NEU1 is the main sialidase expressed in human lung microvascular endothelia.¹⁶ The expression of NEU2 is extremely low in human

cells.¹⁹ NEU3 is the sialidase found conditionally on the plasma membrane of most mammalian cells, and is thought to be a contributor to the net sialylation state of the plasma membrane in eukaryotes, along with NEU1. Sialidase activity has been found in many organs and tissues including liver, kidney and lung as well as in erythrocytes, PMN and platelets.²⁰

Sialidase expression is dynamically regulated in immunocytes. For example, in human T-cells, both NEU1 and NEU3 mRNAs are induced by T-cell receptor stimulation. Several cytokines, including interleukin IL-2 and IL-13, are induced upon the up-regulation of these sialidases, although a direct effect of sialidases on cytokine gene expression has not been established.²¹ While its constitutive location is mainly intracellular, NEU1 has been found to translocate from lysosomes to the plasma membrane during immune activation or differentiation in several cell types, such as macrophages²², EC²³, and erythrocytes²⁴. The expression of NEUs/changes in Sia profile has never before been characterized in pig endothelia or tissues or in human or non-human primate (NHP) blood cells in models of pig-to-human xenotransplantation. This will be a focus of Aim 1 of my thesis.

Xenotransplantation

Xenotransplantation is the process of transplanting organs or tissues from one species into another. It is viewed as an attractive potential solution to the human organ shortage, which currently limits the availability and thus the therapeutic potential of organ, cell, and tissue transplantation. Based on their size and breeding characteristics, pigs have been proposed as the most promising donor species for application in man. However,

Table 1.1 Sialidases and their location and optimum pH

	Primary Location	Optimum pH
NEU1	Lysosome	4.4-4.6
NEU2	Cytosol	6.0-6.5
NEU3	Plasma Membrane	4.5-4.7 or 6.0-6.5
NEU4	Mitochondria	4.5-4.7

humans generally have high levels of preformed "natural" antibodies that recognize the porcine galactose 1,3 α -galactose (1,3 α Gal) antigen as well other carbohydrate antigens expressed by pigs that are not found in humans, including Neu5Gc. Physiologically incongruous coagulation is also another problem seen in transplantation of pig organs. This phenomenon is partially explained by inefficient regulatory inhibition of procoagulant positive feedback loops, which normally downregulate or constrain formation of thrombus through action of endothelial thromboregulatory molecules. This procoagulant phenotype is largely due to molecularly defined incompatibilities between human coagulation pathway molecules and pig thromboregulatory pathways. In addition, binding of anti-Gal α 1,3Gal and other anti-pig antibodies to pig cell surfaces initiates complement activation and endothelial activation, and thus contributes to particularly intense activation of the coagulation cascade. Complement and coagulation pathways each contribute significantly to the "hyperacute rejection" (HAR) phenotype observed when wild type porcine organs are perfused with human or NHP blood.^{25,26,,27,28,29}

Genetic Modification

To overcome this pre-existing, preformed "innate immune" barrier, several modifications have been made to the porcine genome. These include knockout of α -1,3 galactosyltransferase enzyme (GalTKO), removing the 1,3 α Gal epitope from the surface of pig cells. The GalTKO genetic modification was a key step in preventing hyperacute rejection. Other modifications are knock-in of genes encoding human complement regulatory proteins such as CD46 (hCD46, membrane cofactor protein) and CD55 (hCD55, decay accelerating factor); expression of these complement pathway regulatory proteins (CPRP) actively inhibit complement injury to cells on which anti-pig antibody is bound.

Similarly, expression of human endothelial cell protein C receptor (hEPCR), human tissue factor pathway inhibitor (hTFPI), and human thrombomodulin (hTBM), have been genetically introduced into pigs, intending to inhibit non-physiologic activation of clotting mechanisms that are triggered when pig endothelium is exposed to human blood. Each of these modifications improves organ survival in various models. However, alone or even in various combinations, they do not consistently prevent long-term immunologic injury.^{30,31,32}

How does Sia affect Xenotransplantation?

The known mechanisms which contribute to the residual injury of organs during xenotransplantation include binding of "non-Gal" anti-pig ("xeno-reactive") antibodies to other xenoantigens such as Neu5Gc. The human immune system detects Neu5Gc residues expressed on pig cells as a "non-self" carbohydrate antigen (as seen in **Figure 1.1**). Teleologically, perhaps these antibodies contribute as a defense against translocation of gut bacteria expressing this carbohydrate, or against tumors expressing Neu5Gc.^{33,34} Anti-Neu5Gc antibodies contribute to organ injury during xenotransplantation, as demonstrated by Padler-Karavani and Varki where Neu5Gc deficient mice were induced to make anti-Neu5Gc antibodies, which initiated the rejection of transplanted allogenic islets expressing Neu5Gc.^{35,36} This antigenic target became even more relevant recently as studies have shown that knocking out of α -1,3 galactosyltransferase (GalTKO) is associated with increased Neu5Gc levels.³⁷ Lutz et al. recently reported that a significant portion of human IgM and nearly all IgG binding to GalTKO pigs is directed against Neu5Gc and that anti-Neu5Gc antibodies are responsible for significant injury to GalTKO cells and organs.³⁸ Other carbohydrate antigens have been identified as sources of non-Gal antigen including

β 1,4-N-acetylgalactosaminyltransferase (B4GALNT2) which contributes in part to the non-Gal immune responses detected during xenotransplantation along with Neu5Gc.^{39,40} (30,31) Experimental evidence so far strongly supports the recognition of the Neu5Gc xenoantigen as an important trigger in antibody-dependent injury to the pig organ.⁴¹

Limitations of pig lung xenotransplantation

Although all organs are of limited supply for organ transplantation, the lung donor pool is especially small due to the fact that lungs have the lowest recovery and transplantation rates per donor.⁴² Lung xenotransplantation could increase the quantity and potentially improve the quality of lung grafts available for transplantation. There has been significant progress to overcome the barriers of xenotransplantation with survival reaching months or years in some heart and kidney transplant models.^{43,44} However, pig lung injury during *ex-vivo* human blood perfusion and after transplantation into nonhuman primates remains a major problem, one that has to date limited survival to just a few days. The causes of this discrepancy between results with the lung compared to other solid organs remain incompletely understood, and this thesis explores how sialic acid and related pathways may contribute to this phenomenon.

In clinical experience and in many models of injury, inflammation, and infection, the lung is particularly sensitive to damage, which is manifested as alveolar injury, endothelial dysfunction with pulmonary vascular resistance rise, loss of vascular barrier function, bronchoconstriction, and inflammation. Despite genetic modifications that are sufficient to protect pig hearts and kidneys, and the use of specific drug regimens to address residual known pro-inflammatory mechanisms, the pig lung xenograft is still not protected from the immune system. We believe that pig lung xenografts can be injured

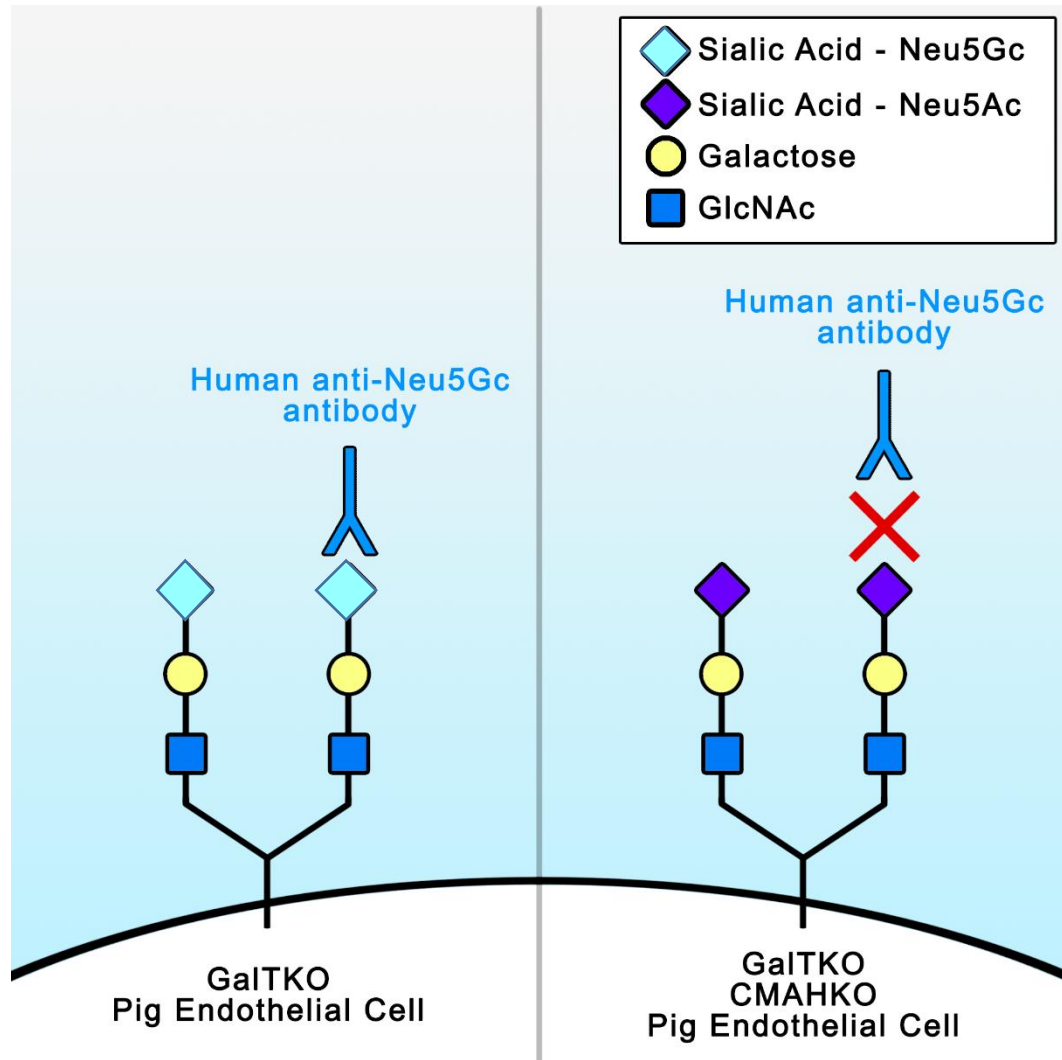


Figure 1.1: Neu5Gc antigen recognition. Neu5Gc is present on wild-type and GalTKO pig cells. Similar to the Gal α 1,3 Gal antigen, Neu5Gc is a carbohydrate that is recognized by preformed “natural” anti-Neu5Gc antibodies, one that contributes to antibody-mediated rejection of pig organ xenografts. Knockout of the gene encoding the enzyme responsible for converting Neu5Ac to Neu5Gc, cytidine monophospho-N-acetylneuraminic acid hydroxylase (CMAH), addresses this problem: GalTKO/CMAHKO pigs do not express Neu5Gc or the Gal α 1,3 Gal antigen. Consequently these pigs only express Neu5Ac, as do humans; therefore the human anti-Neu5Gc antibodies have no target to bind to on the pig cells.

due to antibody-mediated effects, pig pulmonary intravascular macrophage activation, thromboregulatory dysfunction, porcine von Willebrand factor mediated platelet adhesion, histamine release and cytokine synthesis. The main feature of pig lung xenograft injury that I focus on in my thesis project is the fact that leukocyte (specifically PMN) and platelet sequestration occur in our *ex-vivo* human blood perfusion system within minutes, and the mechanisms driving these adhesive interactions are largely unknown. Importantly although the sequestration is delayed in GalTKO.hCD46 lungs, PMN infiltration and platelet sequestration always still occur.^{30,31} They are not prevented by antihistamines, pulmonary vascular macrophage depletion using liposomal clodronate, thromboxane synthase inhibition, pan-selectin blockade (with PSGL-1), Mac-1 blockade, alone or in combination. Therefore, it is important for this limitation to be understood, to understand the contribution PMNs to lung injury.

Neutrophils

Ontogeny

Neutrophils are the most abundant type of white blood cell/granulocyte in most mammals and during the acute phase of inflammation they are one of the first responders of inflammatory cells to the site of inflammation.⁴⁵ Chemokines coordinate the balance between PMN release from or retention in physiologic reservoirs (including the circulating blood pool) and PMN homing to sites of inflammation. Bone marrow stromal cells produce CXCL12 that binds to CXCR4 and leads to PMN retention in the bone marrow. Granulocyte colony-stimulating factor (G-CSF) prompts PMN release from the bone marrow by disrupting CXCL12 and CXCR4 signaling. G-CSF expression produced by macrophages, EC, fibroblasts and related mesenchymal cells as well as tissues such as the

bone marrow stroma and is controlled by a unique feedback loop that senses PMN migration into tissues. Specifically, engulfment of apoptotic PMN initiates a cytokine cascade that includes IL-23, IL-17, and G-CSF.⁴⁶ PMN migration is also mediated by CXCR1 or 2, also known as Interleukin (IL)-8R alpha or beta.⁴⁷ Released PMN from the bone marrow distribute into the periphery in the venous blood which circulate as a non-activated phenotype.⁴⁸ Apoptotic PMN are cleared by macrophages, necessary for homeostasis. The Bcl-2 homologue Mcl-1 is a survival protein that prevents intrinsic apoptosis and when it is gone intrinsic apoptosis occurs. TNF- α drives early PMN apoptosis by the extrinsic pathway. It also promotes late survival (past 24 hour life span) by initiating the NF- κ B pathway. The phenotype of an apoptotic PMN changes to produce 'eat me' signals (annexin 1, phosphatidylserine (PS) residues) to phagocytes which enhances the clearance of the cells. When macrophages interact with the PS residues it induces phagocytosis and increases the production of IL-10 and TGF- β which help to resolve the inflammatory response. Apoptotic PMN also have high LPS-binding capacity and sequester LPS, which reduces the LPS stimulation of viable and responding cells.⁴⁹

Conventional PMN have been believed to have a short life-span, measured in hours, in the circulation or other body sites. However, this notion has been challenged based on recent *in-vivo* labelling studies which found that human PMN have a mean life-span of 5.4 days.⁵⁰ However, there is still no broad consensus yet regarding this time span and how it may differ in response to various infectious or other inflammatory challenges.⁵¹

PMN activation and migration

PMN are the first cells recruited to the site of infection or inflammation. Efficient PMN recruitment to the inflamed tissue first depends upon PMN pre-activation by priming

through exposure to proinflammatory cytokines, such as $\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-8 , leukotriene (LT)B₄ or GM-CSF.⁵² PMN can undergo cycles of priming and de-priming.⁵³ The lung is one location where primed PMN tend to reside during physiologic quiescence.⁵⁴ Once primed PMN are released into the circulation and migrate to the extravascular sites of inflammation, based on chemokine- and adhesion molecule-mediated interactions with the vascular endothelium. L-selectin is expressed on the surface of quiescent PMN and is upregulated after PMN activation. L-selectin tethers to ligands on the surface of EC such as P-selectin glycoprotein ligand (PSGL-1), specifically a SIA sialyl lewis X. Selectin interactions cause PMN (and other leukocytes) to roll along the endothelium. Integrins on the PMN (such as $\alpha 4\beta 1$ -integrin, lymphocyte function-associated antigen-1 (LFA-1; $\alpha L\beta 2$ -integrin, CD11a/CD18) and macrophage antigen-1 (MAC-1; $\alpha M\beta 2$ -integrin, CD11b/CD18)) go through conformational changes from low affinity to high affinity via “inside-out signaling” which occurs when the PMN encounters certain stimuli. Various stimuli, including the bacterial peptide fMLP, complement factor C5a, epithelial cell-derived PMN-activating peptide (ENA-78), growth-regulating oncogene (GRO- α), or IL-8 , activate LFA-1 and MAC-1.^{55,56} These activated integrins then bind with increased avidity to adhesion molecules on the surface of EC such as intercellular adhesion molecule (ICAM)-1 and -2, vascular cell-adhesion molecule-1 (VCAM-1) and mucosal vascular cell-adhesion molecule-1 (MADCAM-1). High avidity binding causes stronger adherence of the PMN and promotes rolling arrest. PMN then migrate into the tissue through junctions between neighboring EC using ligands such as ICAM-2, platelet endothelial-cell adhesion molecule-1 (PECAM-1) and proteins of the junctional adhesion molecule (JAM) family (paracellular) or straight through EC (transcellular). Most found that under *in-vitro*

conditions PMN migration occurs under paracellular conditions. However, researchers have found that transcellular vs paracellular migration under flow conditions depends on the cell types. It was also found that ICAM-1 surface density and distribution and the shape of the endothelial cell shape contributed to what type of migration is used.⁵⁷

Once PMN have passed through the endothelium they travel towards the inflamed tissue along a chemotactic gradient. At a site of bacterial infection, for example, membrane receptors for complement proteins and immunoglobulins on the PMN recognize and bind to the bacteria, leading to phagocytosis. Primed PMN can also synthesize and secrete cytokines, chemokines, leukotrienes and prostaglandins.⁵⁸ Using immunogold staining researchers have found that PMN store some pre-formed cytokines in their secretory granules. Some examples of pre-formed cytokines are TNF- α , TGF- α , IL-6, IL-12 and CXCL2.⁵⁹ PMN have been shown to secrete IL-8 in response to TNF- α and GM-CSF which begins a loop of more PMN being primed and activated and therefore secreting more IL-8.⁶⁰ Activated PMN have also been reported to synthesize IL-1, -6, -12, IL-1RA, TGF- β and TNF- α .⁶¹

How do PMN fight infection?

The main mechanisms that PMN use to fight infections include: phagocytosis with phagosome-mediated lysis of ingested material; release of cytotoxic components into the extracellular space or phagosome (proteases, antimicrobial peptides, oxidants); and formation of PMN extracellular traps (NETs).

For phagocytosis, once PMN bind via one of their surface receptors, such as Fc γ receptors, C type lectins, or complement receptors, the PMN will engulf their targets. They

can internalize and kill many microbes, each phagocytic event resulting in the formation of a phagosome. The next step is the fusion of lysosomes with the phagosome. The result is called a phagolysosome into which reactive oxygen species and hydrolytic enzymes are secreted.

To mediate extracellular killing of invading pathogens, PMN have three types of granules, azurophilic (primary), specific (secondary) and tertiary. Each of these granules have an assortment of proteins that can be released during degranulation. One family of proteins within their granules is serine proteases (PMN elastase and cathepsin G) which directly kill microbes and inactivate bacterial toxins.⁶² When these proteases are released excessively and have uncontrolled activity this can cause increased inflammation and host tissue damage.⁶³ Once granules fuse with the phagosome NADPH oxidase is assembled and reactive oxygen species (ROS) and hydrolytic enzymes are expressed to rapidly kill the pathogens.⁶⁴ The ROS and enzymes can also damage host tissue following their release if PMN are inappropriately activated such as in autoimmune diseases.

Other than phagocytosis and extracellular killing, PMN can also use NETs to capture microbes. NETs utilize autophagy to digest invading pathogens.⁶⁵ The concept of NET induction is a novel discovery. PMN release extracellular DNA traps that are made up of decondensed chromatin, histones and granule proteins. NETosis involves the translocation of elastase from primary granules to the nucleus where it cleaves histones and leads to chromatin decondensation as a form of cell death for the PMN.⁶⁶ Some studies have even found that viable PMN can form NETs. Their NETs are made of mitochondrial or nuclear DNA.⁶⁷ It has also been found that NETs are activators of coagulation. It was

recently discovered that in an in vivo model of infection, NETs-platelets and thrombin together promoted intravascular coagulation in sepsis.⁶⁸

There are several factors that determine which of the PMN functions are activated. The presence of serum drives towards phagocytosis but inhibits the formation of NETs, and transmigration through the endothelium prompts the release of contents from vesicles and granules.⁶⁹ Some pathogens preferentially trigger one pathway; in many circumstances all three are involved to varying degrees.

PMN interaction with other cells

PMN can interact with a variety of immune and non-immune cells including dendritic cells (DCs), macrophages, natural killer (NK) cell, platelets and T cells.^{70,71} PMN and DCs can co-localize at sites of infection.⁷² Th17 cells produce IL-17 and the PMN chemoattractant CXCL8 which recruit PMN and PMN recruit Th17 cells via their release of CCL20 and CCL2.⁷³ Platelets bind to activated PMN in tissue, and in the circulation, to form aggregates.⁷⁴ Activated platelets bind to PMN to form aggregates in a P-selectin dependent manner. Like EC, platelets bind to activated Mac-1 integrin on the surface of adherent PMN.⁷⁵ Activated platelets can also activate PMN to produce NETs by binding to adherent PMN, particularly in liver sinusoids and pulmonary capillaries, a phenomenon associated in particular with transfusion-related acute lung injury.^{76,77}

When PMN activity goes wrong

Several chronic inflammatory disease conditions have a continued inflow of PMN into the site of chronic inflammation, including cystic fibrosis, chronic obstructive pulmonary disease, rheumatoid arthritis, and cardiovascular atherosclerosis. Chemokines,

complement fragments, oxidation pathway byproducts, and necrotic (as opposed to apoptotic) tissue breakdown generate inflammatory signals that promote the movement of PMN into diseased tissues. Infiltrating PMN then release proteases and ROS which cause parenchymal tissue injury (breakdown of collagen, elastin, basement membrane, and other interstitial matrix components) and the production of chemokines which causes additional PMN recruitment in a pathogenic, pro-inflammatory positive feedback loop. PMN can also cleave extracellular matrix into small fragments that can stimulate the immune system and initiate a loop of tissue destruction and other immune cell infiltration.

In ischemia-perfusion injuries there is excessive PMN accumulation which causes tissue injury. $\text{TNF-}\alpha$ is increased after reperfusion inducing the expression of adhesion molecules on EC which that PMN adhesion and stimulates the production of PMN-attracting chemokines. Also in remote organs can be activated by the inflammatory mediators which can result in PMN migrating to those organs as well and causing tissue damage which results in multiple organ dysfunction. In some studies there is less tissue injury when PMN accumulation is prevented^{78,79}. In xenotransplantation studies, hearts and kidneys from GalTKO pigs were transplanted into baboons and histopathology was performed that demonstrated PMN infiltrates in the organ. This partly shows the activation of the innate immune system which causes early or late xenograft rejection.⁸⁰

Mechanisms of Sia-mediated modulation of cell-cell interactions

Sia reduces cell-to-cell interaction through negative charge-mediated repulsion or "veiling" of adhesion molecules. During xenotransplantation, the Sia profile may become altered by an increase in sialidase activity, which may result in a pathological increase in cell-to-cell interactions. Increasing the number, duration, or intensity of cell-cell

interactions, particularly in the context of other pro-inflammatory events, may directly or indirectly contribute to increased human platelet aggregation, PMN/platelet aggregation, and/or PMN adhesion to porcine endothelium.

Selectins and Siglecs

Sias in the sequence of Sia α 2-3Gal β 1-3/4(Fuca1-3/4)GlcNAc β 1-R (Sialyl Lewis X/A) are found expressed on leukocytes, platelets and endothelium. Sialyl Lewis X/A is a component of the ligands for L, P and E-selectins. Therefore, Sia is necessary for lymphocyte homing, platelet binding and PMN migration.⁸¹ Sia is also a ligand for Sia-binding immunoglobulin type lectins (Siglecs). Siglecs are found primarily on the surface of immune cells such as macrophages, B cells, PMN and NK cells. Most Siglecs contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytosolic region which act to down regulate signaling pathways and inhibit immune cell activation. Another category of Siglecs mediate cellular adhesion through the recognition of specific sugar moieties on certain cell types. A member of the latter category of Siglecs, sialoadhesin (Siglec-1, CD169) was found to play a role in xenotransplantation and is found on the surface of macrophages in the lungs, spleen and liver. During an *ex-vivo* perfusion of pig livers with human blood porcine Kupffer cells (macrophages) bind and phagocytosed human red blood cells. Waldman *et al.* found that porcine sialoadhesin expressed on Kupffer cells is responsible for binding Neu5Ac on the surface of human RBCs and the addition of antibody specific for porcine sialoadhesin to a porcine liver xenoperfusion reduced the loss of human RBCs.^{82,83}

Platelets

Platelets contain many pro-inflammatory molecules, and their release can initiate and/or amplify an inflammatory response. If adhesion of either platelets or PMN to endothelium occurs, their aggregates produce an array of molecules known to cause transplant-related tissue damage⁸⁴ including PMN elastase, IL-1 β , thromboxane and other eicosanoids, and ROS. Mandic *et al* believe that lower Sia content on platelets could be directly associated with increased platelet aggregates due to the lower negative surface charge resulting in less repulsion.⁸⁵ Platelets steadily lose surface Sia as they age, at least partially through cleavage by sialidases.⁸⁶ Resting platelets contain an internal pool of sialidase activity which can cause hydrolysis of terminal Sia from platelet glycoproteins on the cell surface; sialidase activity is dramatically accelerated by platelet activation, and by refrigeration⁸⁷.

Platelets express TLR4 which is essential for lipopolysaccharide (LPS)-induced platelet accumulation in the lungs. Feng et al. found that the two components of the TLR4 complex, TLR4 and MD2, express sialyl residues. Cleavage of these sialyl residues by NEU1 heightens LPS-induced TLR4 complex-initiated signaling⁸⁸ which could increase platelet accumulation. We infer that the presence of Sia probably inhibits platelet aggregation during xenotransplantation, while desialylation likely promotes platelet adhesion and activation, and thus thrombus propagation. These interconnected hypotheses are under active investigation by our lab.

Sia removal from the cell surface results in exposure of underlying glycans that can then be recognized by lectin receptors such as galactose-binding proteins of macrophages (e.g. asialoglycoprotein receptor-1 (ASGR-1)). ASGR-1, a receptor expressed by Kupffer cells, hepatocytes and liver sinusoidal EC (LSEC) facilitates phagocytosis of platelets.

ASGR-1 recognizes desialylated platelets and binds them to induce phagocytosis (as seen in **Figure 1.2**). This is thought to be the primary mechanism by which refrigerated platelets are rapidly scavenged by the liver reticuloendothelial system. Paris et al. found that *in vitro* treatment of primary porcine LSEC with siRNA against ASGR-1 transcripts or blocking ASGR-1 with antibodies decreased the ability of porcine LSEC to both bind to and to phagocytose human platelets.⁸⁹ It has also been found that pig ASGR1-mediated binding and phagocytosis by Kupffer cells/LSEC of human platelets can be inhibited by asialofetuin or an anti-ASGR-1 antibody. In addition, they also discovered that there are differences between human and porcine ASGR-1: only 3 amino acid differences between porcine and human ASGR1 but they are found in the binding site and could impact ligand binding affinity. It was also found that human platelets have more exposed galactose β 1-4 N-acetylglucosamine (Gal β) and N-acetylglucosamine β 1-4 N-acetylglucosamine (β GlcNAc) residues, which also play a part in human platelet uptake by porcine livers.^{90,91} Recently it has been shown that ASGR1 knockout pig livers exhibit decreased human platelet uptake.⁹² In summary, these data clearly demonstrate a direct connection between ASGR1 and the human (or baboon) platelet uptake seen during liver xenotransplantation.

PMN

PMN express sialidase activity that influences their adhesion to endothelium. Activation of PMN prompts the translocation of intracellular sialidases to the PMN surface. Such mobilization of sialidases to the surface from the lysosome in activated PMN caused desialylation of the adjacent endothelial surface *in vitro* and increased adhesion of other unactivated PMN to the EC.¹¹⁵ Specifically, the activation of PMN with IL-8 induces translocation of sialidases to the PMN surface.⁹³ In addition, treatment of human EC with

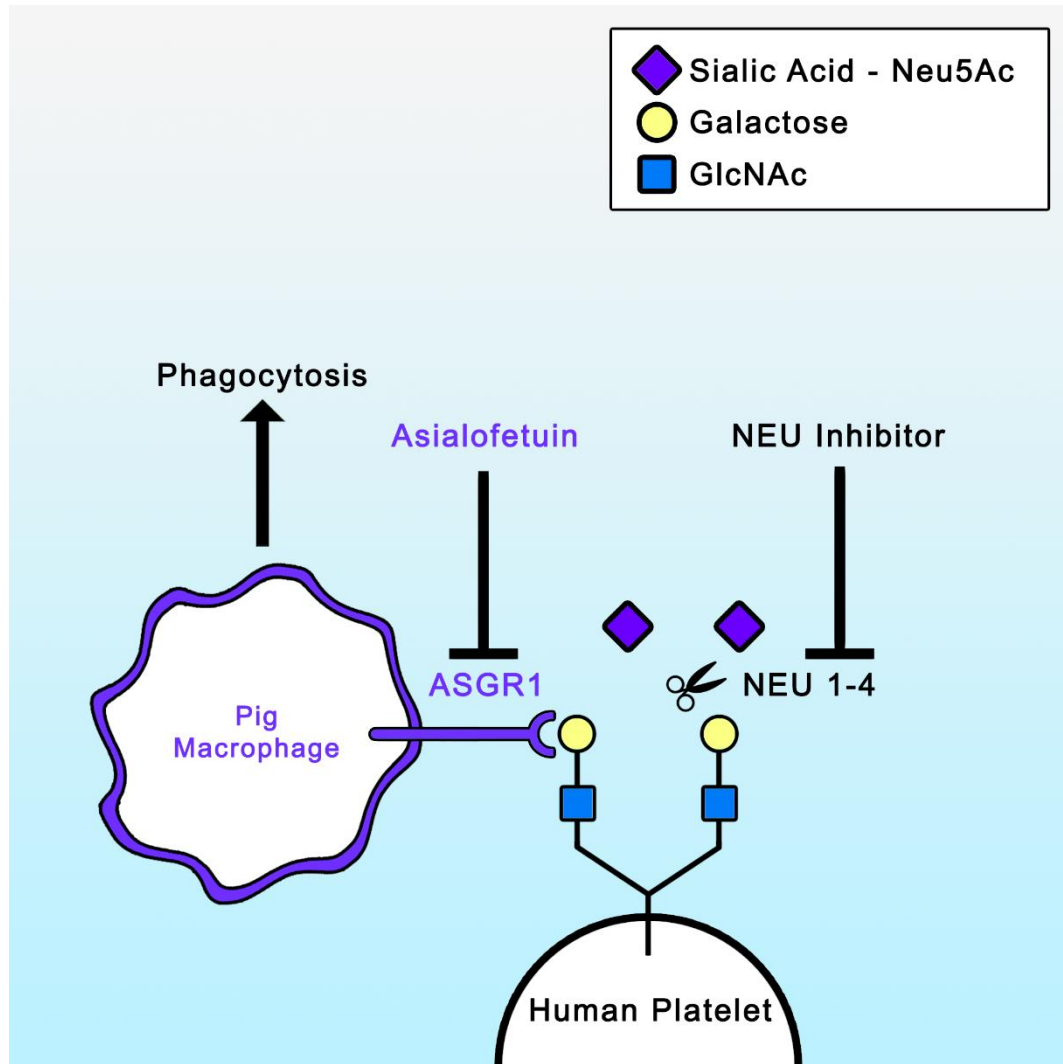


Figure 1.2: Pig ASGR-1 receptors and human platelets recognition.

Asialoglycoprotein receptor-1 (ASGR-1), expressed by Kupffer cells (macrophages), hepatocytes, and liver sinusoidal EC (LSEC), recognizes galactose on human platelets after Neu5Ac has been cleaved by sialidases (NEU 1-4), causing human platelets to be phagocytosed. This interaction can be prevented by inhibition of platelet desialylation using NEU inhibitors, or by targeting ASGR-1, using either a competitive inhibitor such as asialofetuin, or with an antibody that blocks (α ASGR-1).

neuraminidase increased PMN adhesion to and migration across the endothelium. This increase in adhesion/migration can be inhibited by pre-incubation of cells with the sialidase inhibitor 2,3-dehydro-2-deoxy-N-acetyl-neuraminic acid (DANA).⁴⁴ In relation to xenotransplantation, it has been shown that human PMN bind to naïve porcine endothelium more avidly than to human endothelium under static conditions⁹⁴ through a mechanism that may be CD82-dependent, which is a part of the tetraspanin family.⁹⁵ However, the role of desialylation in xenogeneic PMN adhesion is unknown and is a major area of our current focus.

Cellular sialylation also regulates integrin-mediated cellular adhesion on both sides of the receptor/ligand pair. Desialylation of the β 2 integrin (CD11b/CD18) exposed an activation epitope on CD18 and increased binding to the integrin ligand ICAM-1, causing deviated PMN binding to ICAM-1. Similarly, desialylation of ICAM-1 greatly enhanced the binding of PMN under conditions of physiologic shear stress.⁹⁶

Galectins

One of the central mechanisms involved in intercellular adhesion is mediated by galectins. Galectins bind to exposed galactose residues, which become exposed on a wide variety of glycoproteins and glycolipids on cell surfaces after Sia is cleaved. There are twelve known galectins in humans, which bind to many different substrates (such as galactose, integrins, fibronectin, LAMP-1) with varying affinity. Galectins are expressed in a variety of tissues, including EC, alveolar macrophages, PMN and other hematopoietic cells.⁹⁷

Galectins play an important part in PMN recruitment/activation. When Galectin-1 (gal-1) is injected into the peritoneal cavity PMN recruitment is increased, even in the absence of other inflammatory insults. This effect is independent of a G-protein-coupled receptors and instead has been attributed to the sialoglycoprotein CD43. In a model of zymosan-induced acute inflammation, Gal-1 was expressed and co-localized with L-selectin and β 2-integrin both on the plasma membrane and in the cytoplasm of PMN.⁹⁸ Others have found the opposite effect, treatment of peritoneal cells with hrGal-1 inhibited PMN migration and diminished IL-1 β release and expression of adhesion molecules such as β 2-integrin.⁹⁹ Pre-incubation of PMN with hrGal-1 also decreased rolling, capture, and adhesion of PMN on activated endothelial monolayers. Knockdown of endothelial Gal-1 increased the number of PMN captured and rolling.¹⁰⁰ Galectin-3 (gal-3) has also been shown to affect PMN migration and activation, inducing the expression of L-selectin and IL-8 by PMN in vitro, thereby modifying PMN migration and activation.¹⁰¹ During an induced transmigration of IL-8-activated PMN Gal-3 levels in the PMN and EC were elevated, but there was a decrease in the amount of Gal-1.¹⁰² In one Leishmania infection model, extracellularly released gal-3 facilitated PMN migration.¹⁰³

Galectins also been found to play a role in platelet adhesion. Galectin 8 binds glycans on the platelet membrane and prompts spreading, calcium mobilization and fibrinogen binding, while also promoting platelet aggregation, thromboxane generation, P-selectin expression and granule secretion. Gal-1 and -8 promote platelet adhesion by stimulating the transition of α IIb β 3 integrin to a high-affinity/"active" state, which results in unmasking of epitopes for fibrinogen to bind to.¹⁰⁴ Fibrinogen acts as a bridging molecule between platelets to form aggregates. Gal-1, -3 and -8 all strongly induce P-

selectin expression by platelets. Activation of platelets by gal-1 in the presence of PMN results in formation of heterotypic cell aggregates in a dose dependent manner.¹⁰⁵ Similar mechanisms may be responsible for the increased binding of human PMN and platelets to xenoendothelium, further linking sialylation state to the procoagulant phenotype and platelet and PMN activation events that are associated with xenograft injury. We infer that galectin blockade could prevent a clinically significant part of the inflammatory responses to a xenograft (as seen in **Figure 1.3**).

Galectins can also dampen immune responses. Gal-1 inhibits tissue migration of immunogenic, but not tolerogenic, dendritic cells.¹⁰⁶ Gal-1 doesn't affect T cell viability, but it does induce IL-10 production and reduces IFN- γ production in activated T cells. Gal-3 induces phosphatidylserine exposure on activated T cells causing apoptosis.¹⁰⁷ Galectin-8 (gal-8) enables antigen-specific differentiation of Tregs by triggering TGF- β signaling and stimulating sustained IL-2R signaling. Tregs that are differentiated in the presence of gal-8 express elevated levels of IL-10.¹⁰⁸ Promoting mechanisms in which galectins diminish the immune response could be used as therapy to control adaptive immune responses in xenotransplantation.

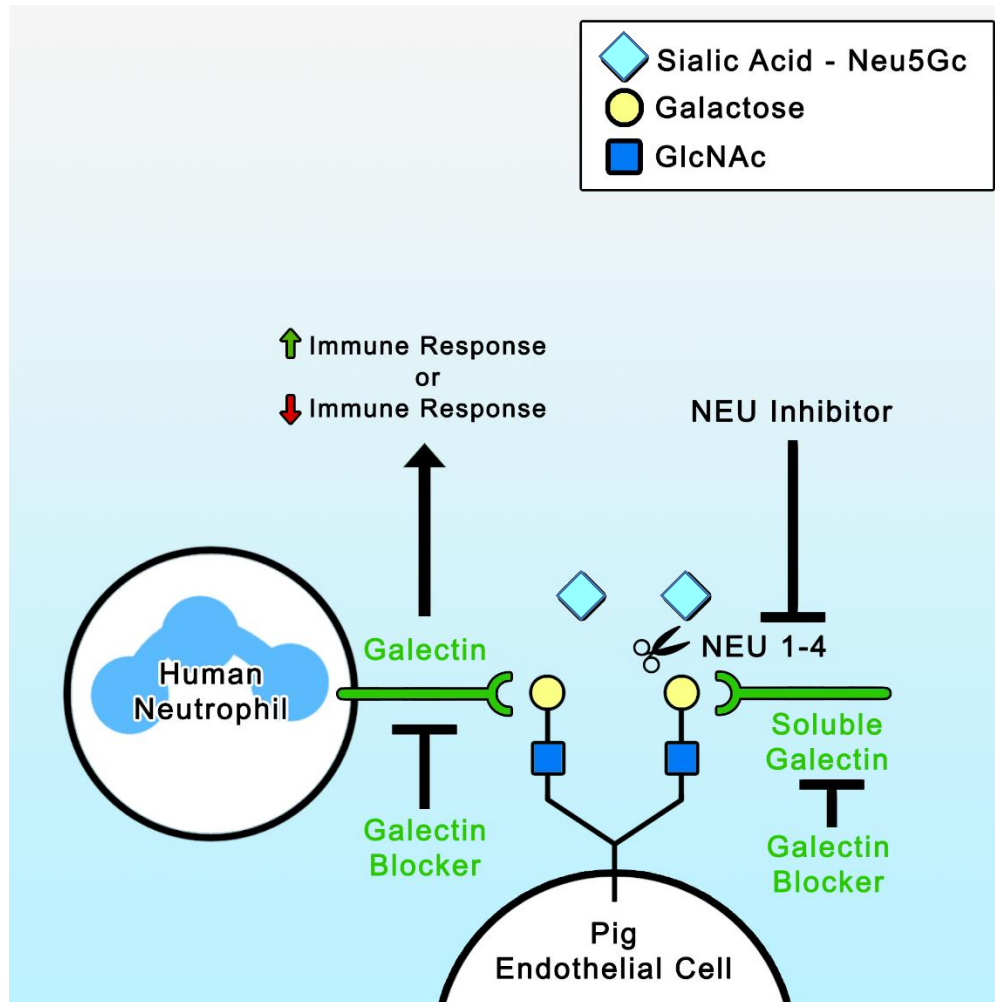


Figure 1.3: Galectins and xenotransplantation. Galectins are galactose binding lectins expressed by many different cell types; they are also found in soluble form. By binding to desialylated cell surfaces as well as to other receptors, they exert pleotropic effects, either increasing or decreasing immune responses depending on the cell types in question, their particular galectin expression profile, and the physiologic environment. Our working hypothesis poses that galectins expressed by PMN bind to galactose residues exposed by desialylated pig EC, thereby enhancing the activation and tissue infiltration of human PMN into pig tissues. This interaction could be prevented by inhibition of cell desialylation using NEU inhibitors, or by selective blockade of the galectin-galactose interaction.

Scope of Dissertation:

Sias play multifarious roles in immunity, including cell-cell adhesion and activation. PMN sequestration is a hallmark of acute lung injury, and is consistently associated with xenograft lung injury, despite genetic modifications of the donor organ. I sought to discover how endothelial and PMN Sias modulate the adhesion of human PMN to human or pig aortic EC (AECs).

In chapter 2, I describe the differences in levels of sialidase activity between pig and human EC as well as the levels and changes in levels of sialidases during our pig xeno-lung perfusions. I will show how knockdown of the highest levels of sialidases affects PMN adhesion of human PMN to pig EC. In chapter 3, I will show that desialylation of pig EC or human PMN increases PMN adhesion and that the mechanism behind this is largely driven by galectins. I will show how blockade of gal-3 decreases the number of PMN adhering after activation and desialylation of pig EC and activation of human PMN. Through these studies I have demonstrated proof-of-concept strategies to prevent the PMN sequestration that is seen during our xeno-lung perfusions.

Chapter 2 : Cellular desialylation promotes xenogeneic PMN adhesion

Introduction

Xenotransplantation is the process of transplanting organs or tissues from one species into another and is viewed as an attractive prospective solution to the human organ shortage for transplantation. To overcome a pre-existing, preformed "innate immune" barrier, several modifications have been made to the porcine genome. These include knockout of the α -1,3 galactosyltransferase enzyme (GalTKO), removing the Gal epitope from the surface of pig cells, transgenic expression of human complement regulatory proteins such as CD46 (hCD46, membrane cofactor protein) and CD55 (hCD55, decay accelerating factor) and other genetic modifications. There has been significant progress to overcome the barriers of xenotransplantation with survival reaching months or years in some heart and kidney transplant models.^{43,44} However, pig lung injury during *ex-vivo* human blood perfusion and after transplantation into non-human primates remains a major problem that has limited survival to just a few days. A main limitation of pig lung xenograft injury is the fact that leukocyte (particularly PMN) and platelet sequestration occurs in our *ex-vivo* human blood perfusion system within minutes, and the mechanisms driving these adhesive interactions are unknown.^{30,31,32}

The sialic acid expression profile on the surface of cells can dynamically change upon cell activation⁵. Moreover, emerging evidence suggests that changes in cell surface sialylation can dramatically affect diverse cellular functions¹⁰⁹. One pathway mediating changes in cell surface sialic acid expression is through the loss of sialic acid by enzymatic cleavage caused by sialidases. There are four mammalian sialidase genes (neuraminidases, NEU) in eukaryotes, NEU1-4. The intracellular location of sialidases varies for each enzyme and

sialidase expression also varies depending on the cell type or tissue. NEU1 is localized primarily in the lysosome, NEU2 in the cytosol, NEU3 in the plasma membrane and NEU4 in the lysosomes, mitochondria and endoplasmic reticulum.^{16,17} In certain cell types, intracellular sialidases were found to mobilize from intercellular reservoirs to the cell surface after activation. For example, NEU1 can translocate from lysosome to the plasma membrane during activation or differentiation in cells such as macrophages¹⁷, EC¹⁸, erythrocytes¹⁹ and polymorphonuclear PMN (PMN)^{6,93}.

A role for sialic acid in PMN adhesion was shown in that PMN express sialidase activity. Activation of PMN stimulates the translocation of intracellular sialidases to the PMN surface. In a model using human EC and human PMN it was found that pre-incubation of activated PMN with resting EC increased PMN adhesiveness. This increase in adhesion/migration was inhibited by pre-incubation of cells with the pan-sialidase inhibitor 2,3-dehydro-2-deoxy-N-acetyl-neuraminic acid (DANA).⁶ The increase in PMN adhesion was associated with the mobilization of sialidases from the lysosome to the cell surface of activated PMN causing *in trans* desialylation of adjacent EC which increased adhesion of other PMN to EC.⁶

In vitro studies using EC from wild type (WT or GalTKO) pigs demonstrated that human serum (a source of xenoreactive natural antibodies and complement) induced endothelial cell activation with consequent leukocyte adhesion under static¹¹⁰ and flow^{111, 112} conditions. Human PMN were also found to directly recognize xenogeneic endothelium, independently of xenoreactive natural antibody and complement, although those results are controversial^{110,113}. In summary, the mechanisms mediating adhesion of human PMN to

pig endothelium, and specifically the role of sialic acids/sialidases in such interaction, have not yet been defined.

The expression profile of NEU enzymes in pig organs and human blood cells and possible variation in their expression levels upon transplantation have never been characterized in models of pig-to-human xenotransplantation. We hypothesize that NEU expression and/or sialidase activity are augmented in the pig organ and/or human cells during xenogeneic injury, and that sialidase activity contributes to increased desialylation of pig tissues (including endothelium) and human PMN. Here we characterize the NEU expression profile and sialidase activity during the *ex vivo* perfusion of pig lungs with human blood. We also evaluate the role of cellular desialylation in the adhesion of human PMN to pig EC *in vitro*, focusing primarily on the NEU1 and NEU3 sialidases.

Materials and Methods

Animals

Genetically engineered pigs lacking the alpha-Gal epitope and expressing human membrane cofactor protein (hCD46) (GalTKO.hCD46) were provided by Revivicor (Blacksburg, VA, USA). All procedures were approved by The Institutional Animal Care and Use Committee (IACUC) at the University of Maryland, School of Medicine, and were conducted in compliance with The National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Lung harvest, *ex vivo* perfusion and transplantation procedures

Anesthesia protocol and the harvest of the lung xenograft were performed as previously described¹¹⁴. Prior to flushing the lungs, 1-benzylimidazole (5 mg/kg BW; a thromboxane synthase inhibitor; Sigma–Aldrich, St. Louis, MO) and synthetic prostaglandin I₂ (0.03 mg/kg BW; Flolan; GlaxoSmithKline, Research Triangle Park, NC) were administered intravenously and allowed to circulate for several minutes. The right and left lungs were separately perfused via the pulmonary artery using side-by-side circuits fashioned from silicon tubing and polyurethane connectors as previously described²⁵. Transgenic pig lungs were perfused with heparinized fresh human blood in an *ex vivo* circuit as previously described.^{30,31,114}.

Endothelial cell isolation and culture

Primary pig aortic EC (PAECs) isolated from pig aortas were grown in culture medium (10% heat-inactivated FBS, 0.15mg/mL gentamicin, 25ng/ml fungizone, DMEM 1g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate). Cells were used after four to ten passages at 80%-95% confluence. HAECs (blood group O or B, Life Technologies, Carlsbad, CA) or HUVECs (Life Technologies, Carlsbad, CA) were also grown in their specific growth factor kit (Thermo Fisher, Waltham, MA, USA) and culture medium (Medium 200).

Fluorometric assay for sialidase activity

Sialidase activity was measured in human umbilical vein EC (HUVECs), human aortic EC (hAECs) and wild Type (WT), α 1,3galactosyltransferase knockout (GalTKO) or α 1,3galactosyltransferase knockout human membrane cofactor protein (hCD46) knock-in (GalTKO.hCD46) pig aortic EC lysates. The lysates were measured in the presence of 2'-

(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid sodium salt hydrate (4-MU-NANA) (Sigma) as the substrate. EC ($\sim 5 \times 10^5$ cells/tube) were suspended in 200 μ l of buffer containing 500 mM sodium acetate, 0.1% Triton X-100, supplemented with protease inhibitor mixture (Roche Applied Science, Indianapolis, IN) and then incubated for 1 h at 37 °C in the presence of 4-MU-NANA, mixing tubes every 15 min. The enzymatic reaction was terminated by the addition of glycine-NaOH buffer, pH 10.3 (133 mM glycine, 60 mM NaCl, and 0.083 M Na₂CO₃), after which fluorescence intensity was measured with a Spectramax3000 fluorometer (excitation at 355 nm; emission at 460 nm). In some experiments EC lysates were pre-incubated with the competitive neuraminidase inhibitor, 2-deoxy-NANA (200 μ g/ml, DANA) (Calbiochem/Millipore, Billerica, MA) or Zanamivir (Relenza, GlaxoSmithKline (Brentford, UK) , a clinical anti-flu treatment). A molecule with comparable molecular mass and charge to 2-deoxy-NANA, 2-keto-3-deoxyoctulosonic acid (KDO) (Sigma), was used as a negative control (200 μ g/ml).

Static PMN adhesion assay

Wild type (WT), GalTKO or GalTKO.hCD46 primary pig aortic EC (pAECs) or human aortic EC (hAECs) were grown to confluent monolayers in 24 well culture plates. After washing with warm DMEM, calcein^{AM}-labeled human PMN (5×10^5 in warm HBSS buffer with Ca⁺⁺ and Mg⁺⁺) were added at 10^4 PMN/ml per well and incubated for 30 min at 37°C. After washing unbound PMN with room temperature PBS, attached PMN were quantified fluorometrically (excitation 485 nm, emission 530 nm) using a Spectramax 3000 plate reader (Sunnyvale, CA, USA). PMN–EC adhesion was expressed as percent adhesion using the amount of PMN that adhere for 10^4 PMN/ml to a culture plate. In some instances,

EC monolayers were activated with TNF α (25 ng/ml for 4 hr) and PMN were treated with IL-8 (1 nM, 30 min) for 30 min at 37°C before incubation with AECs.

Bioflux flow adhesion assay

Primary GalTKO.hCD46 pAECs or hAECs were grown to confluent monolayers on microfluidic channel 24-well plates (Fluxion, South San Francisco, California, USA). Monolayers were treated with DMEM, NA (25mU/ml for 30 minutes at 37 °C), TNF α (25 ng/ml for 4 hours at 37 °C) or both. Freshly isolated calcein^{AM}-labeled human PMN (5×10^5 PMN/ml in HBSS buffer (with Ca⁺⁺ and Mg⁺⁺) were incubated with HBSS, NA or IL-8 (1 nM, 30 min, 37°C). After washing, PMN (10^4 PMN in 300 μ L of HBSS buffer) were introduced into the inflow wells of experimental channels and perfused at 1 dynes/cm² at 37°C for 20 minutes using a Bioflux 200 system (Fluxion, San Francisco, CA). Two pictures (proximal and distal views) of 10x field of view for each of duplicate channels were obtained every 60 seconds for 20min by fluorescent microscopy (Zeiss) using 100 ms exposure. Adhered PMN were counted after processing of the data on the Fluxion system using fluorescence intensity. Counts from both fields of view for each channel at each time-point were averaged for all experiments and SD was calculated.

Real-time RT-PCR

Total cellular RNA was extracted from lung tissue before (“Pre-perfusion”) and after 4 hours of ex-vivo perfusion from untreated lungs (n = 5) using the Qiagen (Valencia, CA) RNA isolation kit (RNeasy Mini kit) according to the manufacturer's protocol. RNA purity was established with the 260/280-nm absorption ratio. For real time quantitative RT-

PCR, 20 µg total RNA was reverse-transcribed using Superscript IV VILO MasterMix (ThermoFisher, Halethorpe, MD). The resulting cDNA (20 ng) was amplified by using real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems/ThermoFisher) and ABI Prism 7900HT cycler. Primers (and probes) for detection of either pig or human NEU1, NEU2, NEU3, and pig RPL32 (pL32) mRNAs were designed and are indicated in Table 2.1. This was achieved by BLAST sequencing the pig and human sequences and choosing primers that were species specific and then testing them in pig only or baboon only samples. Relative gene expression was calculated using the Δ Ct method (Ct gene of interest- Ct housekeeping gene (pL32)), where Ct refers to the cycle number at which the PCR product for a particular gene is detected. The housekeeping gene, pRPL32, was used as an internal control.

Immunoblotting

EC were lysed directly in flasks using cold lysis buffer (50 mM Tris-Cl, 5 mM EDTA, 1 mM PMSF, protease inhibitor cocktail (aprotinin 100 U/ml), 1% Triton X-100 for 1 minute and then scraped from flask. Cell suspensions were centrifuged at for 5 minutes at 4°C and supernatants were frozen at -80°C until use. Lung tissue specimens were homogenized (using sonication) using the same lysis buffer as above. Protein concentration was determined by Bradford Protein assay (Bio-rad, Hercules, CA). Protein lysates (15 µg/lane) were first denatured in Laemmli loading buffer containing 2-βmercaptoethanol before separation on a 4-15% Tris SDS-PAGE (Invitrogen, ThermoFisher, Carlsbad, CA) and transfer to a nitrocellulose membrane (Invitrogen). Western blots were blocked with 3% dry milk in PBS buffer for 1 hour before incubation

Table 2.1: Oligonucleotide primers and probes used for quantitative RT-PCR and sequences for siRNA

F: Forward primer

R: Reverse primer

Pb: probe

RNA target	Species	Accession Number	Primer sequence	siRNA Sequence
NEU1	Pig	NM_00110 1822.1	F:5'CGCCTATGCTG GGATTAGGG 3' R:5'CGGGTGCACCA GATTGAAGT3'	Sense: 5' GGGCAAAGAACGACUCAAU U 3' Antisense: 5' UUGAAGUCGUUCUUUGCCCU U 3'
NEU1	Human	NM_00043 4.3	F:5'CGATGATCATG GTGCCTCCT 3' R:5'GAGCTCATAGG GCTGGCATT 3'	
NEU 2	Pig	XM_01398 4592	F:5'ATTTTGTCTGCA ATGAGCC 3' R:5'GGCGGGTGGAC GCATCGTA 3'	
NEU 2	Human	NM_0053 83.2	F:5'CCTACCTCAACC CGCGACCT 3' R:5'CCCAAACAAGG GGACCA 3'	
NEU 3	Pig	XM_00348 2565.3	F:5'CACGCTGCTGG GTTTCTCA 3' R:5'ACGTGGCTTCC ATTAATGGC 3' Pb: 5'CGTCCTCTGTTCC AGCA3'	Sense: 5'ACACCCAACCAGUAAGAAA UU3' Antisense: 5'UUUCUUACUGGUUGGGUGU UU 3'
NEU 3	Human	NM_00665 6.5	F:5'AGTGCAGAGGT CATGGAAGA3' R:5'CTGGATCCGGT AGGTAATCC3' Pb: 5'AGCCCTCTGTTCC GGGA 3'	

with polyclonal rabbit anti-human NEU1 (Genway Biotech, San Diego, CA; 1:500), rabbit anti-human NEU2 (Invitrogen, 1:500) or rabbit anti-human NEU3 (Santa Cruz, Dallas TX; 1:500) primary antibodies diluted in PBS-T-M (PBS+ 0.01% Tween + 0.1% milk) , overnight at 4°C. Since no commercial antibodies targeting pig NEU enzymes are available, we searched for human specific antibodies that are cross-reactive with pig NEU enzymes. All anti-human NEU antibodies were first verified to cross-react with putative pig NEU enzymes by testing their reactivity on pig tissue lysates in comparison to human positive control tissue lysates (data not shown), as predicted by the strong identity of pig and human NEU enzymes at the protein level (NEU1: 90% identity, NEU2: 80% identity, NEU3: 88% identity). Alignment is seen in Table 2.2. After washing with PBS-T-M goat anti-rabbit secondary antibody labelled to 680 RD (ThermoFisher) was applied for 120 minutes at room temperature (1:10,000 in PBS-T-M). Visualization and quantification was carried out with the LI-COR Odyssey® scanner and software (LI-COR Biosciences, Lincoln, NE). Blots were then stripped, washed with PBS-T and then incubated with mouse anti-human β actin (1:200, Santa Cruz, Dallas, TX) for 2 hours at room temperature. After washing with PBS, goat anti-mouse secondary antibody labelled to 790 RD (ThermoFisher) was applied for 120 minutes at room temperature (1:10,000) and then visualized. Band intensity for NEU was divided by band intensity for β -actin and multiplied by 100 to calculate the ratio of NEU to β -actin.

Table 2.2 Alignment of NEU antibodies to pig

Red: Differences in AA between peptide and pig sequence

Gene	Type of antibody	Alignment
NEU1	Synthetic Peptide	Peptide:VWSKDDGVSWSTPRNLSLDIGTEVFAPGPGSGIQK QREPRKGR LIVCG Pig: VWSKDDGISWSSPRNLSLDIGTEMFAPGPGSGIQK QWAPQKGR LIVCG
NEU2	Synthetic Peptide	Peptide:ANVTLCQVTSTDHGRTWSSPRDLTDIGPAYREWST FAVGPGHCLQLH Pig: TRVTLCQVTSTDHGRSWSWARDLTVIGPAHKDWAT FAVGPGHCLQLH
NEU3	Synthetic Peptide	Peptide:WSEVRDLTEEVI GSEHWATFAVGPGHGIQLQSGRL VIPAYTY YIPSWFFCFQLPKTRPHSLMIYSDDLGV TWHHGR LIRPMVTVECEVAEVTGRA Pig: WSEVRDLTEEVI GPDHWATFAVGPGHGIQLQSGRL VIPAYTY YIPYRFFCFRLYKARPHSLMIYSDDLGA TWHHGR LIKPTVTVECCQVAEVT SRA

Knockdown of NEU1 and NEU3 through small interfering RNA (siRNA) technology

SIRNA for pig NEU1 and NEU3 were designed to specifically target pig NEU1 or NEU3 (Table 1) and synthesized by Dharmacon (Lafayette, CO). GalTKO.hCD46 pAECs were transfected with sense and anti-sense siRNA for NEU1 and/or NEU3, or an irrelevant control siRNA duplex not corresponding to any known sequence in the human and pig genomes (Dharmacon, Lafayette, CO). For transfection, 60-80% confluent pAEC monolayers were incubated with siRNA (10 μ M) and Lipofectamine RNAiMAX transfection reagent (ThermoFisher) for 24 hours and then washed and incubated with culture medium. The transfected cells were cultured for 48 hours after which they assessed for sialidase activity and efficiency of knockdown by immunoblotting, and seeded in the Bioflux plates for PMN flow adhesion assays.

PMN isolation

Venous human blood was collected into EDTA tubes from healthy volunteers under a protocol approved by the University of Maryland Baltimore Institutional Review Board. PMN were isolated by density gradient centrifugation ($500 \times g$, 35 min, brake off) using Lympholyte®-poly Cell Separation Media, (ACL5070 Cedarlane, Burlington, ON, Canada). The PMN layer was collected and residual red blood cells were lysed using RBC lysis buffer (Roche Applied Science, Basel, Switzerland). PMN were washed ($400 \times g$) with Hank's Balanced Salt Solution (HBSS) (Gibco-Life Technologies, Waltham, Massachusetts, USA) without calcium chloride and magnesium chloride and then re-suspended in HBSS with divalent cations at 5×10^5 PMN/ml (purity >86% and viability >95% as measured by flow cytometry (BD FACSVerserTM, Becton Dickinson, Franklin

Lakes, New Jersey, USA)). They were then incubated with 0.5 μ M calcein^{AM} (calcein-acetoxymethyl ester) (Corning, New York, USA) for 30 min with gentle agitation in the dark at 37°C. Labeled PMN were washed two times with HBSS with Ca⁺⁺ and Mg⁺⁺.

PMN adhesion flow assay

GalTKO.hCD46 pAECs transfected with either a scramble siRNA, NEU1 siRNA, NEU3 siRNA or both for 48 hours, or incubated with DANA, Relenza, KDO or DMEM for 72 hours were grown to confluent monolayers in microfluidic channel 24-well plates (Fluxion, San Francisco, California, USA). Monolayers were then treated with DMEM or TNF α (25 ng/ml, 4 hours). Freshly isolated calcein^{AM}-labeled human PMN (5 x 10⁵ PMN/ml in HBSS buffer) were incubated with HBSS, DANA, zanamivir or KDO for 30 minutes in HBSS buffer. In some experiments, PMN were activated with rIL-8 (1 nM, 30 min) before addition of sialidase inhibitors. All incubations were conducted at 37°C. After washing, PMN (10⁴ PMN in 300 μ L of HBSS with Ca⁺⁺ and Mg⁺⁺) were introduced into inflow wells of experimental channels and perfused at 1 dynes/cm² at 37°C for 20 minutes using a Bioflux 200 system (Fluxion). Two pictures (proximal and distal views) of 10x field of view for each of duplicate channels were obtained every 60 seconds for 20min by fluorescent microscopy (Zeiss) using 100 ms exposure. Adhered PMN were counted after processing of the data on the Fluxion system using fluorescence intensity. Counts from both fields of view for each channel at each time-point were averaged for all experiments and SD was calculated.

Statistical analysis

Statistical analyses were performed using the statistical package INSTAT through GraphPad. Unless otherwise indicated, variables were expressed as the mean plus SD. Variables were assessed with the unpaired student t-test. P-values <0.05 were considered statistically significant.

Results

Total sialidase activity is higher in pig EC compared to human EC

We asked whether pig EC express the same level of sialidase activity as human EC. Total sialidase activity was measured for lysates of human umbilical vein EC (HUVECs), human aortic EC (hAECs) and pig aortic EC (Wild Type, GalTKO and GalTKO.hCD46) (Figure 2.1A). Pig aortic EC express a larger amount of sialidase activity (>150 arbitrary fluorescent units, AFU) compared to human (~50 AFU; $p < 0.0001$ vs. hAEC controls). To our knowledge this is the first report of sialidase activity in pig EC and we show that pig EC contain higher levels of sialidase activity than human EC.

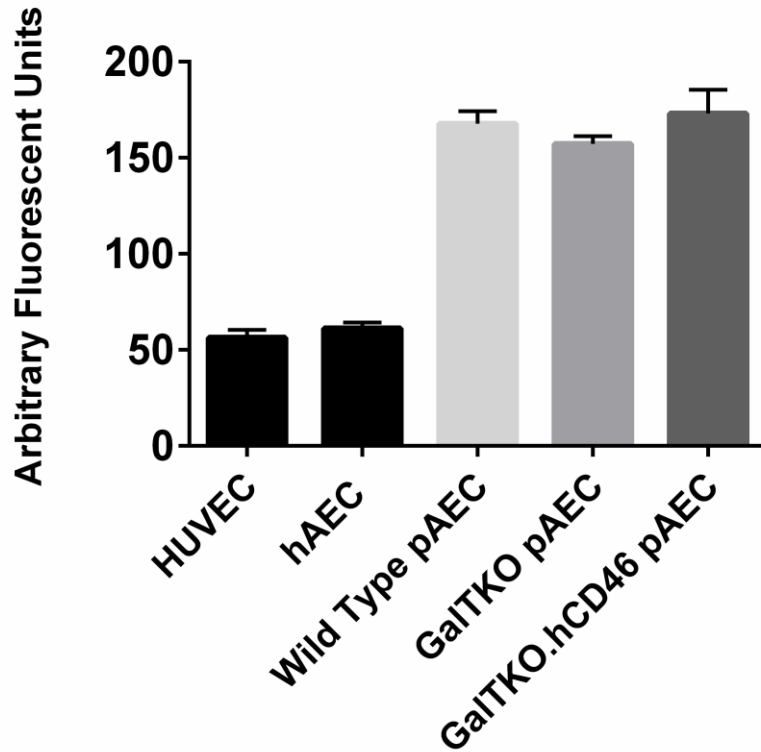


Figure 2.1: Pig endothelial cells express high sialidase activity.

Human umbilical vein EC (HUVECs), human aortic EC (hAECs) and pig aortic EC (wild type, GalTKO and GalTKO.hCD46 pAECs) were assayed for sialidase activity as liberation of sialic acid from the fluorogenic substrate, 4-MU-NANA. Vertical bars represent the mean \pm SD of arbitrary fluorescence units, from four independent experiments with 2-3 different individual pigs.

Human PMN adhere avidly to pig EC after activation

Adhesion of human PMN was measured on hAECs and pig (Wild Type, GalTKO and GalTKO.hCD46 pAECs) aortic EC under static (Figure 2.2A) and flow (Figure 2.2B) conditions. Non-activated PMN adhere similarly to both human and pig EC of all types under static conditions (Figure 2.2A, *left side*). In contrast, we found a slighter higher adhesion of human PMN to pAECs compared to hAECs under flow conditions (Figure 2.2B). As mentioned above, activation of human PMN with IL-8 increases their sialidase activity, inducing *in trans* desialylation of neighboring cells. Therefore, we assessed PMN adhesion under conditions where PMN are activated with IL-8 and EC with TNF α (Figure 2.2A, *right side*). As expected, activation with IL-8/TNF α increased the adhesion of human PMN to hAECs (Figure 2.2A, black bars). Importantly, the adhesion of human PMN on pAECs upon activation with IL-8/TNF α increased even more dramatically on pAECs (Figure 2.2A, *grey bars*) was much higher than on hAECs.

NEU1 is the most abundant NEU enzyme expressed in xenogeneic pig lung tissue

Expression of NEU family members varies by tissue and by species. We measured the levels of human and pig NEU mRNA in pig lung tissues both before and after 4 hours of *ex-vivo* lung perfusion with human blood to determine which NEUs from each species are the most abundant during xeno lung perfusion. NEU1 mRNA expression was the highest in normal pig lung tissue (as reflected by the smallest cycle number (Δ CT) value) followed by NEU3 and then NEU2 (Figure 2.3A). As expected no human NEU was detected (background Δ CT >1) in pre-perfusion pig lung tissue proving primer specificity. We then measured whether expression levels of pig NEUs were modulated by perfusion with human blood (Figure 2.3B).

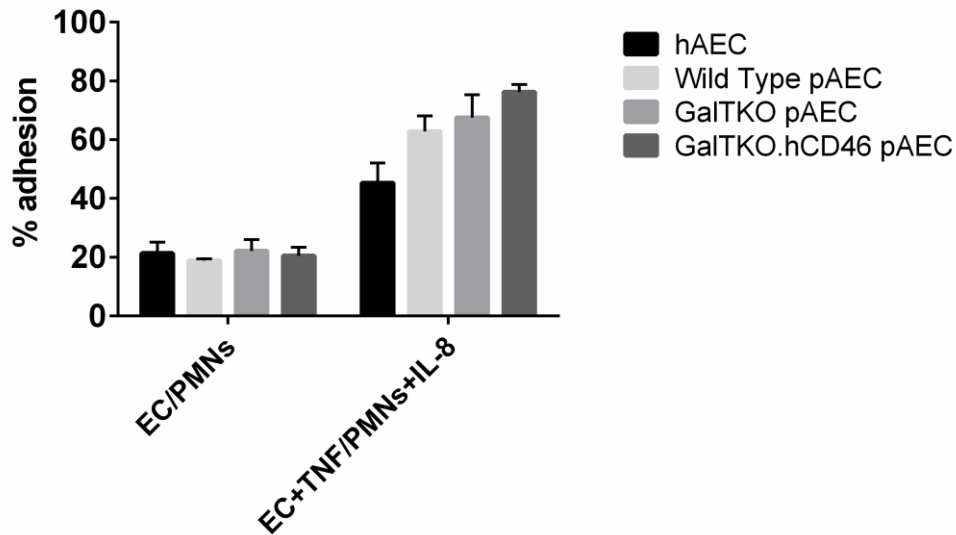
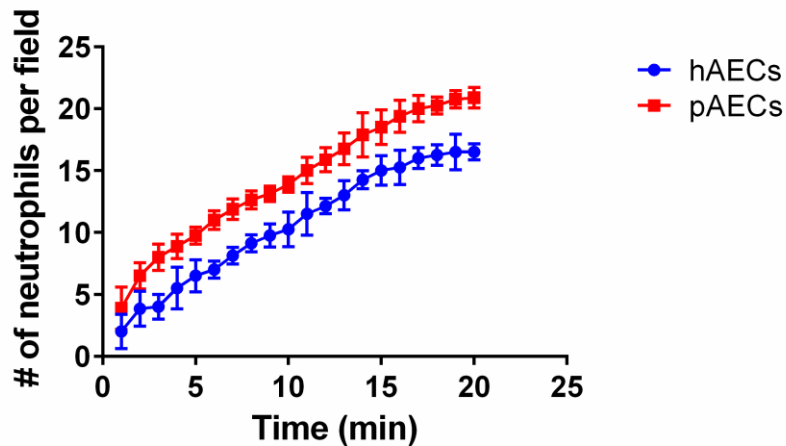
A**B**

Figure 2.2: Human PMN adhere avidly to pig endothelial cells after activation. A: Under static conditions, the adhesion of human PMN was similar on hAECs and pAECs (*left*) but adhesion of activated PMN (with rIL-8, 1nM, 30min) to activated AECs (with TNF α , 25ng/ml, 4hr) (*right*) was higher on pig pAECs. Results were expressed as the mean \pm SD of % adhesion of 2-6 replicates (with duplicate wells) for each cell type. **B:** Adhesion of resting PMN to hAECs or GalTKO.hCD46 pAECs under flow conditions. Results were expressed as the mean \pm SD of the number of PMN per image field from two independent experiments.

After 4hr of perfusion with human blood NEU1 and NEU3 expression was unchanged (fold change ~1), while expression of NEU2 was somewhat increased (~1.5 fold) (Figure 2.3B). Finally, since human blood cells including PMN are sequestered in the pig lung tissue during organ perfusion, we assessed the relative abundance human NEUs at the end of the blood perfusion (Figure 2.3C). Pig NEU1 mRNA levels were by far the most abundant NEU transcripts in the 4 hr samples, with remaining of pig and human NEUs expressed at similar levels (Figure 2.3C).

We then analyzed the expression profile of NEUs at the protein level using immunoblotting analysis of pig lung tissue lysates (Figure 2.4). Using antibodies that recognize both pig and human NEU-1, 2 and 3, we confirmed that NEU-1 is the predominant NEU found in pig lung tissue (Figure 2.4B, Pre, black). However, in contrast to the gene expression analysis above, NEU3 protein levels were almost as high as those of NEU1 in normal lung tissue. Perfusion of pig lungs with human blood for 4hr dramatically increased protein levels of both NEU1 (~3fold) and NEU3 (~2 fold) (Figure 2.4B, grey vs. black). Overall, at the end of the lung perfusion, NEU1 was the most abundant NEU, followed by NEU3. Based on these results, we decided to investigate the role of NEU1 and NEU3 in functional experiments.

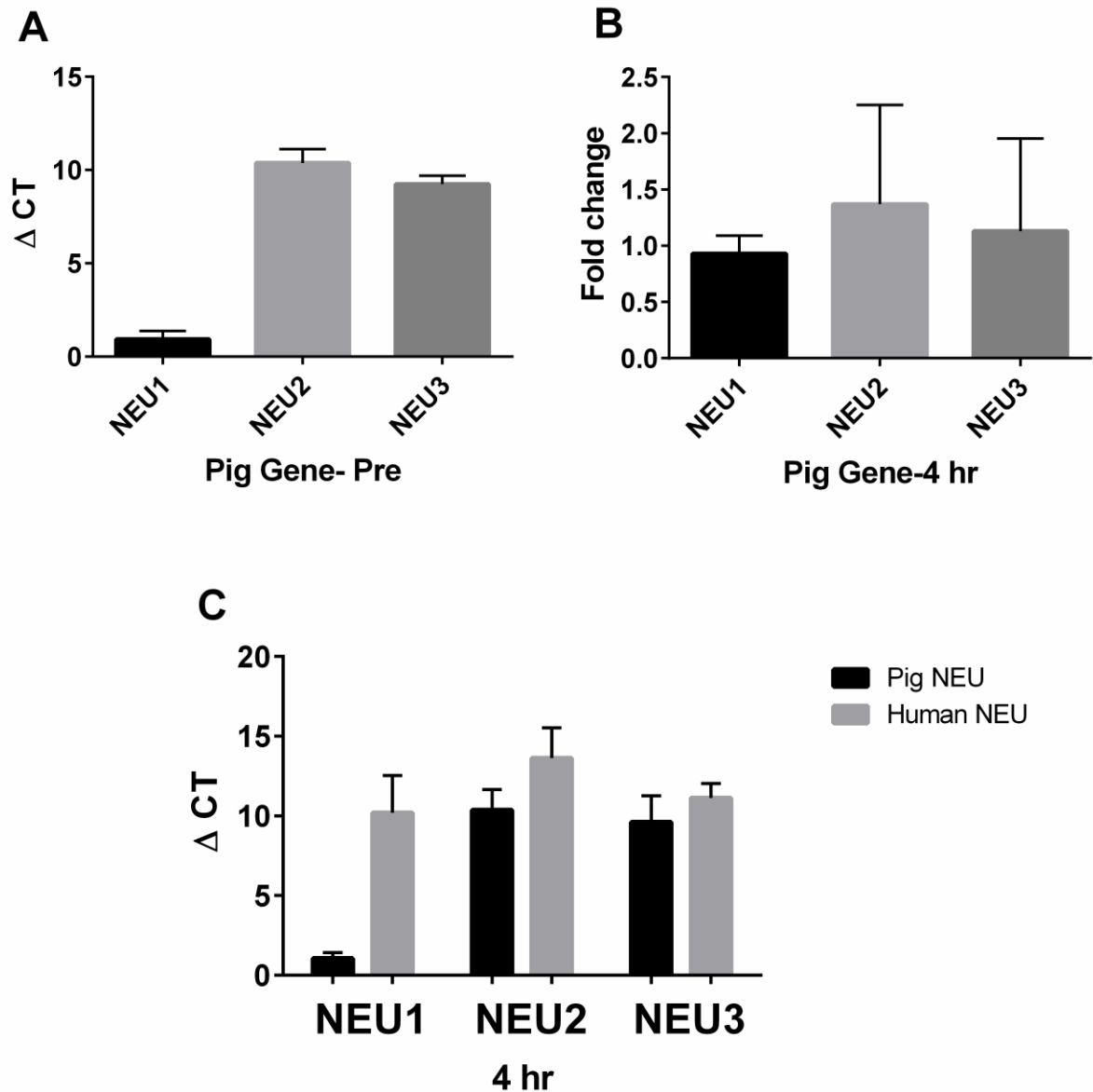


Figure 2.3: NEU1 has the highest mRNA levels present during *ex-vivo* lung xenoperfusions. mRNA levels were assessed by real-time PCR in pig lungs perfused with human blood using species specific primers for human and pig NEUs. **A:** Expression of pig NEU was measured prior to perfusion with human blood (pre) to determine the relative abundance of NEU at the beginning of the experiment. Human NEU were undetectable at this time-point. Results were expressed as ΔC_T using the pig house-keeping gene L32. **B:** Expression of pig NEU was measured after 4 hr of perfusion with human blood (4hr) to determine if xenogeneic stimulation affects NEU levels. Results are expressed as fold change (4hr/pre) after normalization to pig L32. **C:** Relative expression of pig and human NEU 1-3 after 4 hr of xenogeneic stimulation. Results were expressed as ΔC_T using the pig house-keeping gene L32. Data is shown as the mean \pm SD from 5 independent pig lung *ex-vivo* perfusion experiments.

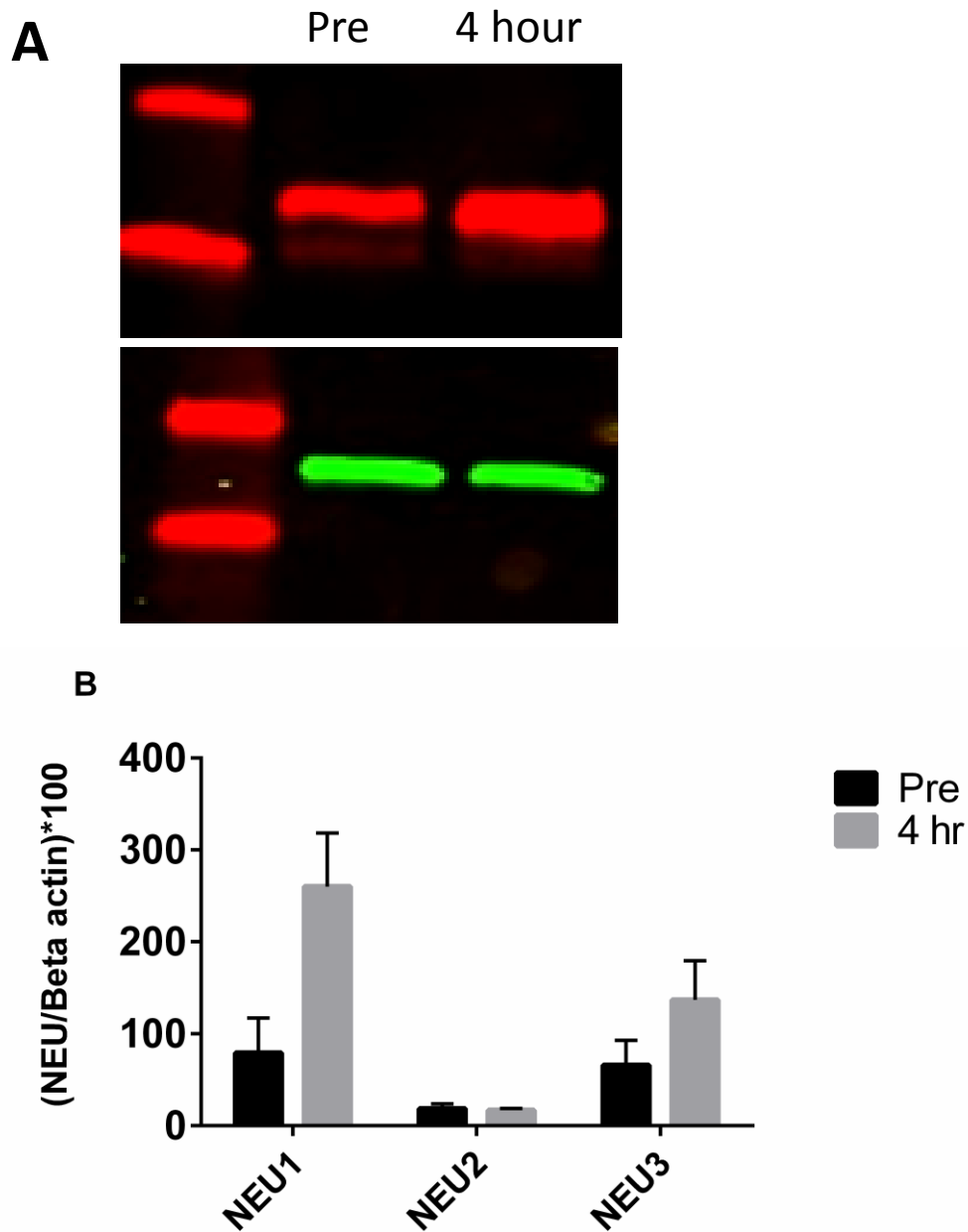


Figure 2.4: NEU1 has highest amount of protein present during ex-vivo lung perfusions. Total NEU protein levels were assessed by immunoblotting in pig lungs perfused with human blood using antibodies recognizing both human and pig NEUs. Expression of NEUs was measured prior to perfusion with human blood (pre) and after 4 hours of perfusion with human blood (4hr). Band intensity was measured by densitometry and results were expressed as the ratio to the loading control (β -actin) multiplied by 100. **A:** Representative immunoblot showing pre and 4 hr samples from 2 experiments; NEU1 blotting (*red, top*) and β -actin (*green, bottom*). **B:** Significantly higher expression of NEU1 at end of experiment ($p < 0.0001$ vs. NEU2 and NEU3). Data is representative of averages (\pm SD) for 5 experiments.

Pharmacologic sialidase inhibition decreases PMN adhesion

In Figure 2.2 activation of PMN with IL-8 (a stimulus previously shown to induce the translocation of sialidases to the PMN surface)^{115,6} increases the adhesion of human PMN to pAECs. Based on the NEU expression profile above, we asked whether increased adhesion of activated human PMN to pAECs is attributable to NEU activity. Pig AECs or human PMN were treated with the competitive sialidase inhibitor, 2-deoxy-NANA (DANA), the neuraminidase inhibitor zanamivir (Relenza), or a similarly charged negative control (KDO) (Figure 2.5). DANA inhibits sialidase activity from all NEUs whereas zanamivir, although developed to inhibit influenza neuraminidases, exhibits inhibitory potency towards eukaryotic NEUs, and particularly NEU3¹¹⁶. In conditions used, those inhibitors reduced sialidase activity in lysates from treated pAECs by ~40% for DANA and ~30% for Relenza (Figure 2.5A). The effect of NEU inhibitors was then tested in a PMN adhesion assay under flow conditions using similar methods as in Figure 2.2B, where PMN were activated with IL-8 and pAECs were activated with TNF α . Treatment of pAECs with DANA almost completely prevented PMN adhesion, whereas treatment of PMN with DANA reduced their adhesion by ~60% (Figure 2.5B). Treatment with Relenza was less potent than DANA, with ~50% inhibition of PMN adhesion, whether or PMN were treated with the sialidase inhibitor ($P < 0.0001$ vs KDO control) (Figure 2.5C).

siRNA knockdown of endothelial cell NEU1 and NEU3 decreases PMN adhesion

Given that pharmacologic inhibition of sialidase activity decreases xenogeneic PMN adhesion, we wished to confirm a NEU-specific mechanism and exclude off-target drug effects. Due to the fact that NEU1 and NEU3 had the highest expression in our lung

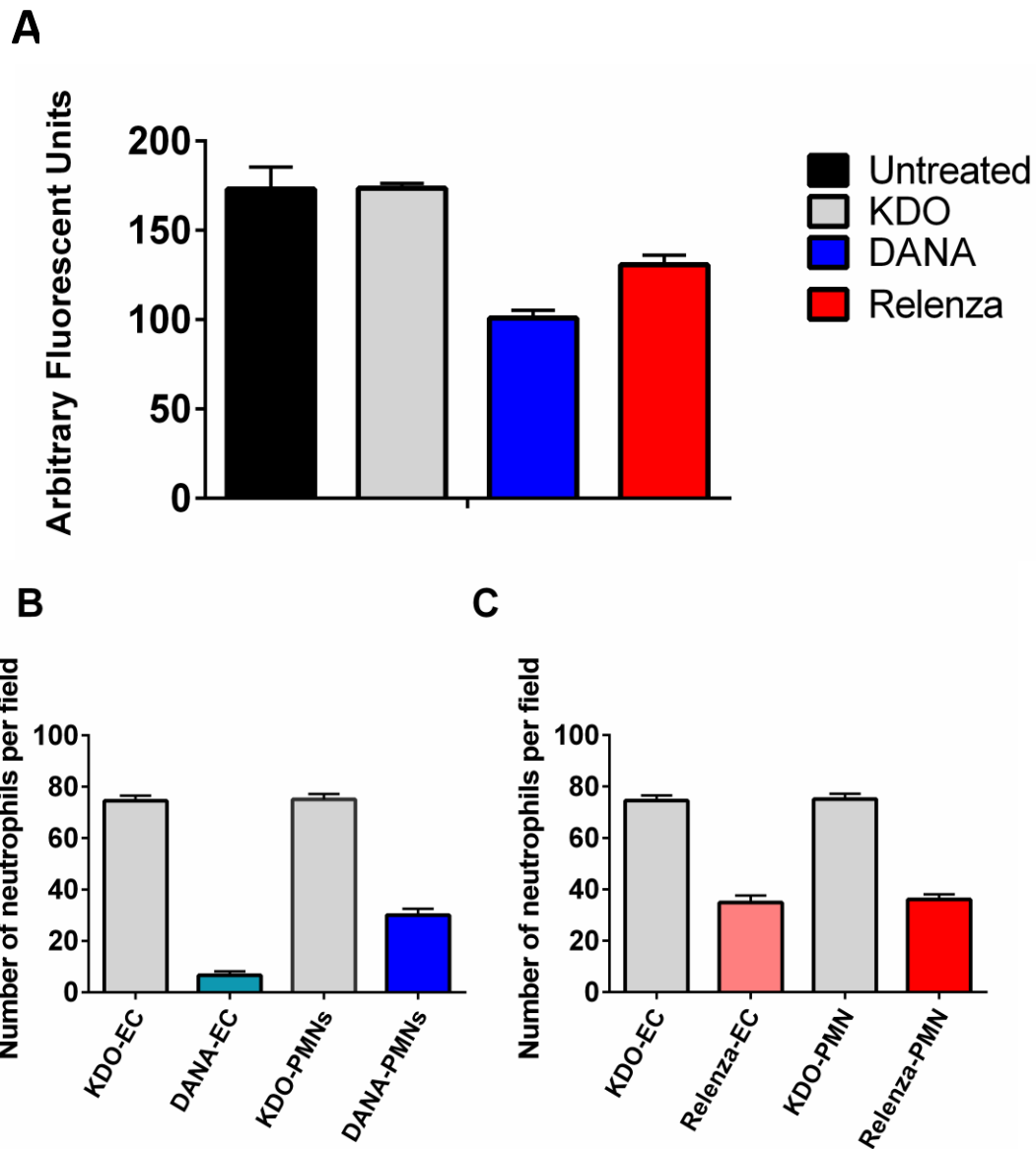


Figure 2.5: Treatment of pAECs or human PMN with pharmacologic sialidase inhibitors decreases xenogeneic PMN adhesion. **A:** Sialidase activity of GalTKO.hCD46 pAEC lysates was measured after incubation of pAECs with sialidase pan inhibitor, DANA or sialidase inhibitor, Relenza (30 min at 37°C). **B, C:** IL-8 activated (1nM, 30min) human PMN were flowed over TNF α activated (25ng/ml, 4hr) GalTKO.hCD46 pAECs monolayers at 1 dyne/cm² for 20 minutes. PMN or pAECs (cells) were also pre-treated with sialidase inhibitors: DANA (**B**) or Relenza (**C**) or KDO internal control. Results were expressed as the mean and standard deviation of the number of PMN per image field from two independent experiments. *: p<0.0001 vs. KDO controls.

tissue lysates from our perfusions we used a siRNA genetic approach to knockdown NEU1, NEU3 or both in GalTKO.hCD46 pAECs, and evaluated the effect of these interventions on PMN adhesion (Figure 2.6). NEU1 knockdown (*A, left*) and NEU3 knockdown (*A, right*) were effective, as measured by protein measurements from multiple Western blots (Figure 2.6A and B). Sialidase activity, measured in cell lysates from control GalTKO.hCD46 pAECs as well as pAECs that have had NEU1 (65% reduction), NEU3 (15% reduction) or both NEU1 and 3 knockdown (85% reduction), confirmed target-specific reduction in NEU activity (Figure 2.6C). When the effect of NEU knockdown was studied under flow conditions (Figure 2.6D), NEU1 or NEU3 knockdown significantly decreased the number of PMN that adhered ($p < 0.0001$ vs. scramble control) although not as low as seen with resting pAECs. NEU1 and 3 combination knockdown decreased the number of PMN adhering even further ($p < 0.0001$ vs. scramble control).

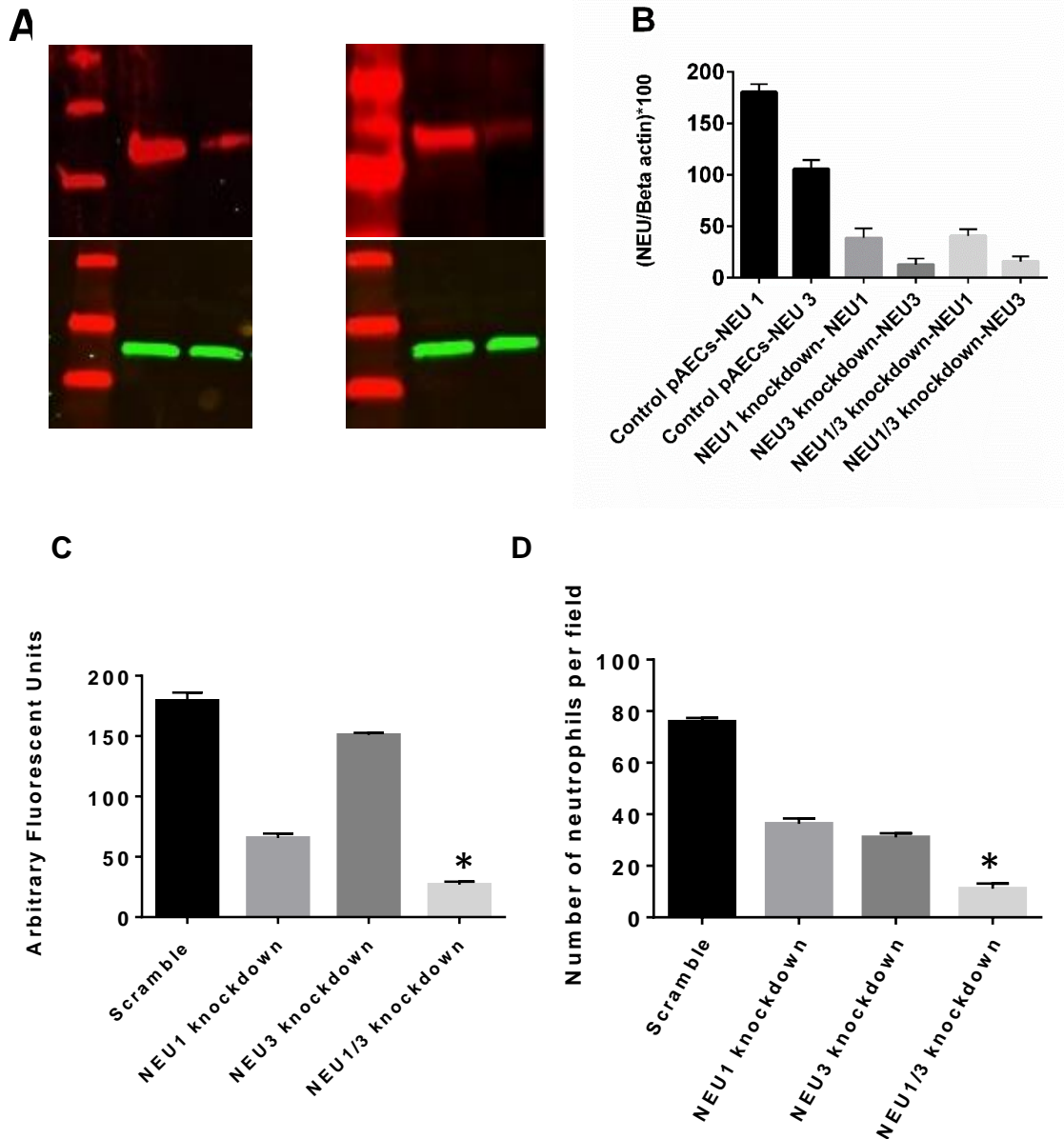


Figure 2.6: NEU1, 3 or both knockdown decreases sialidase activity and neutrophil adhesion. **A:** NEU1 (**A, left**) and NEU3 (**A, right**) siRNA knockdown were both successful in GalTKO.hCD46 pAECs (beta actin controls left and right below, left lane scramble, right lane knockdown). Representative densitometry seen in **B**. **C:** Sialidase activity of GalTKO.hCD46 pAEC lysates was measured after siRNA knockdown of pig NEU1 or NEU3 or both in pAECs. **D:** IL-8 activated (1nM, 30min) human PMN were flowed over TNF α activated (25ng/ml, 4 hr) GalTKO.hCD46 pAECs monolayers at 1 dyne/cm² for 20 minutes. PAECs were transfected with either a scramble siRNA, NEU 1, 3 or both siRNA. Results were expressed as the mean and standard deviation of the number of PMN per image field from two independent experiments. *: p<0.0001 vs scramble controls.

Discussion

Here we show that pig EC have roughly three-fold greater endogenous sialidase activity when compared to human EC. At the transcript (mRNA) and protein levels, NEU1 predominates in pig lung before and after ex-vivo xenoperfusions with human blood, followed by NEU3 and with a minor contribution from NEU2. There is an increase in NEU3 protein expression after 4 hours of perfusion and an even larger increase of NEU1. The difference between NEU1 and NEU3 transcript and protein levels at 4hr is probably related to the influx of human cells in the pig lung tissue, since the staining antibody is presumed to react with both species. In functional experiments, inhibition of endothelial and to a lesser extent of PMN sialidase activity blunts PMN adhesion, suggesting a major contribution of endothelial NEU activity. Accordingly, knockdown of NEU 1 and NEU3 using siRNA significantly impairs PMN adhesion. These results identify for the first time an important role for cellular desialylation in xenogeneic adhesion.

Expression of sialidases has been described in multiple cell types, tissues and organs.³ Our expression results are in line with that of other groups. In human tissues, NEU1 shows the strongest expression at almost 10-20 times greater than NEU3 and NEU4, whereas NEU2 is extremely low. In the lung, NEU1 is the main sialidase expressed in human lung microvascular endothelia¹⁶. NEU1 expression has been found to be increased in lung epithelial and EC and fibroblasts in patients with idiopathic pulmonary fibrosis compared with healthy control human lungs.¹¹⁷ We see a similar increase in NEU1 protein expression in our model which may reflect a progression of inflammation/disease.

PMN sequestration is a consistent feature associated with xenogeneic lung injury. Our findings demonstrate an important role for sialylation and for NEUs in this model, since both pharmacologic and genetic approaches to inhibit NEUs attenuate PMN adhesion, with contributions for both the endothelial and to a lesser extent PMN NEUs. The largest attenuation of PMN adhesion was from DANA pre-treatment or knockdown of NEU 1 and 3. When pAECs were pre-treated with Relenza there was a decrease in neutrophil adhesion, however, to a lesser degree than is seen with DANA which is likely due to the fact that DANA inhibits all NEUs while Relenza mostly targets human NEU3. The fact that the largest decrease in activated PMN adhesion was seen after DANA treatment of activated pAECs or NEU1 and NEU 3 knockdown shows that NEU activity on the endothelium plays a primary role in PMN adhesion after activation. However, treatment of PMN with DANA or Relenza had a similar effect on PMN adhesion, suggesting a primary role for NEU3 in neutrophils. NEU3 is known to be expressed at the membrane level in physiologic conditions, however additional NEU3 expression, possibly by translocation from an intracellular pool, could also be triggered by PMN activation. Although we did not confer this result, our data imply that Relenza inhibits pig NEU3, or other pig NEUs. Since our experimental design was not set out to answer such mechanistic questions, further studies are needed to determine the precise source, type and regulation of NEU activity. We conclude that inhibition of NEU activity decreases PMN adherence under xenogeneic conditions, and by implication that desialylation may act as a major mechanism of PMN sequestration *ex vivo* and *in vivo*, as it does *in vitro*.

Based on our working hypothesis that desialylation drives PMN sequestration to a biologically important degree, and the presumption that PMN are primarily pathogenic in

this context, future experiments will target NEUs with inhibitors. We can investigate the evolution of the Sia expression profile during our ex-vivo xenogeneic lung perfusions, although this is not easy technically. If PMN within the human blood perfusate have been desialylated, serial samples from different time points should demonstrate progressively increased staining with Peanut Agglutinin lectin (PNA) by flow cytometry, which stains for the galactose underneath the Sia after desialylation, as well as Sambucus Nigra lectin (SNA), which stains for Sia in an α 2,6 linkage and Maackia Amurensis lectin (MAA), which stains for Sia in an α 2,3 linkage. Unfortunately, the majority of PMNs are rapidly sequestered in the perfused organ, decreasing the value of this approach. We can also see if the pig lung tissue and the cells that have adhered to porcine endothelium have been desialylated by examining pig lung tissue sections by immunohistochemistry using the lectins mentioned above (methods may not be sufficiently sensitive, but worth looking). This will help give us a clearer picture of what occurs during our lung perfusions when it comes to changes in sialic acid. However, definitive assessment of the role of NEU during lung perfusion will require the perfusion of pig lungs by human blood in presence of selective and potent NEU inhibitors. Our paired lung model is well suited to determine the specific effect of a treatment by adding the inhibitor of interest on one side only of a parallel circuit. The assays described above should be helpful in determining changes in the Sia expression profile in presence or absence of the inhibitor, as correlates to directly testing whether PMN sequestration is inhibited by NEU inhibition.

Others have found similar results to ours that in human cells when NEU1 and NEU3 have siRNA mediated knockdown there is a sialidase activity decrease of

approximately 65 and 17%. Although pig EC have a larger amount of sialidase activity they can be decreased to similar levels of what is seen in human cells.¹⁶ We have explored how knockdown of NEU expression affects neutrophil adhesion. In future experiments we can also see if knockdown affects platelet adhesion which is another limitation that we see in our *ex-vivo* xeno-lung perfusions. It is easier for us to target the pig side vs. the human side with siRNA, however, the use of inhibitors is an easier and broader approach. Researchers have found that NEU1 siRNA inhibited cancer proliferation and induced apoptosis and that it also suppressed invasion of human ovarian carcinoma cell lines (OVCAR3 and SKOV3).¹¹⁸ It has also been found that when LPS binds to TLR4 this induces NEU1 which then causes activation of NF κ B and the production of nitric oxide and IL-6 and TNF α in macrophage cell lines. Mice that have knockdowns of cathepsin A and NEU1 deficiency respond poorly to LPS induced pro-inflammatory cytokines. This shows that TLR activation is dependent on NEU1 activity in macrophages.¹¹⁹ In relation to lung injury it has been found that desialylation through neuraminidase treatment increases the likelihood of LPS-induced lung injury due to enhanced PMN recruitment, edema formation and endothelial cell apoptosis.¹²⁰ These results show that NEU knockdown can have many different effects and can be explored in other avenues of our perfusion system.

Pharmacologic treatment of pAECs or PMN to decrease sialidase activity will be explored because it can be easier to use drug treatments than changes in genetic makeup in our experiments. There is an increase in sialidase activity on the surface of PMN after they are activated, including with IL-8. This increase in sialidase activity also caused increased human PMN adherence in-vivo. Our collaborators have found that the increase

in PMN sialidase expression was blocked by incubation with DANA.^{6,93} We wanted to further expand on this idea and see if blocking sialidase activity on pAECS or human PMN could prevent neutrophil sequestration of activated PMN and found that both these situations do decrease neutrophil adhesion. The next experiment to try would be to use DANA or Relenza as a drug treatment in our ex-vivo xenogenic lung perfusions and see if PMN adhesion/sequestration is decreased. There has been preliminary efforts to target NEUs using oseltamivir (Tamiflu) in *ex vivo* pig lung perfusions (unpublished observations). However, PMN sequestration was not prevented, perhaps because oseltamivir has poor inhibitory activity toward eukaryotic NEUs. In preparation to translate our promising *in vitro* findings with DANA to the *ex-vivo* lung perfusion model, as part of this thesis project, we performed preliminary investigations using *in vitro* assays with whole blood. We were frustrated by the observation that the addition of DANA to whole human blood was pro-thrombotic (data not shown). Dosage curves need to still be done with DANA as well as testing to see if Relenza has the same pro-thrombotic effect. Novel selective, clinically applicable NEU inhibitors may become available soon.^{121,122} Notably, Relenza is used in an aerosol formulation clinically and such formulation may provide a safe and efficient delivery method to limit cellular desialylation in lung organs.

In conclusion, pig EC have more sialidase activity compared to human EC. NEU1 is the predominant sialidase expressed in pig lung tissue during our ex-vivo xeno lung perfusions and it is increased following a 4 hour perfusion with human blood. NEU3 is also relatively abundant 4 hours after lung perfusion. NEU1, 3 or NEU 1/3 knockdown in pAECs or sialidase inhibitor treatment prevents a large portion of the increased

neutrophil adhesion after pAEC activation and PMN activation. As such, this work identifies sialidase activity/expression inhibition as an important treatment that could prevent xenograft neutrophil sequestration.

Chapter 3: Galectin-3 mediates xenogeneic PMN adhesion

Introduction

As discussed in previous chapters, mammalian neuraminidases (called sialidases by convention) cause desialylation of the surface of EC and PMN (PMN) (as well as other cell types). We showed in Chapter 2 that pig EC exhibit higher sialidase activity compared to human EC, and that inhibition of endothelial cell sialidases using pharmacologic (DANA, Relenza) or genetic (siRNA for pig NEU1 and NEU3) approaches significantly inhibit the adhesion of human PMN to pig EC. In this chapter, we investigate further the mechanisms of Sia-related PMN adhesion.

There is evidence that sialidases may exert their effect *in cis* (inducing desialylation of glycoconjugates on the same cell)⁶, or *in trans* (cleaving sialic acid (Sia) residues from neighboring cells)^{6,93}. By cleaving Sia residues on EC, PMN, or both, we determine the role of sialidase activity from each cell type on PMN adhesion. Furthermore, one of the mechanisms by which sialic acid cleavage has been shown to affect cell-cell adhesive interactions is mediated by the binding of galectins⁹⁷. When Sia is cleaved, underlying galactose moieties are exposed in the context of underlying glycoconjugates. Therefore, we characterized the expression of selected galectins in our *ex vivo* lung perfusion model. Finally, by using the competitive inhibitor N-acetyllactosamine (LacNAc) or galectin blocking antibodies, we ask whether galectins are involved in xenogeneic PMN adhesion.

Galectins are carbohydrate-binding proteins named based on their ability to bind galactose residues⁹⁷. Typically, galectins bind β -galactose-containing glycoconjugates. Galactose is displayed at lower densities on the surface of quiescent, healthy cells, but is

exposed under the action of sialidases. Galectins all share a conserved carbohydrate-recognition domain (CRD). There are three types of galectins. The first are prototypic galectins that contain single CRD monomers that non-covalently dimerize to form homodimers. The second are chimeric galectins, in vertebrates only gal-3, which has a single CRD and a large amino-terminal domain which contributes to self-aggregation. This creates prevalent multimers that can then cross link glycoproteins on the cells on which it binds, a property that may lead to intra-cellular signaling in those cells. The third type is tandem repeat galectins that contain at least two non-identical CRDs connected by a linker domain. Galectins regulate cell adhesion and cell signaling by binding to glycan ligands on glycoprotein receptors on the cell surface. Their ability to dimerize or oligomerize allows cross-linking of receptors that control many cellular functions. Based on their structure, tandem-repeat galectins result in the formation of higher-order multimers with increased valency, called the galectin lattice. Galectins can also bind other substrates such as galectin 1 and 3 binding to integrins, fibronectin and LAMP-1. The precise glycan structures on these other substrates that are recognized by galectins are not fully understood.³ Soluble di-saccharide ligands such as lactose, LacNAc and thiodigalactosides exhibit significant binding affinity for galectins with strong inhibitory potency. These compounds can provide effective inhibition of pan-galectin activity, as well as inhibition of individual galectins.^{123,124,125}

There are 12 known human galectins.³ Galectins have been implicated in both innate and adaptive immunity mechanisms.³ Examples of galectins that are known to be involved in inflammation generally, and in lung injury specifically, include gal-1, 3, 8 and 9. These galectins are differentially expressed in various tissues and cells including EC,

alveolar macrophages and PMN.⁴⁹ An increase in the protein level of gal-1 can promote PMN recruitment in a model of acute inflammation induced by zymosan. The authors found that an intra-peritoneal injection of soluble gal-1 increased local PMN recruitment.⁹⁹ Others have found that increase in the protein level of gal-1 can decrease PMN recruitment under flow conditions, which inhibits migration, capture, rolling and adhesion on human endothelial cell monolayers.¹⁰⁰ Therefore the role of gal-1 in inflammation is complex and model dependent.

Gal-3 was also shown to affect PMN adhesion/recruitment. Gal-3 expression is up-regulated during inflammation/infection.^{126,127,128,129} Gal-3 binds to primed PMN, inducing L-selectin shedding and IL-8 production by these PMN which increases their migration and activation.¹⁰¹ Addition of gal-3 promotes adhesion of resting PMN to human EC *in vitro* under static conditions. Sato et al. believe this is due to gal-3 on the endothelium directly cross-linking the PMN to the endothelium, a phenomenon that is dependent on gal-3 oligomerization, based on their observation that truncated gal-3 (lacking N-terminal tandem repeating domain) did not cause PMN adhesion.¹³⁰

As mentioned not only do galectins play a role in inflammation but also specifically in lung injury/inflammation. Galectin-9 diminishes acute lung injury by expanding CD14⁻CD11b⁺Gr-1⁺ plasmacytoid dendritic cell-like macrophages. It does this by downregulating TLR4 and TLR2 and therefore suppressing macrophage function to release pro-inflammatory cytokines.¹³¹ Researchers have also found that after irradiation-induced lung injury there is an increase in the tissue level of gal-3. This was associated with increased gal-3 staining at the surface of newly formed alveolar epithelium. These results suggested that increased release of gal-3 during irradiation induced lung injury and binding

to alveolar epithelial epithelium may promote epithelial expansion and differentiation during injury and repair.¹³² In mice deficient in gal-3, transforming growth factor (TGF)- β and bleomycin-induced lung fibrosis was dramatically reduced.¹²⁹ When an inhibitor of gal-3 was added *in vitro* and *in vivo* late-stage progression of lung fibrosis was diminished. In the bronchoalveolar lavage and serum in patients with idiopathic pulmonary fibrosis there was increased expression of gal-3.¹²⁹ These data show that galectins do play a role in lung injury/inflammation. However, galectins have never been explored in lung xenotransplantation.

A main limitation of pig lung xenograft injury is the fact that leukocyte (specifically PMN) and platelet sequestration occur in our *ex-vivo* human blood perfusion system within minutes, and the mechanisms driving these adhesive interactions are largely unknown. Importantly although the sequestration is delayed in GalTKO.hCD46 lungs, PMN infiltration and platelet sequestration always occur.^{30,31} They are not prevented by antihistamines, pulmonary vascular macrophage depletion using liposomal clodronate, thromboxane synthase inhibition, pan-selectin blockade (with PSGL-1), Mac-1 blockade, alone or in combination. Therefore, it is important for this limitation to be understood, in order for the contribution of these cells to lung injury to be resolved. This study investigates the role of galectin binding, a direct consequence of desialylation, as one primary mechanism that we hypothesized might be causing this phenomenon. We demonstrate that when sialic acid is cleaved from pig EC and/or human PMN this increases human PMN adhesion, and that PMN adhesion is further increased when PMN or EC are pre-activated. We show that galectin binding is the principle mechanism behind this observation. These

findings provide the first mechanistic explanation of how galectin binding, consequent of sialic acid cleavage, affects human PMN adhesion during xenotransplantation.

Materials and Methods

Animals

Pigs of wild type, or genetically engineered genotypes lacking the alpha-Gal epitope (GalTKO), with additional transgenic expression of human membrane cofactor protein (hCD46) (GalTKO.hCD46) were provided by Revivicor (Blacksburg, VA, USA). All procedures were approved by The Institutional Animal Care and Use Committee (IACUC) at the University of Maryland, School of Medicine, and were conducted in compliance with The National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Lung harvest, *ex vivo* perfusion and transplantation procedures

Anesthesia protocol and the harvest of the lung xenograft were performed as previously described¹¹⁴. Prior to flushing the lungs, 1-benzylimidazole (5 mg/kg BW; a thromboxane synthase inhibitor; Sigma–Aldrich, St. Louis, MO) and synthetic prostaglandin I₂ (0.03 mg/kg BW; Flolan; GlaxoSmithKline, Research Triangle Park, NC) were administered intravenously and allowed to circulate for several minutes. The right and left lungs were separately perfused via the pulmonary artery using side-by-side circuits fashioned from silicon tubing and polyurethane connectors as previously described²⁵. Transgenic pig lungs were perfused with heparinized fresh human blood in an *ex vivo* circuit as previously described.^{30,31,114}.

PMN isolation and labeling

Venous human blood was collected into EDTA tubes from healthy volunteers under a protocol approved by the University of Maryland Baltimore Institutional Review Board. PMN were isolated by density gradient centrifugation ($500 \times g$, 35 min, brake off) using Lympholyte®-poly Cell Separation Media, (ACL5070 Cedarlane, Burlington, ON, Canada). The PMN layer was collected and residual red blood cells were lysed using RBC lysis buffer (Roche Applied Science, Basel, Switzerland). PMN were washed ($400 \times g$) with Hank's Balanced Salt Solution (HBSS) (Gibco-Life Technologies, Waltham, Massachusetts, USA) without calcium chloride and magnesium chloride and then re-suspended in HBSS with divalent cations at 5×10^5 PMN/ml (purity $>86\%$ and viability $>95\%$ as measured by flow cytometry (BD FACSVerse™, Becton Dickinson, Franklin Lakes, New Jersey, USA)). They were then incubated with $0.5 \mu\text{M}$ calcein^{AM} (calcein-acetoxymethyl ester) (Corning, New York, USA) for 30 min with gentle agitation in the dark at 37°C . Labeled PMN were washed two times with HBSS with Ca^{++} and Mg^{++} .

Endothelial cell culture and activation

Primary pig aortic EC (PAECs) isolated from pig aortas were grown in culture medium (10% heat-inactivated FBS, DMEM (1 g/L D-glucose), 5 mM L-glutamine, 110 mg/L sodium pyruvate, 1:200 endothelial cell growth factor). Cells were used after four to ten passages at 80%-95% confluence. Human aortic EC (hAECs) (blood group O or B, Life Technologies, Carlsbad, CA) were grown in their specific growth factor kit (Thermo Fisher, Waltham, MA, USA) and culture medium (Medium 200). Primary

microvascular pig lung EC (pMVECs) were isolated from lung wedge biopsies by collagenase digestion and purified through CD31 coated bead isolation and cultured similarly to pAECs (kind gift from Revivicor).

Static PMN adhesion assay

Wild type (WT), GalTKO or GalTKO.hCD46 primary pig aortic EC (pAECs), human aortic EC (hAECs) or GalTKO.hCD46 primary pig lung microvascular EC (pMVECs) were grown to confluent monolayers in 24 well culture plates. After washing with warm DMEM, calcein^{AM}-labeled human PMN (5×10^5 in warm HBSS buffer with Ca^{++} and Mg^{++}) were added at 10^4 PMN/ml per well and incubated for 30 min at 37°C. After washing unbound PMN with room temperature PBS, attached PMN were quantified fluorometrically (excitation 485 nm, emission 530 nm) using a Spectramax 3000 plate reader (Sunnyvale, CA, USA). PMN–EC adhesion was expressed as percent adhesion using the amount of PMN that adhere without washing for 10^4 PMN/ml to a culture plate. In some instances, endothelial cell monolayers were treated with *Clostridium Perfringens* neuraminidase (NA) (25mU/ml for 30 min) to cleave sialic acid from the cells, TNF α (25 ng/ml for 4 hr) to activate EC, both NA and TNF α , or DMEM as control, and PMN were treated with NA, IL-8 (1 nM, 30 min) or HBSS (with Ca^{++} and Mg^{++}) as control for 30 min at 37°C before incubation with AECs. Cleavage of sialic acids from pAECs or PMNs was confirmed by staining with Peanut Agglutinin Lectin (PNA) using flow cytometry as seen in Figure 3.1.

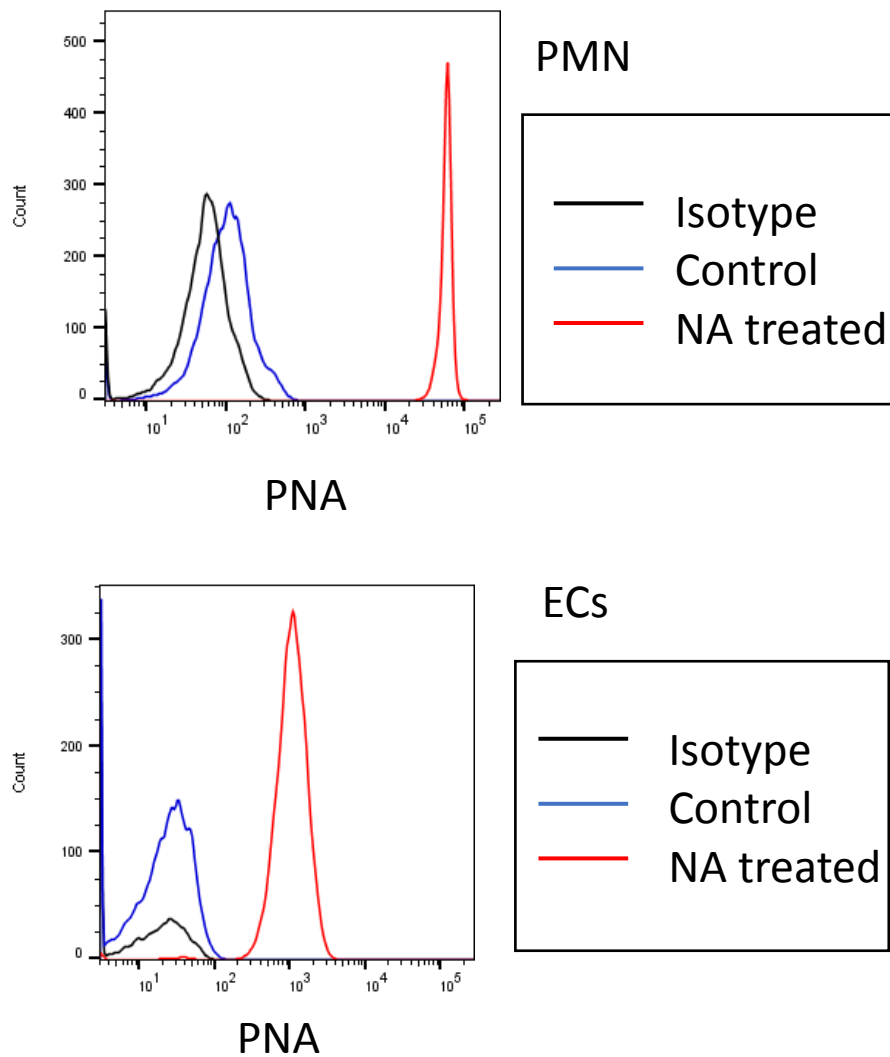


Figure 3.1 *Clostridium Perfringens* neuraminidase (NA) treatment of PMN or EC increased galactose exposure. PMN or endothelial cells were treated with medium (Control) or *Clostridium Perfringens* neuraminidase (NA treated) for 30 minutes. They were then stained with isotype control or peanut agglutinin (PNA) which stains for the exposed galactose after sialic acid cleavage. An increase in PNA staining confirms sialic acid cleavage by NA.

Bioflux flow adhesion assay

Primary pAECs and pMVECs expressing GalTKO.hCD46 were grown to confluent monolayers on microfluidic channel 24-well plates (Fluxion, South San Francisco, California, USA). Monolayers were treated with DMEM, NA (25mU/ml for 30 minutes at 37 °C), TNF α (25 ng/ml for 4 hours at 37 °C) or both. Freshly isolated calcein^{AM}-labeled human PMN (5×10^5 PMN/ml in HBSS buffer (with Ca⁺⁺ and Mg⁺⁺) were incubated with HBSS, NA or IL-8 (1 nM, 30 min, 37°C). After washing, PMN (10^4 PMN in 300 μ L of HBSS buffer) were introduced into the inflow wells of experimental channels and perfused at 1 dyne/cm² at 37°C for 20 minutes using a Bioflux 200 system (Fluxion, San Francisco, CA). Two pictures (proximal and distal views) of 10x field of view for each of duplicate channels were obtained every 60 seconds for 20min by fluorescent microscopy (Zeiss) using 100 ms exposure. Adhered PMN were counted after processing of the data on the Fluxion system using fluorescence intensity. Counts from both fields of view for each channel at each time-point were averaged for all experiments and SD was calculated.

Galectin inhibition

N-acetyllactosamine (LacNAc, 100 nM, 30 min at 37 °C, Sigma, St. Louis, MO) was used as a pan-galectin inhibitor. Gal-3 was inhibited using a mouse monoclonal anti-human gal-3 blocking antibody (B2C10) (Genway Biotech, San Diego, CA) with known cross-reactivity in mouse (and therefore presumed reactivity with pig). Anti-galectin 3 antibody or unconjugated mouse total IgG control (Invitrogen, Waltham, MA) were incubated with pAECs or PMN at different concentrations (5, 10 or 25 μ g/ml) for 30 min at 37°C prior to adhesion assays.

PCR analysis

Total cellular RNA was extracted from lung tissue before (“Pre-perfusion”) and after 4 hours of *ex-vivo* perfusion from pig lungs perfused with human blood (n = 5) using the Qiagen (Valencia, CA) RNA isolation kit (RNeasy Mini kit) according to the manufacturer's protocol. RNA purity was established with the 260/280-nm absorption ratio. 20 µg total RNA was reverse-transcribed using Superscript IV VILO MasterMix (ThermoFisher, Halethorpe, MD). The resulting cDNA was amplified by using real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems/ThermoFisher) and ABI Prism 7900HT cycler. Primers (and some with TET probes) for detection of pig or human galectin 1, 3 and 8, and pig RPL32 (pL32) mRNA were designed and are indicated in Table 3.1. Each galectin primer was designed to specifically recognize pig galectin or human galectin. This was achieved by BLAST alignment of the pig and human nucleotide coding sequences and choosing primers that were in a region of variability between the human and pig species and then verifying species specificity using pig and human samples. Relative gene expression was calculated using the ΔC_t method (C_t gene of interest- C_t housekeeping gene (pL32)), where C_t refers to the cycle number at which the PCR product for a particular gene is detected. The housekeeping gene, pRPL32, was used as an internal control.

Western Blot

EC were lysed directly in flasks using lysis buffer (50 mM Tris-Cl, 5 mM EDTA, 1 mM PMSF, protease inhibitor cocktail, aprotinin, 1% Triton X100 for 1 minute and then scraped from flask. Cell suspensions were centrifuged at for 5 minutes at 4°C and

supernatants were frozen at -80°C until use. Lung tissue specimens were homogenized (using sonication) using the same lysis buffer as above. Protein lysates were denatured in Laemmli loading buffer containing 2-mercaptoethanol and proteins were separated on a 4-15% Tris Gel (Invitrogen, ThermoFisher, Carlsbad, CA) and then transferred to a nitrocellulose membrane (Invitrogen). Blots were then blocked with 3% milk in PBS buffer for 1 hour. Blots were incubated with monoclonal rabbit anti-galectin 1 (Thermo scientific, 1:500), polyclonal rabbit anti-galectin 3 (Aviva 1:500) or rabbit anti-galectin 8 (Aviva, 1:500) antibodies, overnight at 4°C . Each antibody recognizes human and pig based on BLAST sequencing (Gal1: 83% identity, Gal3: 90% identity, Gal8: 93% identity) and testing of antibodies on pig and human specific lysates. Alignment is seen in Table 3.2. After washing with PBS-T-M (PBS+ 0.01% Tween + 0.1% milk) goat anti-rabbit secondary antibody labelled to 680 RD (ThermoFisher) was applied for 120 minutes at room temperature (1: 10,000 in PBS-T-M). Visualization and quantification was carried out with the LI-COR Odyssey® scanner and software (LI-COR Biosciences, Lincoln, NE). Blots were then stripped, washed with PBS-T and then incubated with mouse anti-human β actin (1:200, Santa Cruz, Dallas, TX) for 2 hours at room temperature. After washing with PBS, goat anti-mouse secondary antibody labelled to 790 RD (ThermoFisher) was applied for 120 minutes at room temperature (1: 10,000) and then visualized. Band intensity for NEU was divided by band intensity for β -actin and multiplied by 100 to calculate the ratio of NEU to β -actin.

Statistical analysis

Statistical analyses were performed using the statistical package INSTAT through GraphPad. Unless otherwise indicated, variables were expressed as the mean plus SD. Variables were assessed with the unpaired student t-test. P-values <0.05 were considered statistically significant.

Table 3.1: Oligonucleotide galectin primers and probes used for quantitative RT-PCR

F: Forward primer

R: Reverse primer

Pb: probe

RNA target	Species	Accession Number	Primer sequence
Galectin 1	Pig	NM_00100 1867.1	F: 5'GGTCTGGTCGCCAGCAACCTG 3' R: 5' GGCCACACATTTGATCTTG 3' Pb: 5' CACGTCACCCGCACTT
Galectin 1	Human	NM_00230 5.3	F: 5'GGTCTGGTCGCCAGCAACCTG 3' R: 5' GGCCACACATTTGATCTTG 3' Pb: 5' CACCTCGCCTCGCACTC 3'
Galectin 3	Pig	NM_00109 7501.2	F: 5' CGCTTAACGATGCTTTATCCG 3' R: 5' AGGCATGACTCCTCCAGGC 3'
Galectin 3	Human	NM_00230 6.3	F: 5' CGCTCCATGATGCGTTATCTG 3' R: 5' AGGCACCACTCCCCCAGGC 3'
Galectin 8	Pig	NM_00114 2827.1	F: 5' ATTTCTGAGCAGTTGGAGCCT 3' R: 5' TGCCACACTGTAAGTCCACC 3'
Galectin 8	Human	NM_00649 9.4	F: 5' CGTGGGCATGTTCTTAGTG 3' R: 5' TGCTCTTGGTGTAGACAG 3'

Table 3.2 Alignment of Galectin antibodies to pig

Red: Differences in AA between peptide and pig sequence

Gene	Type of antibody	Alignment
Galectin 1	Synthetic Peptide	Peptide:EQREAFPFQPGSVAEVCITFDQANLTVKLPDGYEF KFPNRLNLEAINY Pig: EQREAFPFQPGSVVEVCISFGQTDLTIKLPDGCEF SFPNRLNLEAIEY
Galectin 3	Synthetic Peptide	Peptide:GASYPGAYPGQAPPGAYPGQAPPGAYPGAPGAYP GAPAPGYPPGPG Pig: GASYPGTYPGQGPPGAYPGQAPPGAYPGQAPGAYP GATAPGYPPGPG
Galectin 8	Synthetic Peptide	Peptide:FPFSPGMYFEMIIYCDVREFKVAVNGVHSLEYKHR FKELSSIDTLEINGD Pig: FPFSPGMYFEMIIYCDVREFKVAINGVHSLEYKHR FRELSNIDTLEIDGD

Results

Cleavage of sialic acid from EC and PMN increases cellular adhesion

We used the static adhesion assay developed in Chapter 2 to study the role of cellular sialylation on PMN adhesion. EC or PMN were treated with *Clostridium perfringens* neuraminidase (NA) to cleave sialic acid from the surface of cells. Adhesion of human PMN to hAECs, pAECs or pMVECs was minimal without cell treatments (Figure 3.1, *left*, black bar). Pretreatment of ECs, or PMN, with NA, significantly increased PMN adhesion to both human and porcine ECs, with no or little additive effect when both cell types were treated with NA (Figure 3.2, *left*, grey bars).

Next we asked if cellular desialylation would also regulate cell adhesion in the context of pro-inflammatory stimuli. We activated PMN with the chemokine IL-8 and ECs with the cytokine TNF α and treated either or both cells with NA. As expected, activation of PMN and ECs with IL-8/TNF α increased cell adhesion (Figure 3.2, *right*, dark gray bar or black bar). Activation and desialylation (with NA) of EC further increased adhesion (Figure 3.2, *right*, medium gray bar). The highest increase in adhesion was seen when EC were desialylated and PMN were activated (Figure 3.2, *right*, light gray bar). Moreover, adhesion to genetically modified (GalTKO or GalTKO.hCD46) aortic or lung microvascular pig ECs (Figure 3.2C-E) was higher than adhesion to human and pig wild-type aortic ECs (Figure 3.2A-B).

Cleavage of sialic acid increases PMN adhesion under flow conditions

To more closely model *in vivo* interactions between human PMN and pig ECs, the same treatment regimens evaluated under static conditions were studied under flow conditions using pAECs (Figure 3.3 A and B) and pMVECs (Figure 3.3 C and D) from

GalTKO.hCD46 pigs. For both pAECs and pMVECs, adhesion of non-activated human PMN was increased when pig ECs of either phenotype were pretreated with NA (red vs. blue) or when PMN were treated with NA (green vs. blue). As in the static assay, activation of ECs with TNF α (orange) or PMN with IL-8 (purple) increased PMN adhesion compared to non-activated cells (blue). Treatment of TNF α -activated ECs with NA lead to an additional increase in cell adhesion (yellow vs. orange). The highest proportion of PMN adhesion was observed when pig ECs were treated with NA and PMN were activated with IL-8 (pink). Collectively these data indicate that desialylation of pAECs or pMVECs enhances human PMN adhesion to pig ECs, an effect which is amplified when ECs or PMN are also activated.

Blocking galectin binding prevents increased PMN adhesion due to desialylation

To evaluate whether galectin interactions are involved in the increase in PMN adhesion observed after cellular desialylation, we used N-acetyllactosamine (LacNAc) as a pan-galectin inhibitor (Figure 3.4). Addition of LacNAc to untreated resting cells had no effect (Figure 3.4A, grey vs. black). In contrast, incubation of PMN with LacNAc before adhesion to NA-treated GalTKO.hCD46 pAECs completely prevented the desialylation-dependent adhesion (Figure 3.4A, pink vs. red). Similarly, adhesion of desialylated PMN to pAECs was prevented when pAECs were pre-treated with LacNAc (Figure 3.4A, light vs. dark green).

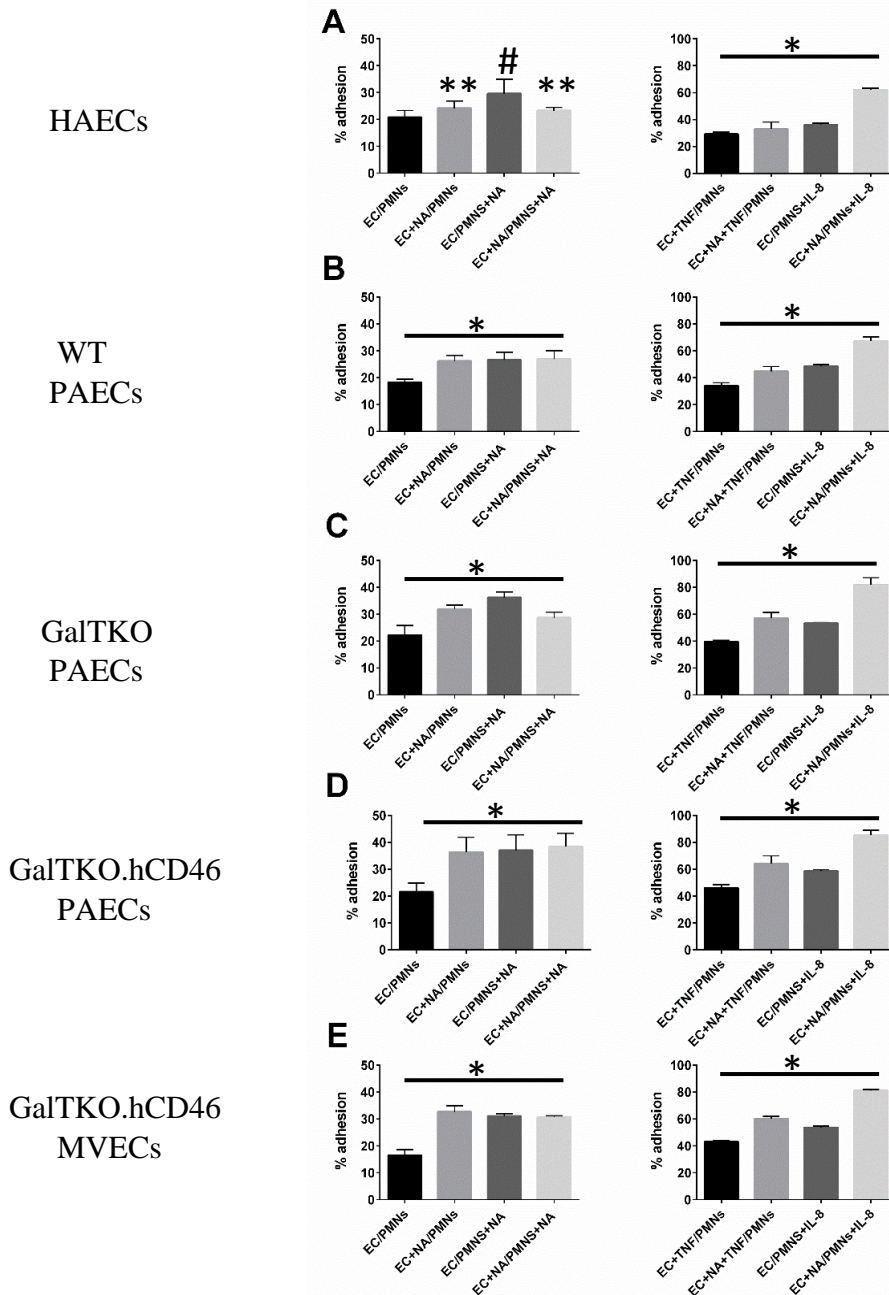


Figure 3.2: Desialylation of endothelial cells or PMN increases PMN adhesion under static conditions. Adhesion of human PMN to human aortic EC (HAECs), wild-type (WT), GalTKO or GalTKO.hCD46 pig aortic EC (PAECs), or GalTKO.hCD46 pig lung microvascular EC (MVECs). PMN and EC were either resting (*left*), or activated with rIL-8 (1 nM, 30 min) and TNF α (25 ng/ml, 4 hr), respectively (*right*). In each panel, PMN or EC or both were pre-treated with *Clostridium perfringens* neuraminidase (NA, 25mU/ml, 30 min) to cleave sialic acid from the cell surface. Results are expressed as the mean \pm SD of the % adhesion from 2-6 independent experiments (each with duplicate wells) for each cell type. *: p<0.0001 vs EC/PMN, **: p=0.02 vs EC/PMN, #: p=0.001 vs EC/PMN.

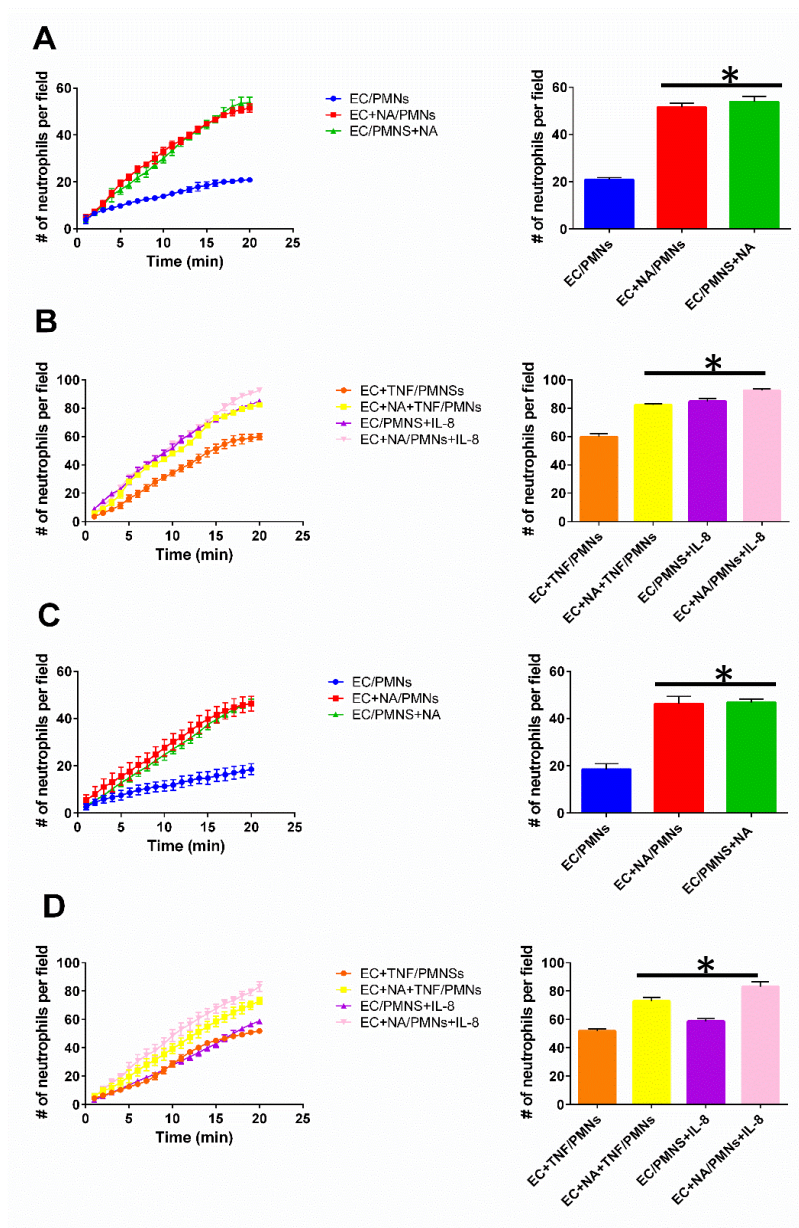


Figure 3.3: Desialylation of endothelial cells or PMN increases PMN adhesion under flow conditions. Adhesion of human PMN to GalTKO.hCD46 pig aortic EC (**A**, **B**), or GalTKO.hCD46 pig lung microvascular EC (**C**, **D**). **A**, **C**: PMN and EC were resting. **B**, **D**: PMN were activated with rIL-8 (1 nM, 30 min) and EC with TNF α (25 ng/ml, 4 hr). PMN, EC or both were pre-treated with *Clostridium perfringens* neuraminidase (NA, 25 mU/ml, 30 min) to cleave sialic acid from the cell surface. Kinetics of adhesion are illustrated on left side of panel. Final counts at endpoint (20 min) are shown on right side of panel. Results were expressed as the mean \pm SD of the number of PMN per image field from two duplicate channels in two independent experiments. *: $p < 0.0001$ vs EC/PMN.

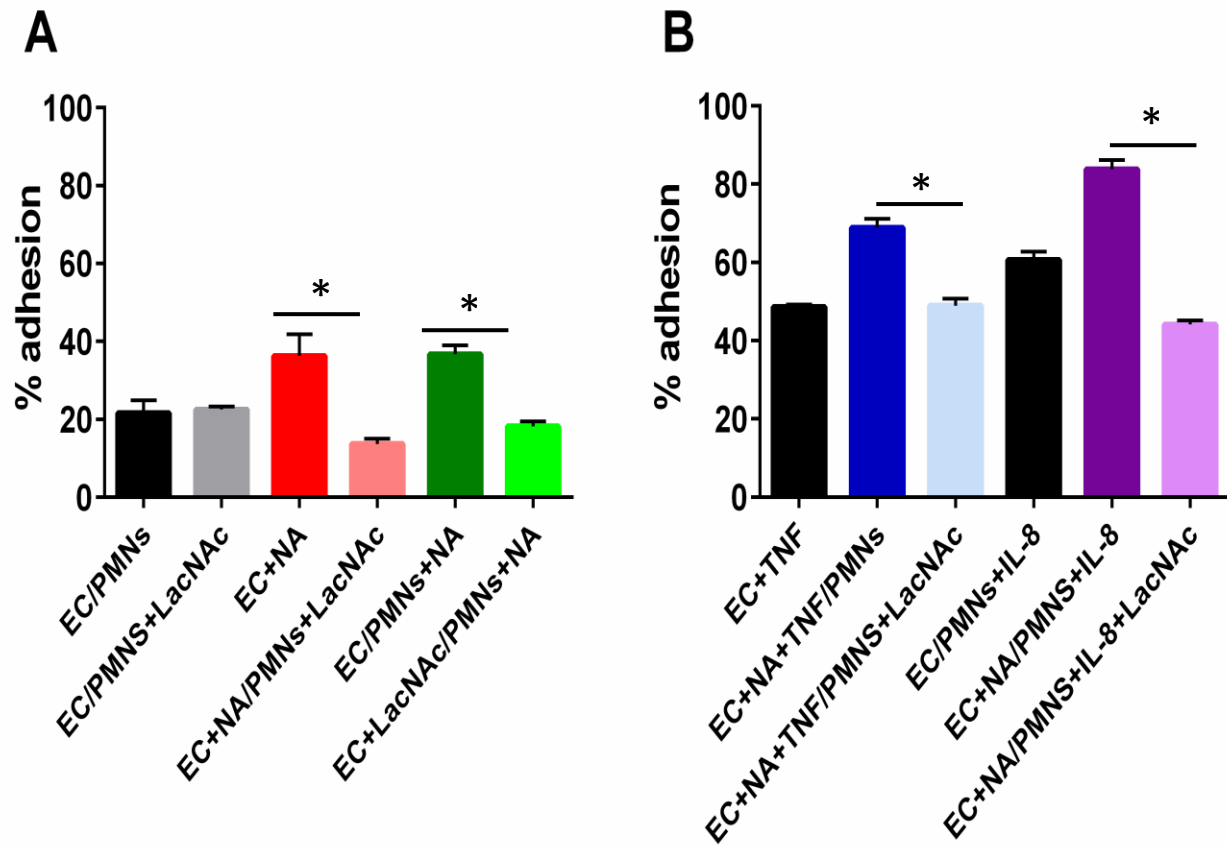


Figure 3.4: Blocking galectin binding using LacNac decreased the desialylation-dependent adhesion of human PMN to pAECs. As seen above, when GalTKO.hCD46 pAECs or PMN are desialylated with NA this increases adhesion of human PMN. We wanted to test to see if this increase in binding is due to galectins. PMN or pAECs were treated with neuraminidase (NA, 25 mU/ml, 30 min) or N-acetyllactosamine (LacNac, 100 nM, 30 min), to prevent binding of galectins to the exposed galactose after sialic acid cleavage. Adhesion of resting (**A**) or activated (**B**) pAECs (TNF α , 25 ng/ml, 4 hr) to resting (**A**) or activated PMN (rIL-8, 1 nM, 30 min) (**B**) was assessed. Results were expressed as the mean \pm SD of duplicate wells from two independent experiments. *: p<0.0001 for LacNac vs. the respective NA-treated cell condition.

Gal-3 is the most abundant galectin in xenogeneic pig lung tissue

Expression of galectin family members varies by tissue and by species. Review of the literature identified that galectins-1, -3 and -8 predominate in hematopoietic stem cell lineage cells, and these family members reportedly have the most effect on PMN and platelet adhesion in other experimental systems. Rapid, prevalent PMN and platelet adhesion are prominent phenomena in our *ex-vivo* xenogeneic perfusion model. Therefore, we set out to determine the relative abundance of those three galectins in our experimental model.

First we used real-time PCR to screen for the presence and determine relative expression levels of galectins-1, -3 and -8 of both pig and human origin by developing species-specific primers for those 6 genes (Table 3.1). Pig lung tissues were analyzed both before and after 4 hours of *ex-vivo* organ perfusion with human blood (Figure 3.5). Before organ perfusion, pig gal-3 showed the highest expression (as reflected by the smallest cycle number (Δ CT) value compared to the pig house-keeping gene L32) (Figure 3.5A). In contrast, pig gal-8 mRNA levels were much lower (10 cycles after the house-keeping gene) and pig gal-1 was expressed at intermediate levels (Figure 3.5A). As expected, human galectins were undetectable in pig lung tissue before perfusion (not shown). We then asked if pig galectins were regulated in response to organ perfusion with human blood (Figure 3.5B). Compared to pre-perfusion levels (fold change), the largest change in gene expression was seen for gal-8 (~6-fold increase), followed by gal-3 (~3-fold increase). Pig gal-1 levels were unchanged after 4 hours of *ex vivo* blood perfusion. Finally, since human blood cells including PMN are sequestered in the pig lung tissue during organ perfusion, we assessed the relative abundance of pig and human galectins at the end of the blood

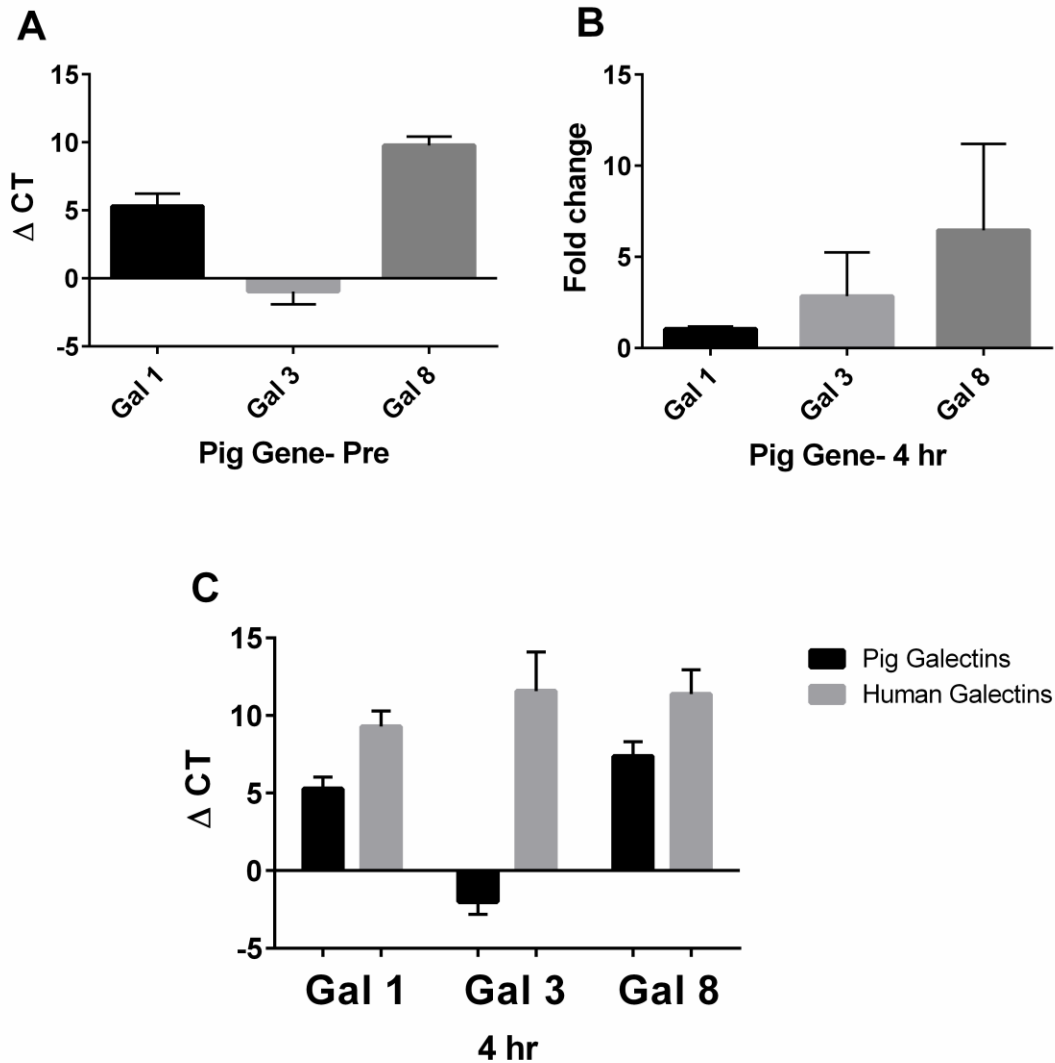


Figure 3.5: Gene expression profile of pig and human galectins-1, -3 and -8 in pig lung tissue during *ex-vivo* xenogeneic lung perfusion. mRNA levels were assessed by real-time PCR in pig lungs perfused with human blood using species specific primers for human and pig galectins. **A:** Expression of pig galectins was measured prior to perfusion with human blood (pre) to determine the relative abundance of galectins at the beginning of the experiment. Human galectins were undetectable at this time-point. Results were expressed as ΔC_T using the pig house-keeping gene L32. **B:** Expression of pig galectins was measured after 4 hr of perfusion with human blood (4hr) to determine if xenogeneic stimulation affects galectins levels. Results are expressed as fold change (4 hr/pre) after normalization to pig L32. **C:** Relative expression of pig and human galectins-1, -3 and -8 after 4 hr of xenogeneic stimulation. Results were expressed as ΔC_T using the pig house-keeping gene L32. Data is shown as the mean \pm SD from 5 independent pig lung *ex-vivo* perfusion experiments.

perfusion (Figure 3.5C). Pig gal-3 mRNA levels were as far the most abundant galectin transcripts in the 4 hour samples, followed by the other two pig galectins. Human galectin transcripts were present after pig lung perfusion with human blood, however, pig galectins were the predominant galectins throughout the experiment (Figure 3.5C).

Next we analyzed the expression profile of galectins at the protein level using immunoblotting analysis of pig lung tissue lysates (Figure 3.6). Using antibodies that recognize both pig and human gal-1, 3 and 8, we confirmed that gal-3 is the predominant galectin found in pig lung tissue (Pre, black). Very little change in total (pig+human) galectin expression was observed over the 4 hour perfusion (4hr, grey). In aggregate, based on the prominent expression of gal-3 in our model, we decided to investigate the role of gal-3 in functional experiments.

Gal-3 blockade decreases the desialylation-dependent adhesion of human PMN to pAECs

Based on above experiments we asked whether increased adhesion of human PMN to desialylated pAECs is primarily attributable to gal-3 binding. We tested PMN adhesion under flow conditions using similar methods as in Figure 3.3. NA-pretreated GalTKO.hCD46 pAECs and human PMN were both incubated with a blocking anti-human gal-3 antibody (known to block mouse gal-3 and therefore presumed to also block pig gal-3 in our system). The anti-gal-3 antibody decreased PMN adhesion to desialylated pAECs in a dose dependent manner, with inhibition beyond the

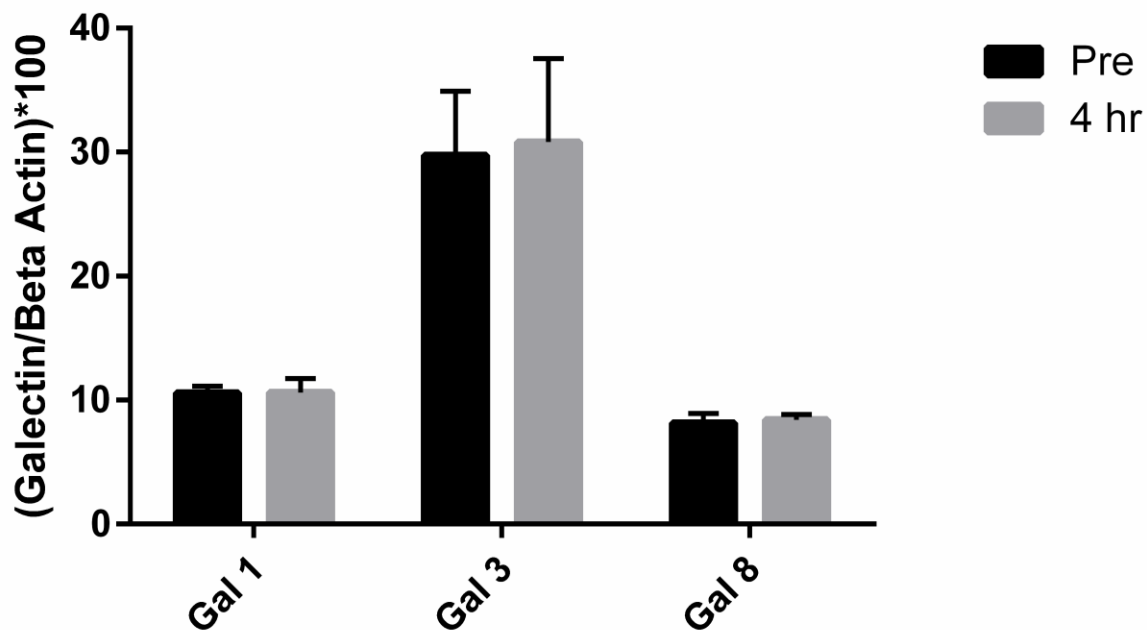


Figure 3.6: Protein expression profile of galectins-1, -3 and -8 in pig lung tissue during *ex-vivo* xenogeneic lung perfusion. Total galectin protein levels were assessed by immunoblotting in pig lungs perfused with human blood using antibodies recognizing both human and pig galectins. Expression of galectins was measured prior to perfusion with human blood (pre) and after 4 hrs of perfusion with human blood (4hr). Band intensity was measured by densitometry and results were expressed as the ratio to the loading control (β -actin) multiplied by 100. Significantly higher expression of gal-3 ($p < .0001$ vs gal-1 or gal-8) lead us to conclude that gal-3 should be the focus of our following experiments. Data is shown as the mean \pm SD from 5 independent pig lung *ex-vivo* perfusion experiments.

level of adhesion seen in absence of desialylation at the concentration of 25 $\mu\text{g/ml}$. (Figure 3.7A).

We also asked the role of gal-3 blockade in the context of $\text{TNF}\alpha/\text{IL-8}$ activated EC and PMN (Figure 3.7B). When desialylated $\text{TNF}\alpha$ -activated pAECs were pretreated with anti-gal-3 antibody (25 $\mu\text{g/ml}$), the desialylation-dependent adhesion of human PMN to pAECs was completely prevented (Figure 3.7B). When pAECs were desialylated and activated and the PMN were activated with IL-8 (conditions that we have shown promote maximal PMN adhesion), again, anti-gal-3 antibody treatment decreased PMN adhesion to levels seen in absence of NA treatment (Figure 3.7B). However, xenogeneic PMN adhesion was not completely prevented. Together, these data provide proof-of-principle that gal-3 mediates desialylation dependent PMN adhesion.

Discussion

In this chapter, we show that sialic acid cleavage from pig EC or human PMN increases human PMN adhesion to pAECs under both under static and flow conditions. This increase in adhesion is amplified by activation of pAECs or PMN. Sialic acid is negatively charged therefore, when it is cleaved from either pAECs or PMN there is now less negative charge to cause cellular repulsion and this could cause an increase in cellular adhesion. In addition, desialylation unveils galactose residues that are capped by sialic acid moieties, and such galactose residues now become available for binding to galectins. We determined that galectin binding was causing the increased PMN adhesion after sialic acid cleavage. Gal-3 is in large amounts in pig lung tissue before and after perfusion by human blood, and an anti-gal-3 antibody decreased the adhesion seen after sialic acid cleavage in

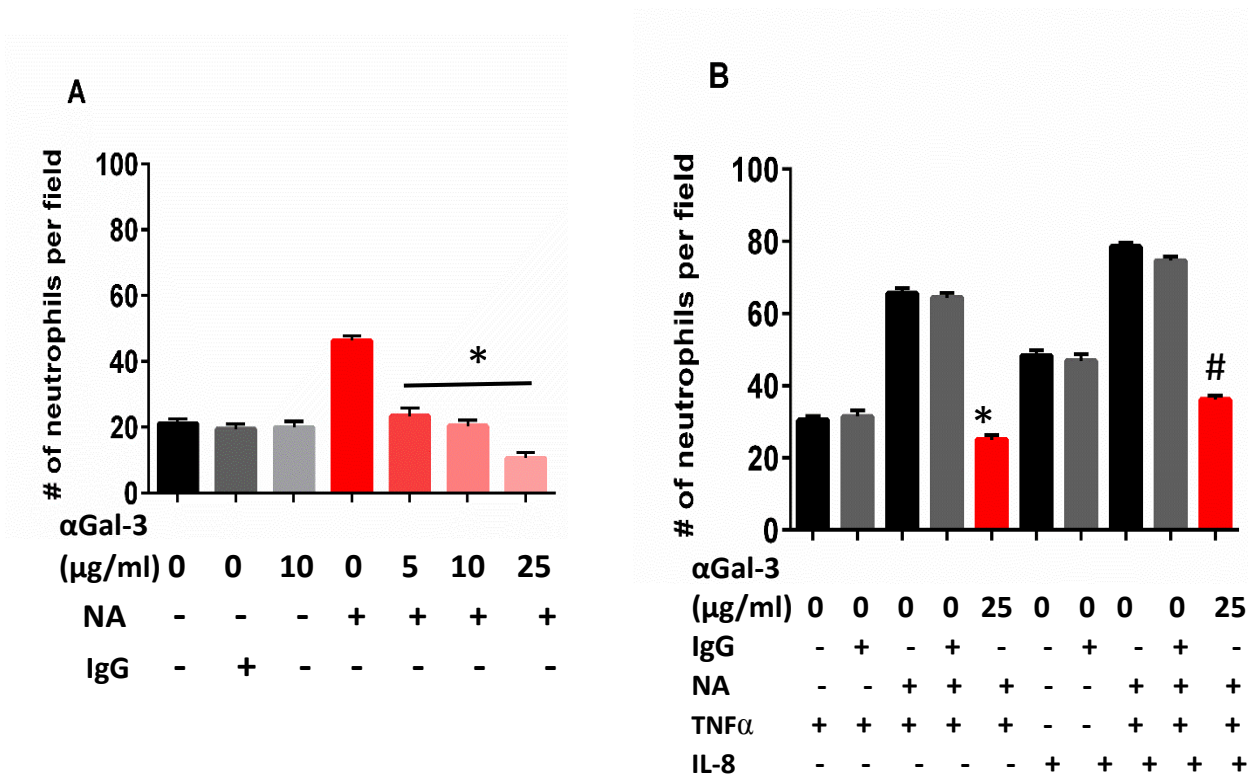


Figure 3.7: Anti-galectin-3 antibody prevents the desialylation-dependent adhesion of human PMN to pAECs. Adhesion of human PMN to pig endothelial cells was studied under flow conditions as in Figures 2 and 3. A: GalTKO.hCD46 pAECs were either treated with DMEM or NA (25 mU/ml, 30 min). Binding of PMN to pAECs was measured in presence of absence of increasing doses of a blocking anti-human gal-3 antibody or IgG control in DMEM or HBSS. B: GalTKO.hCD46 pAECs were all treated with NA and activated with TNF α (25 ng/ml, 4 hr). PMN were incubated with HBSS or IL-8 (1 nM, 30 min). PMN and pAECs were incubated with 25 μ g/ml of anti-gal-3 antibody or IgG control and then washed with DMEM or HBSS. Results were expressed as the mean \pm SD of the number of PMN per image field at endpoint (20 min) from two duplicate channels in two independent experiments. *: p<0.0001 vs. NA treatment, **: p<0.0001 vs. NA and TNF α treatment, #: p<0.0001 vs. NA, TNF α and IL-8 treatment.

the Bioflux assay. Gal-3 blockade also decreased desialylation-dependent PMN adhesion when pig EC and human PMN were activated with TNF α /IL-8.

Gal-3 blockade does not completely abrogate increased PMN adhesion after pAEC desialylation and pAEC/PMN activation. Consequently, other mechanisms will be researched in combination with gal-3 blockade based on previously described mechanisms that regulate leukocyte sequestration. Leukocyte adhesion to EC involves three discrete phases: rolling, firm attachment and migration. Rolling is mediated by selectins, while firm arrest and transmigration both require activated CD18 integrins. One mode of CD18 activation in leukocytes is via activation of outside-in intracellular signaling via various stimuli.¹³³ Once activated in their high-affinity state, integrins bind tightly to complementary receptors expressed on EC, primarily ICAM's, resulting in immobilization of the leukocytes¹³⁴ and subsequent further activation and/or translocation into the tissue. Therefore, projects are underway in our lab to investigate selectin, integrin and gal-3 combination blockade to examine if such a combined approach completely prevents xenogeneic PMN adhesion.

Future experiments to expand on this discovery will test additional galectin inhibitors for gal-3 as well as galectins-1, -8 and -9 (kindly provided by Dr. Gerardo Vasta). These galectin inhibitors will be screened in the Bioflux flow adhesion assay referenced above as well as in a platelet-dependent assay using platelet-rich plasma and whole blood. In particular, we expect to find that gal-8 binds glycans on the platelet membrane, which might promote platelet aggregation, thromboxane generation, P-selectin expression and granule secretion, all of which are prominent features associated with acute

lung injury in our pig lung/human blood xenogeneic perfusion model. Our lab also has an established collaboration and scientific partnership with Glycomimetics, who have provided us with other galectin inhibitors to evaluate in PMN and platelet adhesion flow assays. We also will further investigate the mechanism behind the gal-3 blocking of PMN adhesion by testing if the inhibition is due to blocking of the carbohydrate recognition domain (CRD). This will be tested by incubating different concentrations of biotinylated gal-3 with the anti-gal-3 antibody that we have used. This cocktail will then be incubated on an asialofetuin-coated plate and washed and then the amount of bound gal-3 will be measured. Gal-3's CRD would bind to the asialofetuin. Therefore, if the anti-gal-3 antibody binds to the CRD of gal-3 then it would inhibit gal-3 binding to asialofetuin. The next experiment would be to see if the gal-3 is binding preferentially to the endothelium or to the PMN or to both. So pAECs or PMN would be incubated with biotinylated gal-3 and the amount of gal-3 binding will be measured by flow cytometry. The same experiments will then be performed but with pre-incubation of the gal-3 with the anti-gal-3 antibody we use to see if the antibody will block the gal-3 binding to pAECs or PMN. These experiments will help us define how the gal-3 antibody is blocking gal-3. We will also determine what side the gal-3 is binding to (pig or human or both) and how efficient is the antibody in blocking the binding on each side.

Similar to our research, galectins levels have been measured in many different disease progressions including transplantation. A study found that circulating gal-3 levels were high before transplant and remained elevated in the majority of patients after heart transplant and this was associated with renal dysfunction¹²⁵. We found that gal-3 was a prominent galectin in pig lung tissue. It remains to be determined if this finding is

associated with an increase in circulating gal-3 in blood/plasma, and the potential role of such circulating gal-3 if it exists. Our data so far suggest that pig gal-3 is likely to play a role in human PMN adhesion and perhaps activation, as well as potentially other inflammatory mediators.

Gal-1 levels are reportedly higher in stable liver transplant recipients compared to healthy patients and those with acute rejection after liver transplantation.⁷¹ The authors believe that gal-1 may play a role in suppressing the immune response during liver transplantation.¹³⁵ Tao et al. found that circulating Th17 were increased in the blood of mice with acute rejection after heart transplantation. Galectin-9 could inhibit Th17-mediated acute rejection. The authors believed that use of galectin-9 may be a method to induce tolerance of cardiac allografts.¹³⁶ Hirao et al. have also found that administering galectin-9 protects from inflammation during liver ischemia and reperfusion.¹³⁷ These findings suggest that addition of recombinant galectins (such as gal-1 or -9) could suppress innate and adaptive immune responses in the context of xenotransplantation.

In conclusion, when galectin binding is blocked with LacNAc treatment PMN binding is reduced. There are high levels of gal-3 mRNA and protein expressed in pig lung in our *ex-vivo* pig lung perfusions. Similarly, an anti-galectin 3 antibody decreases binding of activated PMN and desialylated and activated pAECs. As such, this work identifies galectins (specifically gal-3) as an important pathway implicated in xenograft PMN sequestration, and as a candidate for therapeutic interventions.

Chapter 4: Concluding Remarks

Xenotransplantation is viewed as an attractive prospective solution to the human organ shortage for transplantation. Despite genetic modifications of the pig as well as several drug regimens, pig lung injury during *ex-vivo* human blood perfusion and after transplantation into nonhuman primates remains a major problem that has limited survival to just a few days. A rapid innate immune response occurs during a xeno perfusion including infiltration of PMN, monocytes, macrophages and natural killer cells. We hypothesized that the PMN adhesion/sequestration to pig lung endothelium during our *ex-vivo* xeno perfusions is a likely driver of lung injury and aim to understand the role played by those cells by preventing their sequestration. The goal of this thesis was to explore if sialic acid could play a role in xenogeneic PMN adhesion.

Very limited prior *in vitro* studies investigated the mechanisms of human to pig PMN adhesion, with most studies done in the context of absence (WT) or minimal (GalTKO) genetic modifications of the pig organ. Our recent studies using pig lungs expressing multiple transgenes described the consistent sequestration of human PMN in pig lungs, relatively independently of the pig genotype. Overall, the problem of human adhesion to pig EC and organs is not well understood ^{110,111,112}

The sialic acid profile on the surface of cells can dynamically change upon cell activation. Emerging evidence suggests that changes in cell surface sialylation profile can dramatically affect various cellular functions, including PMN adhesion. One pathway mediating change in cell surface sialic acid expression is through the loss of sialic acid by enzymatic cleavage caused by sialidases. One of the mechanisms by which sialic acid cleavage has been shown to affect cell-cell adhesive interactions is mediated by the

binding of galectins⁹⁷. When Sia is cleaved, underlying galactose moieties are exposed in the context of underlying glycoconjugates. We designed our thesis project to address processes related to both sialidases and galectins to learn about the biology of sialic acids in xenotransplantation.

In Chapter 2, we explored if the sialylation status of PMN or EC affect PMN adhesion. We show that pig EC have roughly three-fold greater endogenous sialidase activity when compared to human EC. At the transcript and protein level, NEU1 predominates in pig lung before and after *ex-vivo* xenoperfusions with human blood, followed by NEU3. Our findings demonstrate an important role for sialylation and for NEUs in this model, since both pharmacologic and genetic approaches to inhibit NEU 1 and NEU 3 attenuated PMN adhesion, with contributions for both the endothelial and PMN NEUs. We conclude that inhibition of NEU activity decreases PMN adherence under xenogeneic conditions and that desialylation may drive PMN sequestration *in vivo* and *ex vivo*, as it does *in vitro*.

In Chapter 3, we examined more closely the mechanisms underlying the desialylation dependent adhesion of human PMN to pig EC. We show that sialic acid cleavage from pig EC or human PMN increases human PMN adhesion to pAECs under both under static and flow conditions. We also show that the increase in PMN adhesion is amplified by activation of pAECs or PMN with TNF α or IL-8 respectively, and that such adhesion is further augmented by cellular desialylation. Desialylation unveils galactose residues now able to bind galectin receptors. We set out to ask if galectins could be involved in the xenogeneic adhesion of PMN. First we investigated if galectins are expressed in pig lungs subjected to perfusion with human blood, and found gal-3 is

present in large amounts in pig lung tissue. Second, we asked if galectin binding was causing the increased adhesion of PMN to cultured pig EC using galectin inhibitors. We found that pre-treating with LacNAc (an inhibitor for all galectins) inhibited the increase in PMN adhesion due to desialylation. However, when pAECs or PMN were activated, LacNAc inhibition did not fully abrogate PMN adhesion, indicating that alternative mechanisms, perhaps involving selectin and integrin well-described pathways, are playing a part in PMN adhesion. An anti-galectin 3 antibody decreased the adhesion seen after sialic acid cleavage in the Bioflux assay, identifying gal-3 as a potential therapeutic target.

Our results bring important new insights in the mechanisms of xenogeneic PMN adhesion with important implications for therapeutic targeting of this phenomenon. In theory, one could envision to administer a pan-sialidase inhibitor such as DANA or a more specific sialidase inhibitor such as Relenza to limit cellular desialylation after lung xenotransplantation or xenogeneic perfusion. However, our initial testing of DANA *in vitro* using whole blood suggested that it might be thrombogenic (data not shown), suggesting that selective NEU inhibitors might have a better safety profile for therapeutic use. We showed that PMN adhesion was significantly inhibited by Relenza, a neuraminidase inhibitor targeting influenza neuraminidases, but with also significant potency towards mammalian NEU3. Relenza is used clinically to limit influenza infections, where it is administered in an aerosol formulation (Relenza). This clinical drug could therefore be potentially translated to lung xenotransplantation. Therapeutic benefit may however be limited based on our data showing that NEU1 is the major sialidase in pig lungs, and that endothelial cell NEU1 knockdown was more effective

than NEU3 knockdown in preventing PMN adhesion. Therefore, we began to survey the literature in quest of selective NEU inhibitors, and particularly a NEU1 inhibitor. While there have been recent efforts in the development of novel NEU inhibitors¹²¹, we could not identify selective compounds to inhibit NEU1. Our collaborator Dr. Simeon Goldblum is evaluating a novel potential inhibitor of a NEU1, such inhibitor would have a high potential therapeutic value for lung xenotransplantation as well as in many other pathologies.¹²² Very recently, we identified a monovalent antibody fragment (Fab or scFv) targeting human NEU1. Future experiments will test if this antibody fragment inhibits pig NEU1, and it interferes with xenogeneic PMN adhesion. Use of an antibody as therapeutic agent has potential advantages over a compound because it targets only surface expressed

NEU1 activity, leaving unopposed intra-cellular NEU1 activity with the expectation of maybe less toxicity to the cells.

Based on our data, another approach to limit the desialylation dependent adhesion of PMN consists in targeting galectins. As described above, among galectins previously involved in inflammation (1,3 and 8), gal-3 was as far the most abundant in pig lungs and a blocking anti-human gal-3 antibody significantly prevented PMN adhesion. These findings prompted us to look for therapeutic gal-3 inhibitors, and studies are beginning in the lab to evaluate such inhibitors on xenogeneic PMN adhesion.

In addition to opening new avenues for therapeutic interventions, our work raise many questions about the mechanisms underlying our observations. One question is how much does desialylation play a part in the mechanism behind increased PMN adhesion? Does desialylation and mechanisms, such as selectin or integrin binding, play dual roles

in the increase in adhesion? This can be tested by inhibiting desialylation along with selectin and integrin binding and seeing the effect of PMN adhesion. Another question that is raised is gal-1 and -8 are also present during our *ex-vivo* xeno lung perfusions. What role do they play in our system?

While this dissertation focuses on the adhesion of PMN to pig EC, as explained in the Introduction, many other cellular adhesive interactions occur during xenogeneic lung perfusion. Human platelets, monocytes and NK cells, particularly are rapidly sequestered by pig lungs. The role of cellular sialylation for those cells was beyond the scope of this thesis, but our results in the context of PMN suggest that sialic acid may play a similar role in the adhesion of other cell types. Future experiments may expand on our discovery to test if inhibitors of gal-3 as well as galectins-1, -8 and -9 play a role in platelet adhesion. For this purpose, platelet dependent assays could be developed using human platelet rich plasma and whole blood to determine the effect of galectin blockade on platelet adhesion. We also will further investigate the mechanism underlying gal-3 blockade to determine if the inhibitory effect is mediated at the level of the carbohydrate recognition domain (CRD). Additional studies are needed to determine the cell source of gal-3 and it acts *in cis* or *in trans* in our model. These experiments will inform the development of new therapeutic strategies or additional reagents to target this pathway.

In conclusion, our data shows for the first time that pig EC have more sialidase activity compared to human EC and that pharmacological or genetic modifications of NEU levels prevent a large portion of the increased PMN adhesion seen after pAEC and PMN activation. As such, this work identifies sialidase activity/expression inhibition as an important treatment that could prevent xenograft PMN sequestration. Our data also

show for the first time that desialylation of pAECs increases adhesion of human PMN, and such adhesion is inhibited by a pan-galectin inhibitor, implying galectins as a potentially important mechanism of adhesion. This work further identifies gal-3 as an important pathway implicated in xenogeneic PMN adhesion, and as a candidate for therapeutic interventions to limit the adhesion of PMN in xenotransplantation models.

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