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Molecular Characterization and Inhibition of Antibodies Elicited Against Galactosyltransferase Knockout Pig Xenografts

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by

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A Dissertation submitted in partial satisfaction of
the requirements for the degree
Doctor of Philosophy in Pharmacology

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

FVIII	clotting factor VIII
α-Gal	Galalpha1-3Galbeta1-4GlcNAc-R
GTKO	α1,3-galactosyltransferase gene knockout
nonGal	non-Gal-α-1,3-Gal
Neu5Gc	N-glyconeuraminic acid
B4GALNT2	β1,4 N-acetylgalactosaminyl transferase
HEK	human embryonic kidney
IBMIR	instant blood mediated inflammatory reaction
hHT	human α 1,2 fucosyltransferase
AVR	acute vascular rejection
CDR	complementarity determining region
DAF	decay accelerating factor
DXR	delayed xenograft rejection
FWR	framework region
HAR	hyperacute rejection
HRF	homologous restriction factor
Ig	immunoglobulin
IGHJ	immunoglobulin heavy chain joining region
IGKV	immunoglobulin kappa chain variable region
NICC	neonatal islet cell clusters
NHP	non-human primate
T1D	type 1 diabetes

MFI	mean fluorescence intensity
scFv	single-chain variable fragment
vWF	von Willebrand factor
Fv	variable fragment

ABSTRACT OF THE DISSERTATION

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by

John M. Stewart

Doctor of Philosophy, Graduate Program in Pharmacology
Loma Linda University, June 2015
Dr. Mary Kearns-Jonker, Chairperson

On average, 21 people in the United States die every day while waiting for a transplantable organ. Moreover, at the end of 2014, nearly 130,000 additional individuals were still on the transplant waiting list. Xenotransplantation, the transplantation of cells and/or organs from a member of another species, is both a viable supplement for, and bridge to, allotransplantation. Pigs are the favored organ donor because they: 1) breed and grow rapidly, 2) can be genetically modified, and 3) present few ethical concerns. However, due in part to their phylogenetic distance, several barriers must be successfully overcome before clinical xenotransplantation can be fully realized. Antibody deposition is presently a barrier to successful cardiac, renal, and non-encapsulated islet xenotransplantation; although all xenografts are likely susceptible to antibody-mediated damage. The purpose of this dissertation is twofold: 1) to provide further molecular characterization of the elicited antibodies which mediate rejection of porcine xenografts, and 2) to identify clinically applicable small molecules capable of selectively inhibiting these antibodies. We have found that vascular pig xenografts, and isolated porcine pancreatic islets, both elicit antibody responses encoded by structurally related antibody gene progenitors. These structurally related antibody gene progenitors are both known to

encode human antibodies capable of inhibiting clotting factor VIII (FVIII). Therefore, we subsequently identified FVIII as a novel xenoantigen, and provided a preliminary molecular analysis of the interaction between FVIII and the antibodies elicited after xenotransplantation. Additionally, our analyses strongly suggested that it was feasible for a single reagent to inhibit the majority of the antibodies elicited against transplanted xenografts. Consequently, we screened for, and identified, a single clinically relevant small molecule drug which, when tested *in vitro*, inhibited elicited antibody from multiple xenotransplant settings. However, it was only when utilized in combination with a previously identified experimental reagent that it was possible to reduce the post-transplant antibody to levels below, or comparable to, those present before transplantation. These results challenge long-standing presumptions with regard to the nature of xenoantigens and their role in antibody-mediated xenograft rejection. Furthermore, identification of a clinically relevant selectively inhibitory small molecule should expedite transition into large animal work.

CHAPTER ONE

INTRODUCTION

Introduction to Xenotransplantation

In 1906, shortly after introducing a procedure for arterial anastomosis, Mathieu Jaboulay performed the first organ transplantation (1). However, it was not until after Frank MacFarlane Burnet and Peter Medawar's discovery of acquired immunological tolerance in 1953 (for which they received the Nobel Prize 1960)(2), that successful transplantation was feasible. Thus, the first successful human organ transplantation took place in Boston on December 23, 1954, when a healthy monozygotic twin donated one of his kidneys to his brother (3). In the intervening decades, allotransplantation has been increasingly successful and is currently the favored treatment for end stage organ failure. In fact, it has been so successful that in 2013 in the United States, 28,473 people received at least one donor organ (4). Soberingly, over four times this number of individuals were still waiting for a transplant in February of 2015. On average, 21 people on the waitlist die every day. Moreover, the number of patients included on the waitlist is misleading given that it is limited by the treatment modalities available to sustain patients while waiting for a suitable donor organ. For instance, kidney transplant patients typically account for about 80% of the waitlist because they can be sustained for years by hemodialysis. Moreover, individuals over 65 years of age make up approximately half of the waitlist but receive only one fifth of the available transplants. This growing discrepancy between supply and demand has spurred investigation into alternative treatments for end stage organ failure.

Xenotransplantation, the transplantation of cells and/or organs from a member of another species, is both a viable supplement for, and bridge to allotransplantation. In New Zealand, encapsulated pancreatic porcine islets have recently demonstrated safety and efficacy in treatment for type 1 diabetes in early phase clinical trials (5). Furthermore, non-life supporting models of pig-to-primate cardiac xenotransplantation have demonstrated survival up to almost 600 days (6). Pigs have been considered the favored xenotransplant organ donors for over two decades. Unlike primates (which are phylogenetically closer to humans), they breed and grow rapidly making it possible to meet the growing demand for donor organs (7). Furthermore, pigs can be genetically modified and present fewer ethical concerns. However, due in part to their phylogenetic distance, several barriers must be successfully overcome before clinical xenotransplantation can be fully realized.

Knocking Out the Galactosyltransferase Gene

When he performed his first transplantation experiment in 1906, Mathieu Jaboulay found that humans reject porcine organs almost immediately (1). It wasn't until 1988, that Uri Galili et al. demonstrated old world monkeys and humans differ from other mammals in that they do not express the Galα1,3-Galβ1,4GlcNAc-R (Gal) terminal carbohydrate (8). This led several group in the early 1990's to provide preliminary evidence (9, 10) that anti-Gal antibodies, which make up as much as 1% of circulating IgG in humans (11, 12), were responsible for hyperacute xenograft rejection of porcine xenotransplants (10, 13). Over the next decade, several methods were employed in attempt to prevent hyperacute rejection.

One of the most successful methods utilized pig donors expressing transgenes for human complement regulatory proteins (14-16). These transgenes were considered necessary to compensate for the molecular incompatibility between human serum complement proteins and pig cell membrane-bound complement regulatory proteins. However, enhanced regulation of complement activation, initiated by anti-Gal antibody bound to the graft vasculature, only sustained xenograft survival long enough to endure a newly elicited anti-Gal antibody response (17).

The process of cloning using somatic cell nuclear transfer, first utilized to generate Dolly the sheep (18), enabled the production of pigs which did not express Gal (19-21). These pigs, which did not carry a functional allele of the GGTA1 gene responsible for the synthesis of the Gal epitope (galactosyltransferase gene knockout; GTKO), were generated in 2003 by Revivicor (a spin-out from the UK company PPL Therapeutics which produced Dolly). To the excitement of scientists in the field in 2005, when baboons were transplanted with GTKO pig hearts (22, 23) or kidneys (13, 24) they did not succumb to hyperacute rejection nor did they develop anti-Gal antibodies after cessation of pharmacologic immunosuppression. However, these grafts still showed signs of antibody mediated rejection, albeit with kinetics and pathology consistent with delayed humoral xenograft rejection.

Anti-Non-Gal Antibodies

Even before the era of GTKO pigs was ushered in, anti-pig antibodies directed at antigens other than Gal (non-Gal) were under investigation. In 1998, experimental evidence from Mauro Sandrin's group using GTKO mouse recipients demonstrated that

anti-non-Gal antibodies could also mediate damage to xenografts (25)[Confirmed in (13, 26, 27)]. Additionally, work performed by Leo Buhler and David Cooper in 2003 provided early evidence that antibodies against non-Gal antigens elicited in baboons after cardiac xenotransplantation were not donor specific (28) [Confirmed in (29)].

In 2006, pre-existing anti-non-Gal antibodies were characterized as distinct from antibodies directed against human allografts (30, 31). Thus, sensitivity to allotransplantation would not preclude xenotransplantation, nor would sensitivity to xenotransplantation preclude allotransplantation. Therefore, xenotransplantation could be utilized as a short-term bridge to allotransplantation or, eventually as a long-term treatment option for patients with high alloantibody titers.

Fortunately, it is well documented that the majority of individuals have anti-non-Gal antibodies which pre-exist at levels too low to initiate xenograft damage (32, 33). Consequently, newly elicited anti-non-Gal antibodies present the greater barrier to successful clinical xenotransplantation. Work by Muhammad Mohiuddin, using non-life supporting models of cardiac xenotransplantation, best illustrates the impact of elicited anti-non-Gal antibodies on xenograft survival. In 2012, the singular addition of a B-cell depleting anti-CD20 antibody to the immunosuppressive regimen extended median xenograft survival from 8 days to 71 days, now with a maximum survival time of 236 days (34). Furthermore, modifying the pharmacologic method of inhibiting B-cell co-stimulation by T cells extended the xenograft survival of one animal to approximately 600 days at the time of publication in 2014 (6). However, in the context of transplantation, B cell depletion is known to result in a greater risk of infection and infection-related death (35-37).

Non-α-1,3-Gal Antigens

Identification and characterization of the antigens present in GTKO pig tissues which initiate antibody-mediated rejection should expedite the development of strategies to enhance xenograft survival. For instance, the gene which encodes the antigen could be deleted. Alternatively, a soluble version of the antigen could be utilized to selectively inhibit/deplete xenograft-rejecting antibodies and B cells. Moreover, analysis of the functional impact of antibodies which mediate rejection may reveal complement-independent antibody-mediated pathology.

Even before the first knockout pigs were generated, there were several candidate non-Gal antigens. In addition to anti-Gal antibodies, humans are also known to have pre-existing antibodies against N-glyconeuraminic acid (Neu5Gc), α–lactosamine, and the Forssman antigen, among others (38, 39). It was also conceivable that deletion of the GGTA1 gene may have significantly altered glycosylation patterns or exposed internal carbohydrate moieties creating novel immunogens (40). Additionally, early evidence suggested that anti-non-Gal antibodies might bind protein epitopes (28, 41). Over the last decade, several new candidates have been identified while others have been deemed unlikely. However, the identification of the relevant immunogen(s) present on GTKO organs and cells is still an ongoing area of investigation.

After the knockout of the GGTA1 gene, Mauro Sandrin's group was concerned that GTKO pigs might still express residual Gal epitope. GTKO mice had only recently been determined to express the Gal carbohydrate in the form of a glycolipid, named isoglobotrihexosylceramide, due to the presence of a functional GGTA2 gene (40, 42, 43). However, thorough analyses of glycolipids in GTKO pig intestine, pancreas, heart,

and kidney have observed an absence of isoglobotrihexosylceramide present in porcine tissues (44-46).

In 2010, David Cooper's lab investigated the most plausible non-Gal carbohydrate antigens using naïve human, baboon, wt pig, and GTKO pig sera (47). This study found that GTKO pigs did not express the Forssman carbohydrate antigen while anti-lactosamine antibodies were not elicited against GTKO xenotransplants. Thus, neither α–lactosamine nor the Forssman carbohydrate were considered likely non-Gal antigens. However, perhaps the most important finding of this study was that human serum, but not baboon or pig serum, contained antibodies which bound Neu5Gc indicating that Neu5Gc is only pertinent in pig-to-human xenotransplantation.

Initial suspicions that disrupting the GGTA1 gene would alter glycosylation patterns were well founded. Compared to wt pigs, GTKO pigs were determined to have a higher expression level of sialic acid residues, including Neu5Gc (46, 48). By 2013, Joseph Tector's group had generated pigs in which both the GGTA1 and CMAH genes were knocked out, and therefore expressed neither Gal nor Neu5Gc (49). As expected, a smaller proportion of naïve human IgM bound to double knockout pig cells than to GTKO pig cells (50). Furthermore, erythrocytes from double knockout pigs were less susceptible to damage mediated by antibodies in human sera when compared to red cells from GTKO pigs (51). However, erythrocytes from double knockout pigs were more susceptible when using baboon sera because unlike humans, baboons express Neu5Gc. Thus, illustrating that while GGTA1/CMAH double knockout pigs will be important for clinical xenotransplantation, they will likely complicate experiments using non-human primates.

Interestingly, a portion of pre-existing anti-Neu5Gc antibodies also bind fibronectin from GTKO pigs (52). However, not all pre-existing antibodies against fibronectin from GTKO pigs bind Neu5Gc. Fibronectin was first identified as a non-Gal antigen in 2008 by Guerard Bryne and Christopher McGregor (53). They utilized proteomic techniques, including western blotting, 2-D gel electrophoresis, and mass spectroscopy, to identify a number of targets which bound IgG antibody eluted from rejected GTKO pig hearts. Many of the proteins bound by the eluted IgG were deemed unlikely to initiate xenograft rejection given their intracellular location. However, given that fibronectin is an extracellular matrix protein expressed by endothelial cells, antibodies against fibronectin could plausibly initiate xenograft rejection. Importantly, in 2013, further study by Joseph Tector's group revealed that pre-existing human antibodies against pig fibronectin can elicit cytotoxicity of GTKO pig endothelial cells (52).

In recent years, Guerard Byrne and Christopher McGregor have provided evidence that the porcine β1,4 N-acetylgalactosaminyl transferase (B4GALNT2) is responsible for the synthesis of a novel carbohydrate non-Gal antigen (54, 55). This gene was first identified in 2011 using an expression library screening strategy in which mRNA from GTKO pig aortic endothelial cells was expressed in human embryonic kidney (HEK) cells (54). Expression of the porcine B4GALNT2 was identified to enhance binding of IgG from sensitized cardiac xenograft recipients. Further research in 2014 demonstrated that compared to normal HEK cells, HEK cells expressing porcine B4GALNT2 showed increased sensitivity to antibody-dependent complement-mediated cytotoxicity assays which made use of serum from GTKO xenograft sensitized baboons

(55). Notably, absorbing antibodies on GTKO pig aortic endothelial cells beforehand dramatically reduced this effect.

Antibody-Mediated Xenograft Rejection

When pig organs are transplanted into non-human primates, rejection is a complicated process that results from interplay among a multitude of intricately related factors, including: the method and/or site of transplantation [reviewed in (56)], the organ being transplanted [reviewed in (57-60)], the immunosuppressive regimen utilized [reviewed in (61)], the donor genetics [reviewed in (62)], the donor source (63), the remaining molecular incompatibilities, the innate immune system [reviewed in (64)], activation of the adaptive immune system (see below), and coagulative dysregulation [reviewed in (65)]. However, experimental manipulation and histological evaluation of both wt and GTKO vascularized xenografts has indicated that vascular antibody deposition is a prime initiator of xenograft rejection in certain settings (13, 22, 34, 58, 60, 66-68). Although all xenografts are likely susceptible to antibody-mediated damage, there are only a few organs whose survival is currently limited by humoral mechanisms. Generally, xenografts must survive long enough for adaptive immunity to be relevant. Therefore, antibody-mediated rejection of vascular xenografts is currently most pertinent in cardiac and renal xenotransplantation. Additionally, it has recently been accepted that antibody-mediated damage is detrimental to engraftment of non-encapsulated pancreatic islets (26, 59, 69). Therefore, when developing novel interventions designed to enhance xenograft survival and function, an understanding of the mechanisms of antibody-mediated xenograft rejection are of the utmost importance.

The kinetics of antibody-mediated xenograft rejection are dependent on the abundance of graft-reactive antibody present in the recipient. In the absence of immunosuppression, xenotransplantation of wt pig organs results in hyperacute rejection which takes place within minutes to hours (70, 71). This is because pre-existing anti-Gal antibody is exceedingly abundant. In similar circumstances, xenotransplantation of GTKO pig organs only succumb to accumulation of newly elicited anti-non-Gal antibodies within 3-14 days, depending on the sensitivity of the graft (27, 72). Unfortunately, even with a robust immunosuppressive regimen, rejection associated with *de novo* production of anti-pig antibodies (termed delayed humoral xenograft rejection), can take place weeks, or even months after transplantation (68). Thus, suppressing the majority of the adaptive immune system has become the prevailing approach to xenotransplantation. However, the relationship between the rejection kinetics and the abundance of graft-reactive antibody can also be altered by modifying antibody-antigen interactions (73-75), as well as downstream components of rejection (14, 16, 72, 76, 77).

Histologic examination after antibody-mediated vascular xenograft rejection reveals that in addition to vascular antibody deposition there is complement deposition and platelet-rich fibrin thrombosis (22, 78, 79). Of note, serial biopsies of GTKO cardiac xenografts reveal that vascular antibody and complement deposition precede histologic and systemic evidence of damage. Furthermore, the extent of antibody and complement deposition parallels the degree to which indications of damage are apparent.

Vascular antibody and complement deposition cause endothelial cell activation by catalyzing the formation of the membrane attack complex (27, 66, 67, 79) (Figure 1). Regrettably, membrane bound porcine complement regulatory proteins do not adequately

regulate soluble primate complement cascade components [reviewed in (80)]. However, transgenic expression of human complement regulatory proteins (CD59 Figure 1A, CD46, CD55, Figure 1B-C) can raise the threshold of antibody required to elicit complement-mediated vascular damage (14-16). For this reason, it is currently accepted that vascular GTKO xenografts will need to express at least one of these human complement regulatory transgenes.

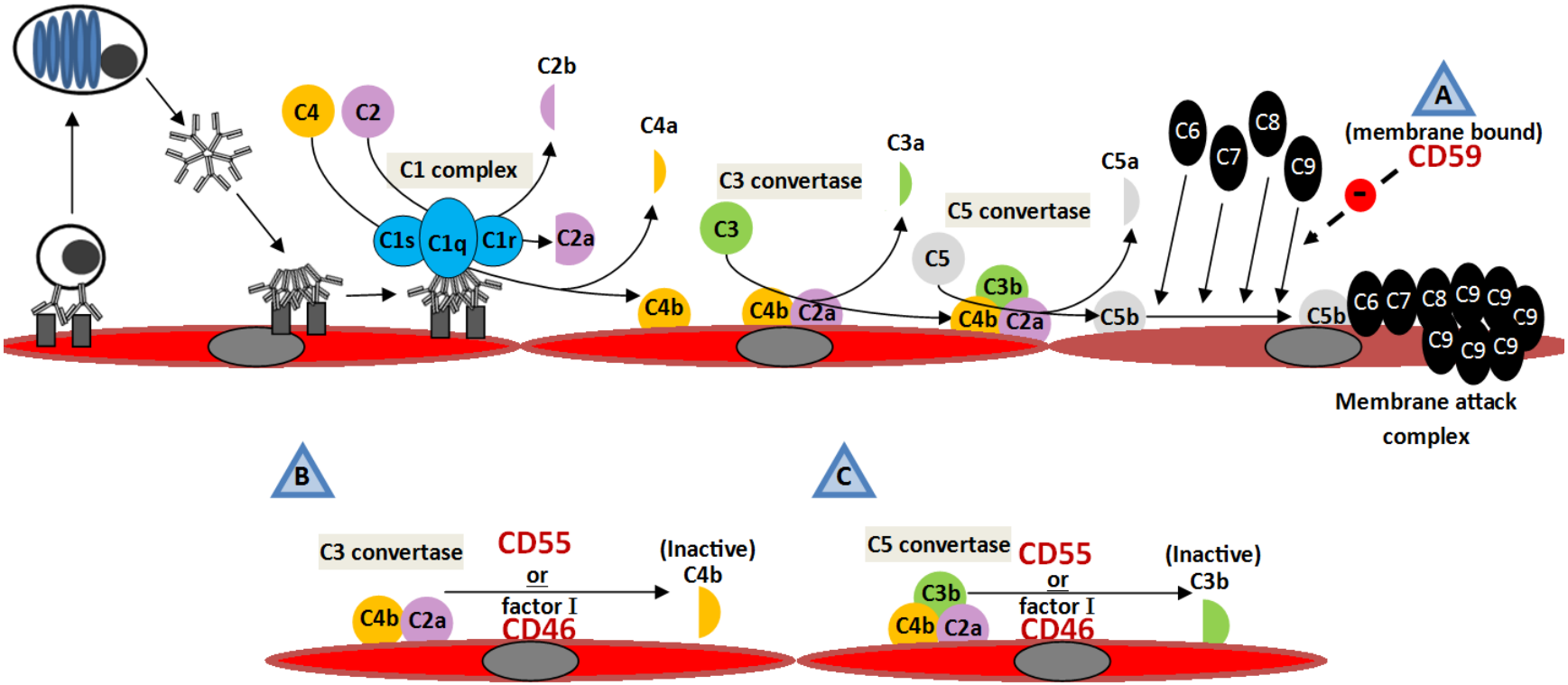
C4
C2
C2b
C1 complex
C1s
C1q
C1r
C2a
C4a
C4b
C3 convertase
C3
C4b C2a
C3a
C5 convertase
C5
C3b
C4b C2a
C5a
C5b
C6
C7
C8
C9
A
(membrane bound)
CD59
C5b C6 C7 C8 C9 C9 C9 C9 C9 C9
Membrane attack complex
B
C3 convertase
CD55
or
factor I
CD46
(Inactive)
C4b
C4b C2a
C
C5 convertase
CD55
or
factor I
CD46
(Inactive)
C3b
C3b
C4b C2a

Figure 1. Vascular antibody deposition causes activation of endothelial cells. Cross-linking of the B cell receptors (membrane bound IgM) upon binding non-Gal antigen causes activation of xenoreactive B cells. Newly differentiated plasma cells secrete soluble antibody. Antibody bound to the graft vasculature ultimately causes the formation of the membrane attack complex which is composed of C5b, C6, C7, C8, and multiple C9 proteins. The membrane attack complex is a transmembrane channel which disrupts the integrity of the cell allowing free diffusion of Ca^{2+} and other ions across the cell membrane. Ca^{2+} signaling triggers activation of endothelial cells. Overexpression of human complement regulatory transgenes on the graft vasculature raises the threshold of antibody required to elicit endothelial activation. **(A)** CD59 inhibits polymerization of C9 required for a functional membrane attack complex. **(B)** The C3 convertase and **(C)** C5 convertase complexes can be disrupted by either CD55 or CD46 (a cofactor for factor I).

Once activated, endothelial cells express tissue factor (TF) and von Willebrand factor initiating fibrin deposition and platelet activation (Figure 2A-B). Even if formation of the membrane attack complex is averted, byproducts of antibody-mediated complement activation (C3a and C5a) can also activate and recruit host neutrophils and monocytes (81). In turn, induction of recipient TF on neutrophils and monocytes can contribute to development of consumptive coagulopathy (Figure 2C) characterized in part by thrombocytopenia (low platelet count), declining fibrinogen (fibrin precursor) levels, and prolonged clotting time (82, 83). It has long been postulated that these effects could be tempered by transgenic expression of human thromboregulatory proteins such as tissue factor pathway inhibitor or thrombomodulin. GTKO pigs expressing such transgenes have only recently been utilized as experimental organ donors. However, they are typically present in the context of complement regulatory transgenes. At this time, *in vivo* experimentation has yet to tease out the contribution to survival with the addition of each transgene. However, recent findings suggest that transgenic overexpression of human tissue factor pathway inhibitor (84), or thrombomodulin (6), can attenuate coagulative dysregulation and may set the threshold for rejection even higher.

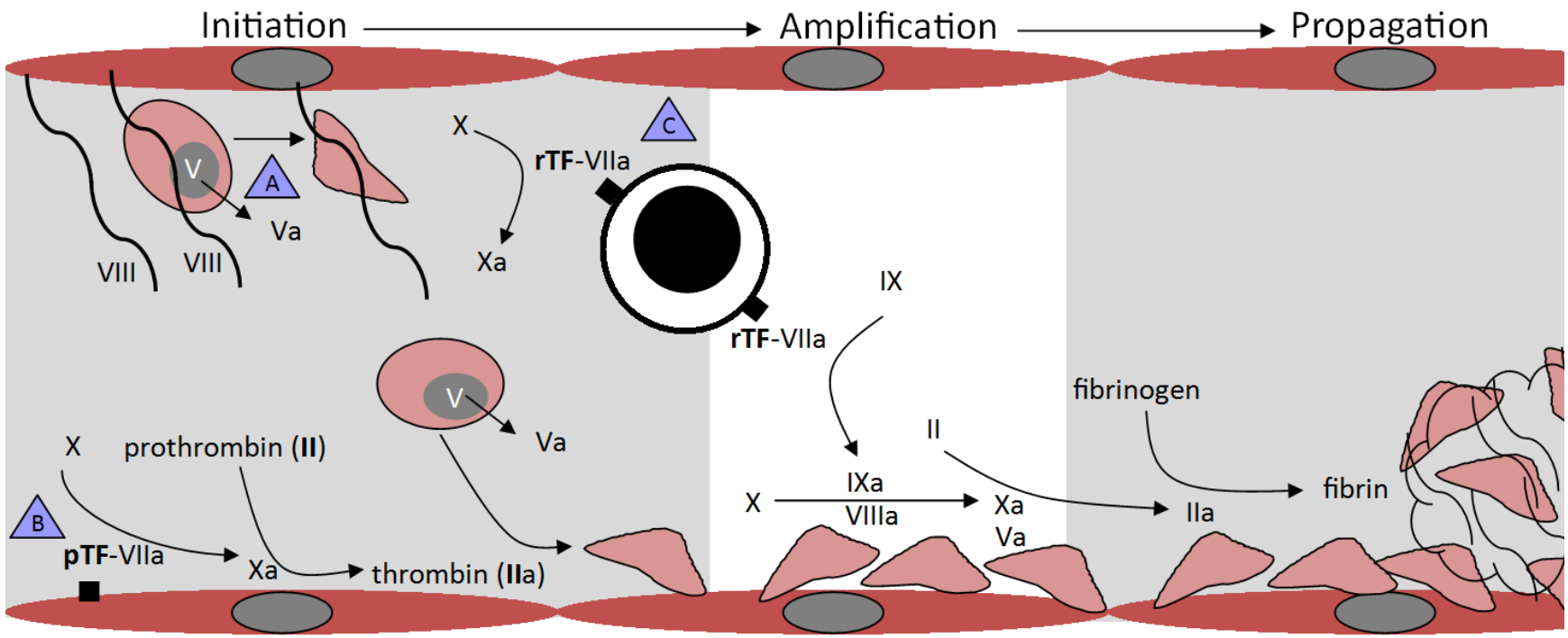
Initiation
Amplification
Propagation
V
A
Va
VIII
VIII
X
Xa
C
rTF-VIIa
rTF-VIIa
IX
V
Va
X
prothrombin (II)
B
pTF-VIIa
Xa
thrombin (IIa)
X
IXa
VIIIa
Xa
Va
II
IIa
fibrinogen
fibrin

Figure 2. Activated xenograft endothelial cells trigger the clotting cascade. Activated GTKO xenograft endothelial cells display porcine tissue factor (pTF) and von Willebrand factor (~~) on the cell surface. In this setting thrombosis can be **initiated** three ways. Initially, pTF cleaves Factor X. Activated factor X (Xa) can convert small amounts of prothrombin (II) to thrombin (IIa). **(A)** Recipient platelets can be activated directly by porcine von Willebrand factor in the absence of shear stress due to molecular incompatibility. **(B)** Platelets can also be activated by cleavage of membrane receptors by IIa downstream of pTF or **(C)** recipient tissue factor (rTF) on activated immune cells. Once initiation takes place the hemostatic cascade both **amplifies** and **propagates** the signal at the site of endothelial activation and vascular damage.

Islet Xenotransplantation

Similar mechanisms contribute to destruction of non-vascular xenografts such as non-encapsulated islets. Anti-pig antibodies, along with TF expressed on isolated islets, induce what is called instant blood-mediated inflammatory reaction (IBMIR)(26, 59, 69, 85). IBMIR dramatically reduces the proportion of isolated islets which remain viable after transplantation. Therefore, a greater number of donors is required to pool enough islets to ensure enhanced control of blood glucose. Graft TF initiates fibrin deposition and platelet activation. Additionally, antibody-mediated complement activation triggers complement-mediated graft damage through the terminal complement membrane attack complex and activation and recruitment of neutrophils and monocytes via complement cleavage products. Just as with vascular grafts, activated recipient neutrophils expressing TF can exacerbate local coagulative dysfunction by contributing to fibrin deposition and platelet activation. Fortunately, recent evidence suggests that expression of complement regulatory (85), and thromboregulatory transgenes (86), diminish IBMIR and enhance survival of islet xenografts. After cessation of IBMIR, destruction of xenotransplanted islets is considered to be a primarily T cell-dependent processes. Of note, several groups have reported xenograft survival over 1 year in non-human primates (86-88)(Table 1) and transplantation of encapsulated pig pancreatic islets is currently in clinical trials (5).

Renal Xenotransplantation

When co-transplanted with pig thymic tissue, GTKO kidney xenografts have a reported record survival time of 83 days (24, 78, 89)(Table 2). However, the record median survival is only 49 days, even though recipients develop donor-specific

hyporesponsiveness. Histologic examination in these cases reveals antibody-mediated glomerulopathy, with thrombosis of the glomerular microvasculature (thrombotic microangiopathic glomerulopathy) and effacement (lengthening/thinning) of podocyte foot processes (90). Pre-existing anti-pig antibodies have recently been reported to disrupt podocyte morphology and function leading to rejection of GTKO kidney xenotransplants (91). Thus, in some settings, antibody-mediated damage more directly effects xenograft function. Approaches which stabilize podocyte integrity or inhibit/neutralize anti-pig podocyte antibodies will likely extend renal xenograft survival.

Cardiac Xenotransplantation

Recent advancements in the field of cardiac xenotransplantation have provided new hope that clinical xenotransplantation is on the horizon (6, 34, 92). The record median survival of non-life supporting cardiac xenografts was previously 96 days with a maximum of 137 days (73)(Table 3.1). This record was set in 2005 utilizing hCD46 transgenic organs. However, refinements of the immunosuppressive regimen, donor genetics, and quality of post-transplant monitoring have lengthened median survival to over 200 days, with at least one graft reported be survive for approximately 600 days at the time of publication in September of 2014 (6)(Table 3.11). At this time, life supporting models of xenotransplantation have yet to catch up and survival is limited to a maximum of 57 days (93)(Table 3.A). However, this record will likely be surpassed following suit with non-life supporting models. In spite of this success, antibody-mediated damage plays a large role in cardiac xenograft rejection and possibly chronic damage of cardiac xenografts. Antibody was found on the explanted xenografts in each of the

aforementioned settings. Additionally, biopsies from surviving hearts still demonstrated vascular antibody deposition, in addition to fibrosis as early as postoperative day 182. This suggests that chronic vascular deposition of antibody, even at low levels, may steadily contribute to loss of function (6). The relationship between antibody deposition and graft damage in these animals is still under investigation.

Table 1 GTKO and record islet xenotransplantation experiments since 2005

	First Author (year)	Donor genetics	Recipient (n)	Immunosuppression (anti-coagulation)	Survival in days (median)
1	Rood (2007)	GTKO adult	Cynomolgus monkey (n=2)	ATG, tacrolimus, rapamycin +/- anti-CD20mAb (n=1), (heparin)	<5
			Cynomolgus monkey (n=3)	ATG, anti-CD154mAb, MMF, CVF (heparin, +/- prostacyclin, aspirin)	5 to 7
			Cynomolgus monkey (n=2)	ATG, anti-CD154mAb, MMF (dextran sulfate, prostacyclin, aspirin)	> 58 (partial function)
2	Rogers (2007)	WT	rhesus monkey (n=3)	No IS [multiple Txs; mesenteric]	78 to 409
3	van der Windt (2009)	WT and GTKO	cynomolgus monkey (n=2)	ATG, anti-CD154mAb, MMF, CS (dextran sulfate, prostacyclin)	7, 20, 31, 46
		CD46	cynomolgus monkey (n=5)	ATG, anti-CD154mAb, MMF, CS (dextran sulfate, prostacyclin)	planned 3 months [87, 91, 92, ~90 (partial)]; selected for long term follow up [396]
4	Thompson (2011)	GTKO	rhesus monkey (n=5)	Anti-CD154mAb, anti-LFA1mAb, MMF, CTLA4-Ig (heparin)	n/a, 50, 91, 99, 249 [4/5 insulin independence]
		Hemizygous GTKO (effective WT)	rhesus monkey (n=5)	Anti-CD154mAb, anti-LFA1mAb, MMF, CTLA4-Ig (heparin)	4x n/a; 137 [1/5 insulin independence]
5	Bottino (2014)	GTKO/CD46/CD39	cynomolgus monkey (n=1)	CS, ATG, MMF, anti-CD154mAb (dextran sulfate, prostacyclin, aspirin)	5
		GTKO/CD46/TFPI/CTL4-Ig	cynomolgus monkey (n=2)	CS, ATG, MMF, anti-CD154mAb (dextran sulfate, prostacyclin, aspirin)	0, 365
		GTKO/CD46/TFPI/CTL4-Ig/CD39	cynomolgus monkey (n=2)	CS, ATG, MMF, anti-CD154mAb (dextran sulfate, prostacyclin, aspirin)	3, 160

ATG - anti-thyroglobulin, CS - corticosteroids, CVF - cobra venom factor, IS - immunosuppression, MMF - mycophenolate mofetil

Table 2 GTKO kidney xenotransplantation experiments since 2005

	First Author (year)	Donor genetics	Recipient (n)	Immunosuppression	Survival Range in days (median)
1	Yamada (2005)	GTKO	Baboon (n=6)	(thymokidney) +/- WBI, thymectomy, splenectomy, anti-CD2mAb, anti-CD154mAb, MMF, CS, +/- CVF	4 to 68 (32)
2	Chen (2005)	GTKO	Baboon (n=3)	ATG, tacrolimus, CS	8, 10, 11
			Baboon (n=3)	ATG, tacrolimus, CS, MMF, CVF	9, 13, 16
3	Griesemer (2009)	GTKO	Baboon (n=7)	thymectomy, splenectomy, TBI (n=1), ATG +/- anti-CD2mAb, anti-CD154mAb, tacrolimus, MMF, anti-CD20mAb	18 to 83 (49)
4	Lin (2010)	GTKO/CD46	Baboon (n=1)	no IS	2
5	Griesemer (2010)	GTKO	Baboon (n=2)	TBI, TI, splenectomy, ATG, anti-CD2mAb, tacrolimus, GTKO BMTx, +/- CVF (n=1)	8, 11
6	Nishimura (2011)	GTKO	Baboon (n=2)	(thymokidney) thymectomy, splenectomy, anti-CD3IT, +/- anti-CD2mAb, ATG, anti-CD20mAb, tacrolimus, anti-CD154mAb, MMF	15, 15
7	Le Bas-Bernardet (2011)	GTKO/CD55/CD59/CD39	Baboon (n=2)	No IS	3, 4
		GTKO/CD55/CD59/CD39/HT	Baboon (n=4)	splenectomy (n=2), CyP, tacrolimus, MMF, CS, C1-INH	4, 12, 13, 15
8	Shimizu (2012)	GTKO	Baboon (n=2)	ATG, anti-CD2mAb, anti-CD154, MMF, CS	20, 33
			Baboon (n=1)	thymectomy, splenectomy, TBI, ATG, anti-CD2mAb, anti-CD154, MMF, CS	34
			Baboon (n=4)	(thymokidney) thymectomy, splenectomy, ATG, anti-CD2mAb, antiCD154mAb, MMF, CS, +/- TBI (n=1)	56, 68, 81, 83
9	Pintore (2013)	GTKO/CD55/CD59/CD39	Cynomolgus monkey (n=5)	CyP, CsA, MMF, CS	18 +/- 3.2 (16)
		GTKO/CD55/CD59/CD39/HT	Cynomolgus monkey (n=3)	Anti-CD20mAb, CsA, MMF, CS	13 +/- 2.3 (12)
10	Spiezia (2013)	GTKO/CD55	Cynomolgous monkey (n=2)	CyP or anti-CD20mAb, CsA, MMF, CS	12, 13
		GTKO/CD55/CD59/CD39/HT	Cynomolgus monkey (n=2)	CyP or anti-CD20mAb, CsA, MMF, CS	8, 8
11	Ezzelarab (2014)	GTKO	Baboon (n=1)	Anti-CD154mAb, ATG, MMF	6
		GTKO/CD46	Baboon (n=3)	Anti-CD154mAb, ATG, MMF	9, 10, 10
12	Hwang (2014)	GTKO (heart and kidney)	Cynomologus monkey (n=2)	ATG, anti-CD20mAb, anti-CD154mAb, CVF, tacrolimus, steroid	24, 25
13	Sekijima (2014)	GTKO (MGH-Nippon)	Cynomolgus monkey (n=3)	splenectomy, ATG, anti-CD20mAb, tacrolimus, MMF, anti-CD154mAb, prostacyclin, sTBM, ganciclovir	27, 28, 30
		GTKO (Meji U)	Cynomolgus monkey (n=5)	splenectomy, ATG, anti-CD20mAb, tacrolimus, MMF, anti-CD154mAb, prostacyclin, sTBM, ganciclovir	7 to 15, 9.2 average
14	Bottino (2014)	GTKO	Baboon (n=6)	**pre-treatment with anti-CD20mAb**, (thymokidney) thymectomy, splenectomy, ATG, anti-CD2mAb, antiCD154mAb, MMF, CS	average > 12.5
15	Le Bas-Bernardet (2015)	GTKO/CD55/CD59/CD39/HT	Babon (n= 2)	splenectomy	4, 3
		GTKO/CD55/CD59/CD39/HT	Babon (n= 3)	MMF, CS, tacrolimus, CyP (n=2), rhC1-INH	15, 13, 12
		GTKO/CD55/CD59/CD39/HT	Babon (n= 3)	splenectomy, plasma exchange (d4, 1), MMF, CS, tacrolimus, bortezomib, rhC1-INH	9, 9, 11
		GTKO/CD55/CD59/CD39/HT	Babon (n= 4)	splenectomy, plasma exchange (d4, 1, 3), MMF, CS, tacrolimus, bortezomib, rhC1-INH	12, 14, 12, 14
		GTKO	Babon (n= 1)	splenectomy, plasma exchange (d4, 1, 3), MMF, CS, tacrolimus, bortezomib, rhC1-INH	11

anti-CD3IT - anti-CD3 immunotoxin, ATG - anti-thymocyte globulin, BMTx - bone marrow transplant, CS - corticosteroids, CsA - cyclosporine, CVF - cobra venom factor, CyP - cyclophosphamide, IS - immunosuppression, mAb - monoclonal antibody, MMF - mycophenolate mofetil, rhC1-INH - recombinant human C1 inhibitor, sTBM - soluble thrombo modulin, TI - thymic irradiation, TBI - total body irradiation

Table 3 GTKO and record cardiac xenotransplantation experiments since 2005

	First Author (year)	Donor genetics	Recipient (n)	Immunosuppression	Survival time in days (median)
1	McGregor (2005)	hCD46	Baboon (n=7)	Splenectomy, ATG, anti-CD20mAb, tacrolimus, rapamycin, CS, TPC	15, 38 , 54, 64, 96, 99, 137 (96)
2	Kuwaki (2005)	GTKO	Baboon (n=8)	ATG, anti-CD2mAB, TI, CVF, anti-CD154mAb, MMF, CS	>16, >23, >56, 59, 67, 78, 110, 179 (63)
3	Byrne (2008)	GTKO	Baboon (n=8)	splenectomy, ATG, anti-CD20mAb, tacrolimus, rapamycin	0 to 128 (25)
4	Ezzelarab (2009)	GTKO	Baboon (n=9)	ATG, CVF, anti-CD154mAb, MMF, CS	0, 1, 6, 6, 7, 12, 12, 35, 56 (7)
5	Bauer (2010)	GTKO/CD46	Baboon (n=2)	ATG, anti-CD20mAb, tacrolimus, rapamycin, MMF, CS, bortezomib, immunoadsorption (n=1)	<1, 50
6	Tazelaar (2011)	GTKO +/- CD55	Baboon n=5)	ATG, anti-CD20mAb, tacrolimus, rapamycin	18 to 71 (26)
7	Mohiuddin (2012)	GTKO/CD46	Baboon (n=2)	No IS	<1, <1
			Baboon (=2)	ATG, CVF, anti-CD154mAb, MMf, CS	8, 8
			Baboon (n=9)	ATG, anti-CD20mAb, CVF, anti-CD154mAb, MMf, CS	>36, >39, >42, >58, > 71, 75, >115, >179, >236 (71)
8	McGregor (2012)	GTKO	Baboon (n=6)	ATG, anti-CD20mAb, tacrolimus, rapamycin, CS	0, 2, 21, 22, 79, 128 (21)
		GTKO/CD55	Baboon (n=5)	ATG, anti-CD20mAb, tacrolimus, rapamycin, CS	15, 27, 28, 41, 52 (28)
9	Kim (2013)	GTKO	Cynomologus monkey (n=4)	ATG, anti-CD20mAb, CVF, anti-CD154mAb, tacrolimus, CS	11, 14, 14, 24 (14)
10	Mohiuddin (2013)	GTKO/CD46	Baboon (n=3)	ATG, anti-CD20mAb, CVF, anti-CD40mAb **(3A4)**, MMF, CS	21, 21, 28 (21)
			Baboon (n=6)	ATG, anti-CD20mAb, CVF, anti-CD40mAb **(2C10R4)**, MMF, CS	>30, >40, 60,107,146, 149 (84)
11	Mohiuddin (2014)	GTKO/CD46/TBM	Baboon (n=5)	ATG, anti-CD20mAb, CVF, anti-CD40mAb **(2C10)**, MMF, CS, steroids	146, 159, >200, >380, >500 [3 ongoing >200 to > 500 days](>200)
12	Ezzelarab (2014)	GTKO	Baboon (n=2)	Anti-CD154mAb, ATG, MMF	56, 35
		GTKO/CD46	Baboon (n=1)	Anti-CD154mAb, ATG, MMF	26
13	Hwang (2014)	GTKO (heart and kidney)	Cynomologus monkey (n=2)	ATG, anti-CD20mAb, anti-CD154mAb, CVF, tacrolimus, steroid	24, 25
A	Byrne (2011)	CD46 or CD55 or GTKO/CD55	Baboon (n=14) non life supporting	ATG or Cyp, tacrolimus, rapamycin +/- anti-CD20mAb +/- GAS914 or TPC	0 to 57 (6)

ATG - anti-thymocyte globulin, CS - cyclosporine, CVF - cobra venom factor, IS - immunosuppression, mAb - monoclonal antibody, MMF - mycophenolate mofetil

Preamble

As demonstrated above, the impact of antibodies elicited against GTKO xenografts cannot be overstated. Currently, most xenotransplantation laboratories favor inhibition of the majority of the adaptive antibody response utilizing a combination of B cell depletion and inhibition of T cell-mediated B cell activation (costimulation blockade). Unfortunately, while therapies such as these, which indiscriminately disarm B cells, can significantly prolong xenograft survival (6, 34, 92), they also significantly heighten recipient susceptibility to infectious complications (35-37). More selective inhibition of the anti-non-Gal antibody response may enhance xenograft survival while preserving a greater portion of B cell-mediated adaptive immunity to ward off infection. The following chapters describe original research which provides further molecular characterization of antibodies elicited by xenotransplantation in addition to identification of small molecules capable of selectively inhibiting antibodies elicited by xenotransplantation.

Chapter 2 - Rationale and Hypothesis

Our collaborators at St. Vincent's hospital in Melbourne have generated GTKO pigs expressing the human transgenes CD55, CD59, and HT (GTKO/hCD55.hCD59.hHT)(16, 72, 94). In addition to human complement regulatory proteins (CD55 and CD59), these animals express the enzyme α-1,2-fucosyltransferase (H-transferase, HT). This modification has been thought to reduce the immunogenicity of the graft glycocalyx by reducing surface expression of immunogenic terminal

carbohydrates by replacing them with the immunologically inert terminal α-1,2-fucose (human blood group O antigen) (95).

Porcine neonatal islet cell clusters from these animals have been isolated and transplanted into baboons (Papio hamadryas) at Westmead laboratories. Baboons were treated with a typical allotransplantation immunosuppressive protocol. However, the presence of a strong anti-non-Gal antibody response (85)(see chapter 2), as reported in other GTKO islet xenotransplantation experiments (96), provided the opportunity to analyze the heavy and light chain usage in GTKO xenoislet transplantation. We have previously identified the antibody heavy chain elicited against GTKO pig endothelial cells (33). However, amino acid residues which contribute to the binding site of anti-non-Gal antibodies are likely to be conserved between settings. **We hypothesized that the baboon antibody response elicited against GTKO/hCD55.hCD59.hHT porcine islets would be encoded by genes which would demonstrate a high degree of sequence and modeled structural homology with previously identified primate anti-pig antibodies.**

This chapter provided three key pieces of information which prompted additional inquiry outlined in chapters three and four: 1) the antibody response to GTKO pig islets, like that to GTKO pig endothelial cells (33), is primarily composed of antibodies which have a high degree of sequence, and modeled structural homology; 2) the heavy chain elicited against porcine islets has a high degree of sequence, and modeled structural homology with the antibody heavy chain elicited against pig endothelial cells; and 3) both of the heavy chain genes which encode these elicited antibodies also encode human antibodies which inhibit the function of clotting factor VIII.

Chapter 3 – Rationale and Hypothesis

Endothelial cells are known to express several of the currently known non-Gal porcine antigens (52, 53, 97). FVIII is also known to be synthesized and released by endothelial cells in the dermis, aorta, heart, lung, liver, and kidney (98-100). As noted above, our lab has previously intravenously injected rhesus monkeys with GTKO porcine aortic endothelial cells to stimulate an anti-non-Gal antibody response without the procedural complications of islet or solid organ xenotransplantation (101). Our preliminary data demonstrated that the elicited antibodies inhibit FVIII cofactor activity significantly more than those present before immunization (see chapter 3). Of interest, this level of inhibition is consistent with an inhibitor titer of approximately 16 Bethesda units (Bu); where an increase in 5 Bu in humans can indicate pathology (102). However, the "normal" coagulation parameters after xenotransplantation are currently under investigation (103, 104).

On occasion, acquisition of FVIII inhibitors has been traced to the mutation of a single amino acid of FVIII (105-107). Of note, there is only 76-90% amino acid homology between porcine and human FVIII for those domains commonly inhibited (108). Characterization of the antibody-FVIII interactions that are likely to participate in antibody-mediated injury after xenotransplantation of genetically modified porcine cells and organs should enhance our ability to rationally design methods directed at mitigating humoral rejection. **We hypothesized that elicited anti-pig antibody would inhibit cofactor activity of FVIII by binding to domain segments which are not homologous between pigs and primates.**

Chapter 4 – Rationale and Hypothesis

While selective inhibition of anti-non-Gal antibodies may at first seem to be a tall order, work from our lab published in 2008 demonstrated its plausibility (33). We determined that the heavy chain of anti-non-Gal antibodies elicited by rhesus monkeys against GTKO pig endothelial cells is encoded by a germline gene most similar to the human gene IGHV3-21. Furthermore, 72-85% of antibody-producing peripheral blood mononuclear cells utilized this gene by 8 days after immunization compared with 1-3% on day 0. Thus, therapies which target antibodies encoded by this gene could preemptively inhibit at least 72-85% of the anti-non-Gal xenoantibody response. The spirit of this approach is comparable to several methods previously utilized to inhibit anti-Gal antibody-mediated hyperacute rejection (39). These methods utilized structural derivatives of the Gal carbohydrate to selectively inhibit anti-Gal antibodies. These treatment modalities had varying levels of success (74, 75, 109) and went out of favor with the production of GTKO pigs. However, unlike anti-Gal antibodies, antibodies against non-Gal pig antigens pre-exist at low levels (32, 33) and do not initiate rejection until after the initiation of a vigorous humoral response (22, 24, 110). Therefore, selectively, and preemptively, disrupting the B cell receptor (antibody)-antigen interaction would inhibit the formation of a productive B cell response and antibody-mediated rejection of GTKO porcine cells and organs. To our knowledge, small molecules which can selectively inhibit anti-non-Gal antibodies have not been reported by others. **We hypothesized that the combination of screening a clinically relevant small molecule library *in silico* and *in vitro* would allow us to identify drugs which selectively inhibit elicited anti-non-Gal antibodies.**

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CHAPTER TWO

XENOANTIBODY RESPONSE TO PORCINE ISLET CELL TRANSPLANTATION USING GTKO, CD55, CD59, AND FUCOSYLTRANSFERASE MULTIPLE TRANSGENIC DONORS

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TITLE: Xenoantibody response to porcine islet cell transplantation using GTKO, CD55, CD59, and fucosyltransferase multiple transgenic donors

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RUNNING HEAD: XAb to GTKO/hCD55/hCD59/hHT Pig Islets

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Abstract

Background: Promising developments in porcine islet xenotransplantation could resolve the donor pancreas shortage for type 1 diabetic patients. Using GTKO donor pigs with multiple transgenes should extend xenoislet survival via reducing complement activation, thrombus formation, and the requirement for exogenous immune suppression. Studying the xenoantibody response to GTKO/hCD55/hCD59/hHT islets in the pig-to-baboon model, and comparing it with previously analyzed responses, would allow the development of inhibitory reagents capable of targeting conserved idiotypic regions.

Methods: We generated IgM heavy and light chain gene libraries from ten untreated baboons and three baboons at 28 days following transplantation of GTKO/hCD55/hCD59/hHT pig neonatal islet cell clusters with immunosuppression. Flow cytometry was used to confirm the induction of a xenoantibody response. IgM germline gene usage was compared pre and post transplant. Homology modeling was used to compare the structure of xenoantibodies elicited after transplantation of GTKO/hCD55/hCD59/hHT pig islets with those induced by GTKO and wild type pig endothelial cells without further genetic modification.

Results: IgM xenoantibodies that bind to GTKO pig cells and wild type pig cells were induced after transplantation. These anti-nonGal antibodies were encoded by the IGHV3-66*02 (Δ28%) and IGKV1-12*02 (Δ25%) alleles, for the immunoglobulin heavy and light chains, respectively. IGHV3-66 is 86.7% similar to IGHV3-21 which was elicited by rhesus monkeys in response to GTKO endothelial cells. Heavy chain genes most similar to IGHV3-66 were found to utilize the IGHJ4 gene in 85% of V-D regions

analyzed. However, unlike the wild type response, a consensus complementarity determining region 3 was not identified.

Conclusions: Additional genetic modifications in transgenic GTKO pigs do not substantially modify the structure of the restricted group of anti-nonGal xenoantibodies that mediate induced xenoantibody responses with or without immunosuppression. The use of this information to develop new therapeutic agents to target this restricted response will likely be beneficial for long term islet cell survival and for developing targeted immunosuppressive regimens with less toxicity.

Introduction

Allotransplantation has provided an effective treatment for patients with type 1 diabetes (T1D) and its debilitating chronic complications [1, 2]. Due to the current shortage of donor pancreatic islets, however, transplantation of porcine islets is being considered as a potential alternative [3-6]. Survival for over 1 yr with diabetes reversal has now been reported in diabetic non-human primates (NHPs) using porcine islets in combination with chronic immunosuppression [3, 4, 6].

Immune-mediated rejection remains a challenge to the survival of genetically modified porcine xenografts [5-9]. In preclinical studies, various immunosuppressive regimens have been developed to facilitate porcine to primate islet cell transplants, but the most successful regimens represent an immunosuppressive burden that is greater than that currently used in human allotransplantation [7, 10-12]. The use of α1,3-galactosyltransferase gene knockout (GTKO) neonatal porcine islets reduces the immune response and improves the rate of return to normoglycemia [5]. Genetic modifications such as the expression of human complement regulatory proteins, hCD55, and hCD59 in transgenic pig donors reduce the rate of complement activation [6, 13-15], and the introduction of additional transgenes may make it feasible to further prolong graft survival. Nevertheless, genetic modification alone is not likely to be sufficient to mitigate rejection given the profound immune barrier existing between the human and pig species. Combination therapies, including those that are directed at xenoantibodies, will need to be developed to improve xenograft survival beyond what is currently achievable using existing strategies.

Our laboratory has defined a selected, restricted usage of immunoglobulin heavy chain variable (IGHV) and immunoglobulin kappa light chain variable (IGKV) genes that encode xenoantibodies in multiple settings [16-19]. Although we have previously identified the IGHV utilized in response to GTKO endothelial cells in non-immunosuppressed rhesus monkeys, this report identifies both the immunoglobulin (Ig) heavy and light chain genes that encode the xenoantibodies induced in baboons responding to transplantation with GTKO/hCD55/hCD59/hHT transgenic porcine neonatal islet cell clusters (NICC). Amino acid residues which contribute the binding site of anti-non-Gal xenoantibodies are likely to be conserved between systems. Thus, the goal of our study was to identify the structure of xenoantibodies that initiate antibody-mediated injury after genetically modified porcine islet cell xenotransplantation, to compare these with previously identified antibody sequences, and ultimately to use this information to rationally design selective immunosuppressive interventions directed at mitigating humoral rejection.

Materials and Methods

Preparation of cDNA libraries and analysis of immunoglobulin gene usage

α1,3-Galactosyltransferase gene knockout donor pigs (Sus scrofa) transgenic for hCD55, hCD59, and hHT were generated by Cowan et al. [8, 13, 14], and porcine NICC from these animals were produced and transplanted (10 000 IEQ/kg) into baboons (Papio hamadryas) at the Westmead laboratories. Prior to NICC isolation and transplantation, expression of Gal, CD55, and CD59 on peripheral blood leukocytes from GTKO/hCD55/hCD59/hHT was analyzed by flow cytometry (Fig. 1) as previously

described [13] with the exception that Gal expression was detected using a fluorescein isothiocyanate (FITC)-conjugated anti-Gal monoclonal antibody (GT6-27-23) which was prepared from a hybridoma kindly provided by Dr. Guerard Byrne, Nextran, Princeton NJ, USA.

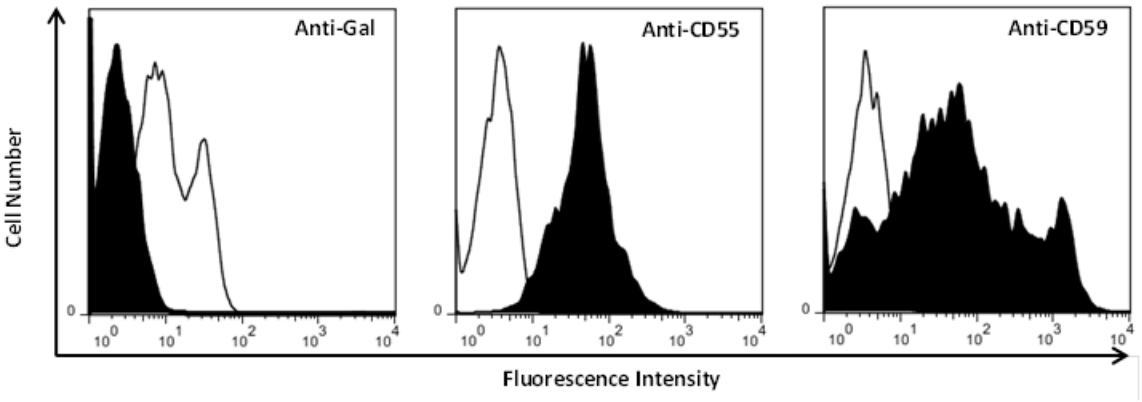

Anti-Gal
Anti-CD55
Anti-CD59
Cell Number
Fluorescence Intensity
0
10^0
10^1
10^2
10^3
10^4

Figure 1. Flow cytometric analysis of transgene expression of peripheral blood from a GTKO/hCD55/hCD59/hHTF pig (black), compared with a wild type non transgenic pig (white). GTKO/hCD55/hCD59/hHTF pigs do not express α Gal and strongly express human CD55 and human CD59.

Baboons were treated with a typical allotransplant immunosuppressive protocol, including a combination of induction with anti-thymocyte globulin (ATG) and ongoing treatment with mycophenolate mofetil and tacrolimus. However, because antibodies developed after transplantation, we sought the opportunity to analyze this response. Serum and peripheral blood cell samples from a total of 10 baboons were used to prepare cDNA libraries for the analysis of the immune repertoire in control and transplanted animals. cDNA libraries representing genes encoding xenoantibodies before and after transplantation were constructed as previously described [16]. Nested PCRs were performed to amplify the product of somatic recombination of either the heavy chain V, D, and J antibody gene progenitors or the light chain V and J antibody gene progenitors [17-20]. Ig heavy chain libraries were prepared by amplifying cDNA using a μ-chain-specific primer (Cμ; 5′-GGG AAA AGG GTT GGG GCG GAT GCA-3′) and a framework region (FWR) specific primer for the IGHV 3 family (VH3; 5′-GAG GTG CAG CTG GTG GAG TCT GG-3′). The first PCR for the IgM libraries was then subjected to one additional nested reaction. VH primer (5′-TCT GGG GGA GGC TTG GTC-3′) and Cμ primer pairs were used in the nested PCR. The primers used to amplify immunoglobulin kappa light chain genes were kappa 5 (5′-CAG ATG GCG GGA AGA TGA AG-3′) and CR50 (5′-AGC TCC TGG GGC T(GC) CT(AG)(AC) TGC-3′), which is aligned to the FWR1 of the light chain. The first nested PCR was performed using the CR50 and kappa 4 (5′-ACA GAT GGT GCA GCC ACA G-3′) primers. The second nested PCR was amplified using kappa 4 paired with the primer (5′-TCT GCA TCT GTA GGA GAC-3′). The PCR was performed in a TECHNE TC-4000 PCR System Thermocycler (Bibby Scientific Limited, Staffordshire, UK) for one cycle of 94 °C 5 min

followed by 35 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 60 s and one cycle of 72 °C for 7 min. The PCR products were verified by size on a 2% agarose gel, cloned into the TA 2.1 vector (Invitrogen, Carlsbad, CA, USA), transformed into MAX Efficiency DH5α™ T1R competent cells (Invitrogen); DNA was isolated, and the clones were sequenced by the City of Hope DNA sequencing core facility. A minimum of 40 heavy and 40 light chain sequences were obtained per transplanted animal. Sequencing results were analyzed using the NCBI website (http://0-www.ncbi.nlm.nih.gov.catalog.llu.edu/igblast/) to identify the closest human germline progenitor genes. Pre- and post-transplant Ig gene usage was compared in each animal and in a series of control animals, allowing us to define the normal repertoire of Ig gene usage in baboons and to identify changes in the relative frequency of usage of specific IGHV and IGKV genes.

Flow Cytometry

Xenoantibody levels in the sera of recipient baboons were determined at 28 days after transplantation of genetically modified porcine NICC. Heat-inactivated baboon serum samples were diluted 1/10 and were incubated at room temperature with endothelial cells from both GTKO and wild-type pigs. Cells were washed twice with cold FACS buffer and incubated with FITC-conjugated goat (Fab') anti-human IgM (Southern Biotech, Birmingham, AL, USA) or FITC-conjugated goat anti-human IgG (γ-chain specific) (Sigma, St. Louis, MO, USA). The cells were washed, resuspended in cold FACS buffer, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA, USA) using FlowJo software (Tree stars, Ashland, OR, USA).

Homology Modeling

Homology modeling, along with further *in silico* refinement, was used to compare post-transplant anti-non-Gal xenoantibodies induced by rhesus monkeys in response to GTKO pig endothelial cells without additional genetic modifications [19] and by baboons in response to GTKO/hCD55/hCD59/hHT porcine islets. Antibody models were prepared using the Discovery Studio 3.5 software suite (Accelrys, San Diego, CA, USA) using representative sequences derived from post-transplant IgM xenoantibodies. Each antibody FWR was modeled based on homology using Modeller and crystal structures deposited in the Protein Data Bank (RCSB.org). Antibody complementarity determining regions (CDRs) were modeled separately using the three crystal structures with the highest degree of sequence homology available for each CDR. Ab initio structural refinement and molecular dynamic simulations were used to optimize prediction of the heavy chain CDR3 which had the lowest percent homology in each case. For visual comparison, heavy chain models were aligned by the α-carbons and colored by amino acid.

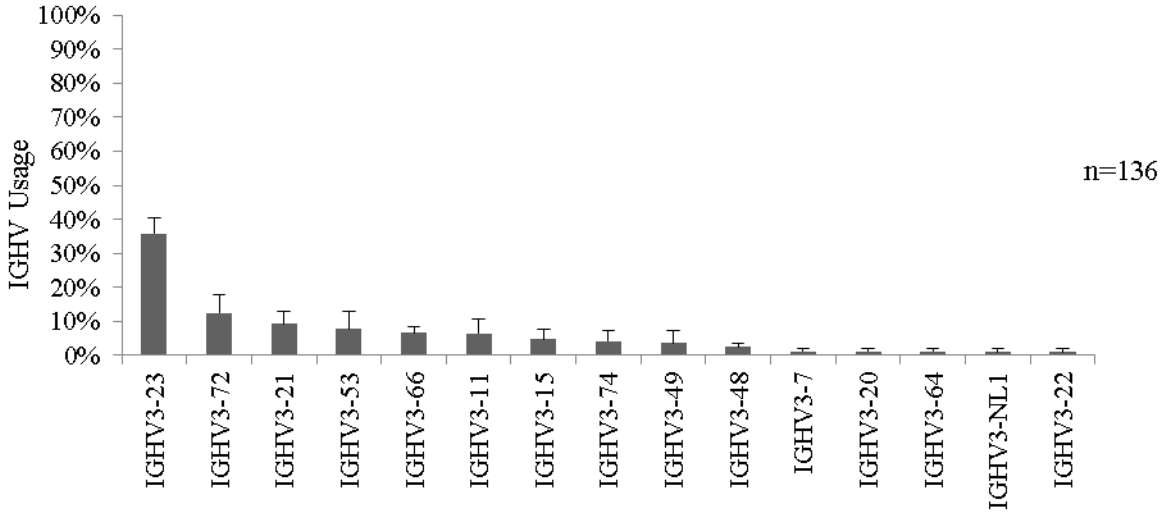
Results

Immunoglobulin heavy and light chain gene usage in untreated baboons

The distribution of heavy and kappa light chain Ig germline gene usage in 10 untreated baboons including the three recipient animals was analyzed to identify normal variability within the baboon colony (Fig. 2). The Ig heavy chain gene that was used most frequently in untreated baboons most closely resembled human IGHV3-23. The light chain genes used most frequently were most similar to human IGKV1D-16 and IGKV1-9

germline progenitors. There is little information currently available reporting the sequence of baboon germline genes; however, in our experience, the sequences encoding immunoglobulin genes in baboons are very similar to human immunoglobulin gene sequences.

A


IGHV Usage
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
n=136
IGHV3-23
IGHV3-72
IGHV3-21
IGHV3-53
IGHV3-66
IGHV3-11
IGHV3-15
IGHV3-74
IGHV3-49
IGHV3-48
IGHV3-7
IGHV3-20
IGHV3-64
IGHV3-NL1
IGHV3-22

B

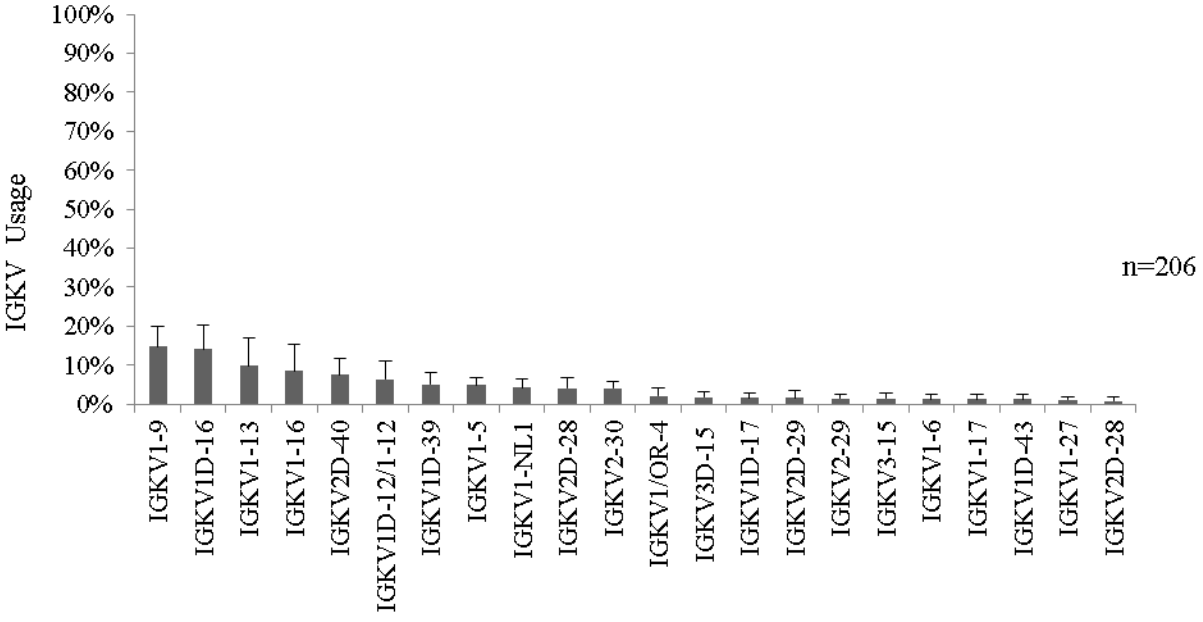
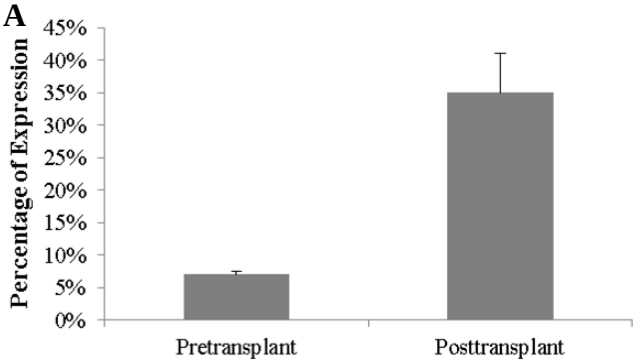

IGKV Usage
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
n=206
IGKV1-9
IGKV1D-16
IGKV1-13
IGKV1-16
IGKV2D-40
IGKV1D-12/1-12
IGKV1D-39
IGKV1-5
IGKV1-NL1
IGKV2D-28
IGKV2-30
IGKV1/OR-4
IGKV3D-15
IGKV1D-17
IGKV2D-29
IGKV2-29
IGKV3-15
IGKV1-6
IGKV1-17
IGKV1D-43
IGKV1-27
IGKV2D-28

Figure 2. The frequency of IGHV3 and IGKV gene usage in untreated baboons. (A) The IGHV3-23 germline gene is most frequently utilized in the normal baboon repertoire. The distribution of IGHV3 germline gene usage was determined by sequencing IGHV3 family genes in ten untreated baboons including 3 which later received transplants. (B) IgKV germline gene usage in untreated baboons was determined by sequencing immunoglobulin light chain gene libraries in ten baboons. The IGKV1D-16 and IGKV1-9 germline genes were most frequently used in these animals. Data is represented as average usage per animal ± the standard error of the mean. N= the number of colonies sequenced.

Restricted Xenoantibody Response to Porcine Islet Transplantation

At 28 days after transgenic porcine NICC transplantation, we observed a >2-fold increase in the usage of the IGKV1-12*02 and IGHV3-66*02 light and heavy chain genes relative to pre-transplant levels (Fig. 3A). Both pre- and post-transplant levels were determined independently in each baboon. The results were consistent in all animals. Figure 3 shows representative cDNA sequences from libraries that were prepared pre- and post-transplantation. The germline gene used most frequently to encode xenoantibodies in this model was IGHV3-66, a gene that is closely related but not identical to IGHV3-21, and the germline progenitor that encodes xenoantibodies elicited by non-immunosuppressed rhesus monkeys after immunization with GTKO pig endothelial cells without additional genetic modifications. The similarity in the Ig gene sequence when comparing xenoantibodies to GTKO islet cells and IGHV3-21 is shown in Fig. 3B,C. These genes are 86.7% similar at the amino acid level. The xenoantibodies induced in response to transplantation of genetically modified porcine islets are also 92.0% similar to the human IGHV3-11*01 allele encoding human xenoantibodies to wild-type pig solid organs or islet cells [17].

A



B

```
                <-------------------------------------FWR1--------------------------
IGHV3-21*01     GAGGTGCAGCTGGTGGAGTCTGGGGGAGGCCTGGTCAAGCCTGGGGGGTCCCTGAGATTCTCCTGTGCAG
H.11            -------------------------------T-----C--------------------C------------
H.13            -------------------------------T-----C--------------------C------------
H.18            -------------------------------T-----C--------------------C------------
H.19            -------------------------------T-----C--------------------C------------

                -------------------><-----CDR1----><--------------------FWR2-----------
IGHV3-21*01     CCTCTGGATTCACCTTCAGTAGCTATAGCATGAACTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTG
H.11            -------------------------CTA-----G-------------------------------------
H.13            -------------------------CTA-----G-------------------------------------
H.18            -------------------------CTA-----G-------------------------------------
H.19            -------------------------CTA-----G-------------------------------------

                ------>  <-------------------------CDR2---------------------> <---------
IGHV3-21*01     GGTCTCA  TCCATTAGTAGTAGTAGTTACATATACTACGCAGACTCAGTGAAGGGC CGATTCACC
H.11            -------  -A----...------G--G--AG--C---------------C---------- ---------
H.13            -------  -A----...------G--G--AG--C---------------C---------- ---------
H.18            -------  -A----...------G--G--AG--C---------------C---------- ---------
H.19            -------  -A----...------G--G--AG--C---------------C---------- ---------

                ---------------------------------------FWR3----------------------------
IGHV3-21*01     ATCTCCAGAGACAACGCCAAGAACTCACTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCTG
H.11            ---------------T-A------A-G--C-CC-------------------------------------
H.13            ---------------T-A------A-G--C-CC-------------------------------------
H.18            ---------------T-A------A-G--C-CC-------------------------------------
H.19            ---------------T-A-G----A-G--C-CC-------------------------------------

                ---------------->
IGHV3-21*01     TGTATTACTGTGCGAGA
H.11            -C--C------------
H.13            -C--C------------
H.18            -C--C------------
H.19            -C---------------
```

C

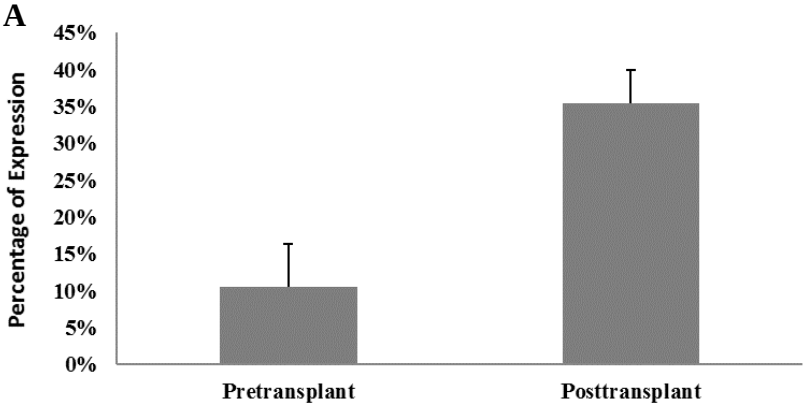
```
                              FWR1       CDR1         FWR2
                                25  30      35      40   45
                                |    |       |       |    |
HumanIGHV3-21*01               SCAASGFTFS   SYYMN   WVRQAPGKGLEWVS
Pretransplant                  ----------   ----S   --------A-----
Posttransplant                 ----------   ----S   --------A-----

                              CDR2                  FWR3
                             50    55   60  65      70    75   80  85   90  95
                             |     |    |   |       |     |    |   |    |   |
HumanIGHV3-21*01              SISSSSSTIYYADSVKG   RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR
Pretransplant                 Y-.--GGST--------   --------S--T-S------------------
Posttransplant                Y-.--GGST--------   --------S--T-S------------------
```

Figure 3. The anti-nonGal xenoantibody response to genetically modified porcine islets is restricted. (A) The usage of the immunoglobulin heavy chain gene IGHV3-66*02 was elevated from 7%+0% in pretransplant baboon serum to 35% ±6% post-islet cell transplantation. (B) The nucleotide sequence of four representative IGHV genes encoding IgM anti-nonGal xenoantibodies is shown. The IGHV genes encoding anti-nonGal porcine islet xenoantibodies is shown compared to the sequence of the IGHV3-21*01 germline gene which encodes anti-nonGal xenoantibodies to GTKO pig endothelial cells without multiple genetic modifications. (C) The amino acid sequence of IGHV genes encoding anti-nonGal xenoantibodies to genetically modified pig islets is shown. The regions of the CDR1 and CDR2 which are conserved may be relevant for contact with nonGal xenoantigen. (-) Identical residues when compared with the closest human germline gene. FWR (framework region) CDR (complementarity determining region).

The light chain gene encoding xenoantibodies to either wild-type or genetically modified GTKO cells is identical [21]. Representative amino acid sequences from pre-transplant and post-transplant samples are shown aligned to human IGKV1-12*02 (IGKV1D-12*02) (Fig. 4). As demonstrated by flow cytometry in Fig. 5, induced xenoantibodies present in the serum at 28 days after transplantation with genetically modified GTKO porcine islets also bind to wild-type pig cells. The fact that the response induced by NICC transplantation can be measured using endothelial cells indicates there is likely a prominent common non-Gal antigen expressed on both of these cell types. Furthermore, the high degree of sequence similarity in the induced xenoantibodies that bind Gal and non-Gal targets supports the concept of an induced, structurally related xenoantibody response initiated after pig cell xenotransplantation.

A



B

```
          <-------------FWR1--------------------->   <--------------CDR1------------->
IGKV1-12*02 CTTTCTGCATCTGTAGGAGACAGAGTCACCATCACTTGT   CGGGCGAGTCAGGGTATTAGCAGCTGGTTAGCC
K.2         ----------------------A---------------C   -AT--C----------------------------
K.7         ----------------------A---------------C   -AT--C----------------------------
K.10        ----------------------A---------------C   -AT--C----------------------------
K.12        ----------------------A---------------C   -AT--C---------------------------G

          <---------------------FWR2------------------>  <--------CDR2------->  <----
IGKV1-12*02 TGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTAT  GCTGCATCCAGTTTGCAAAGT  GGGGT
K. 2        ---------------------------------------------  AAG------------------  -----
K. 7        ---------------------------------------------  AAG------------------  -----
K.10        ---------------------------------------------  AAG------------------  -----
K.12        ---------------------------------------------  AAG------------------  -----

          <--------------------------------FWR3------------------------------------->
IGKV1-12*02 CCCATCAAGGTTCAGCGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGCCTGCAGCCTGAAGATTT
K. 2        -------------------------------A-----------------------------------------
K. 7        -------------------------------A-----------------------------------------
K.10        -------------------------------A-----------------------------------------
K.12        -----------------------------------A----------------C-------------------G-

          --------------->
IGKV1-12*02 TGCAACTTACTATTGT
K. 2        ----G----T--C---
K. 7        ---------T--C---
K.10        ---------T--C---
K.12        ---------T--C---
```

C

```
                                FWR1            CDR1          FWR2
                               15    20       25  30        35   40   45
                                |    |        |   |         |    |    |
IGKV1-12*02                   SASVGDRVTITC   RASQGISSWLA    WYQQKPGKAPKLLIY
Pretransplant                 ------K-----   H----------    ---------------
Posttransplant                ------K-----   H----------    ---------------

                              CDR2         FWR3
                             50   55      60   65  70   75   80   85
                             |    |       |    |   |    |    |    |
IGKV1-12*02                  AASSLQS   GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC
Pretransplant                K------   --------------------------------
Posttransplant               K------   --------------------------------
```

Figure 4. The immunoglobulin light chain germline genes encoding anti-nonGal xenoantibodies and anti-gal xenoantibodies is identical (A) The usage of the IGKV1-12*02 germline gene was 11%+6% in pre-transplant serum and 36%+5% in post-transplant serum. (B) The nucleotide sequence of four representative IGKV genes encoding IgM xenoantibodies following porcine islet transplantation is shown compared to the closest human progenitor, the IGKV1-12*02 germline gene. (C) The amino acid sequence of representative pre and post-transplant samples is shown aligned with the amino acid sequence of the human IGKV1-12*02 gene. The residues which contribute to the gal carbohydrate binding sites of anti-gal xenoantibody are reported in ref (20). Many of these sites are conserved on both the heavy and light chain suggesting anti-nonGal and anti-gal xenoantibodies may have similar antibody-antigen interactions. (-)Identical residues when compared with the closest human germline gene. FWR (framework region) CDR (complementarity determining region).

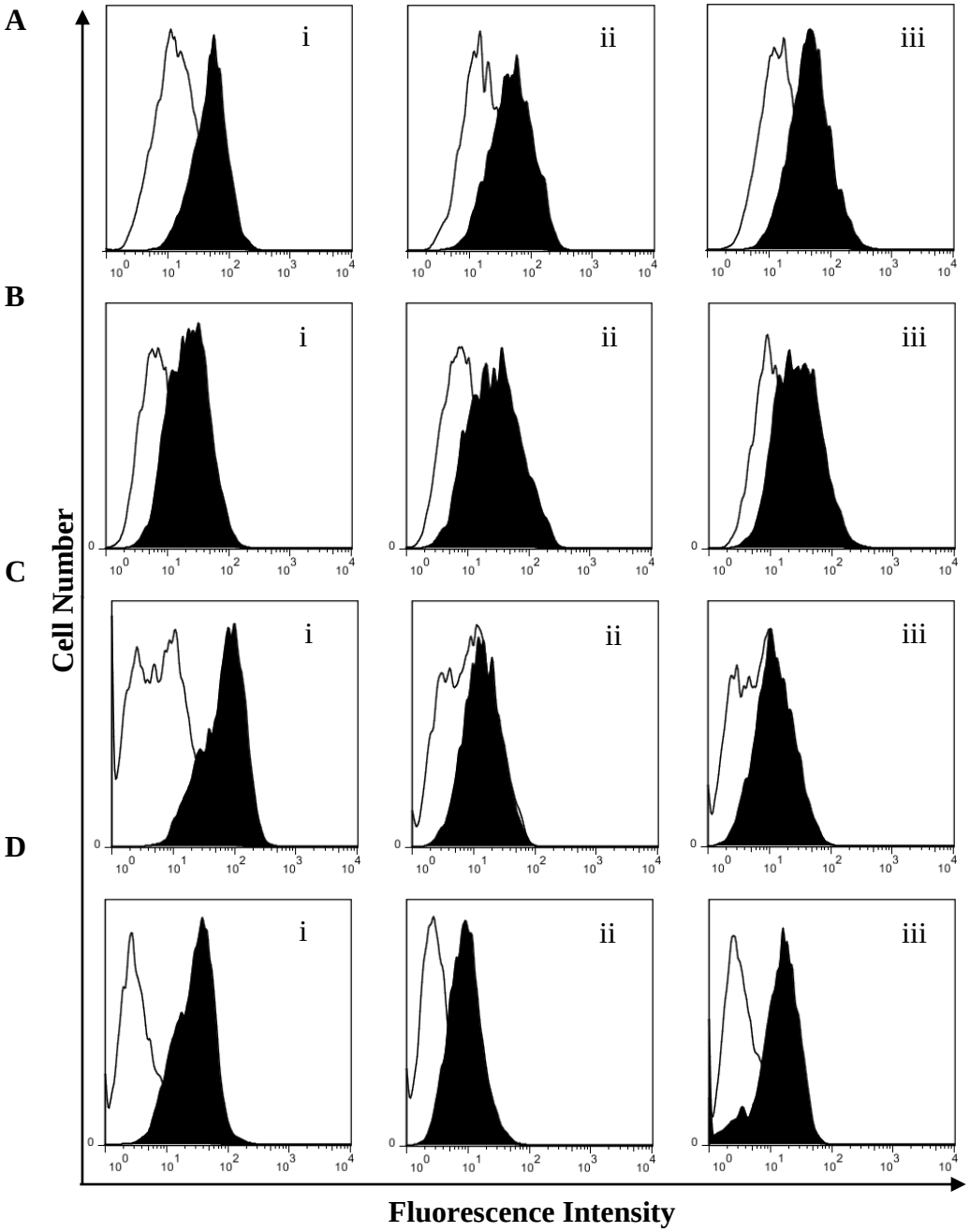

A
B
C
D
i
ii
iii
Cell Number
Fluorescence Intensity

Figure 5. Xenoantibody responses pre (white) and 28 days post (black) transplantation in the sera of three baboons (i, ii, iii) which received genetically modified GTKO porcine islets. Induced IgM (A&B) and IgG (C&D) xenoantibodies bind to (A&C) wild type pig (1.4-8.5 fold increase in Mean Fluorescence Intensity or MFI)and (B&D) GTKO pig endothelial cells (1.7-5.5 fold increase in MFI).

J Segment Usage

A restricted usage of heavy chain joining (IGHJ) genes was noted when comparing post-transplant cDNA libraries with those prepared from pre-transplant animals. A diverse selection of immunoglobulin kappa joining (IGKJ) genes was represented pre-transplant. The IGHJ4*02 allele accounted for 85% of J segment usage in 40 sequenced cDNA samples per animal examined with a functional IGHV gene (Fig. 6). Heavy chain genes most similar to the human IGHV3-66*2 germline gene were found to utilize the IGHJ4*02 allele in 85% of V-D regions analyzed. However, unlike the heavy chain utilized in response to wild-type pig solid organs or islet cells [17], our analysis of the encoded antibody sequences indicates that there is no consensus CDR3 amino acid sequence utilized in the anti-non-Gal xenoantibody response.

Comparison of Heavy Chain Structure in GTKO Xenotransplantation

There is predicted to be a high degree of structural homology between the heavy chains utilized in the xenoantibody responses of rhesus monkeys responding against GTKO endothelial cells [19] and baboons responding against GTKO/hCD55/hCD59/hHT transgenic porcine islets (Fig. 7). Within the heavy chain variable region, the CDR1 segments are nearly identical (Fig. 7B), while only moderate differences exist when comparing the CDR2 segments (Fig. 7C). No structural homology is evident when comparing the CDR3 segments (Fig. 7D). Thus, amino acid residues which are conserved are likely to make contact with a common antigen.

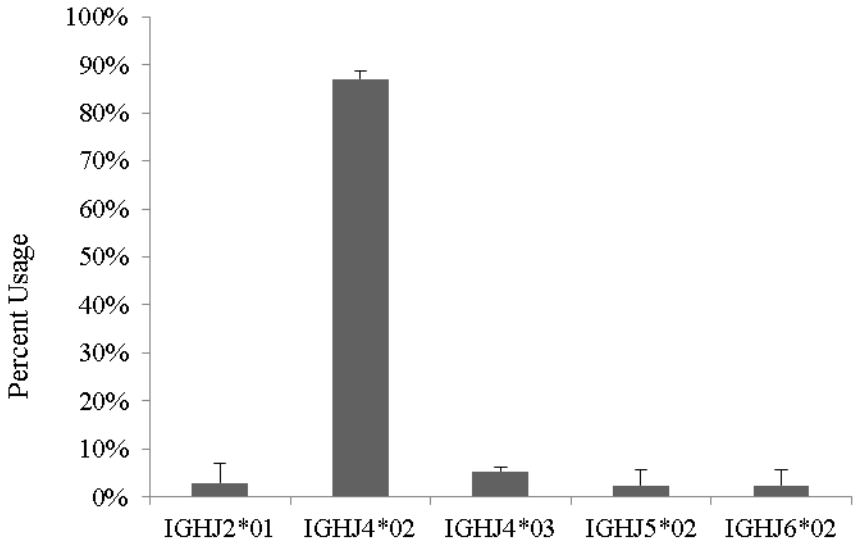

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percent Usage
IGHJ2*01
IGHJ4*02
IGHJ4*03
IGHJ5*02
IGHJ6*02

Figure 6. Immunoglobulin J gene usage following islet cell cluster transplantation in baboons. The IGHJ gene usage was restricted to IGHJ4*02 in all baboons after porcine islet cell transplantation (87%+2%).

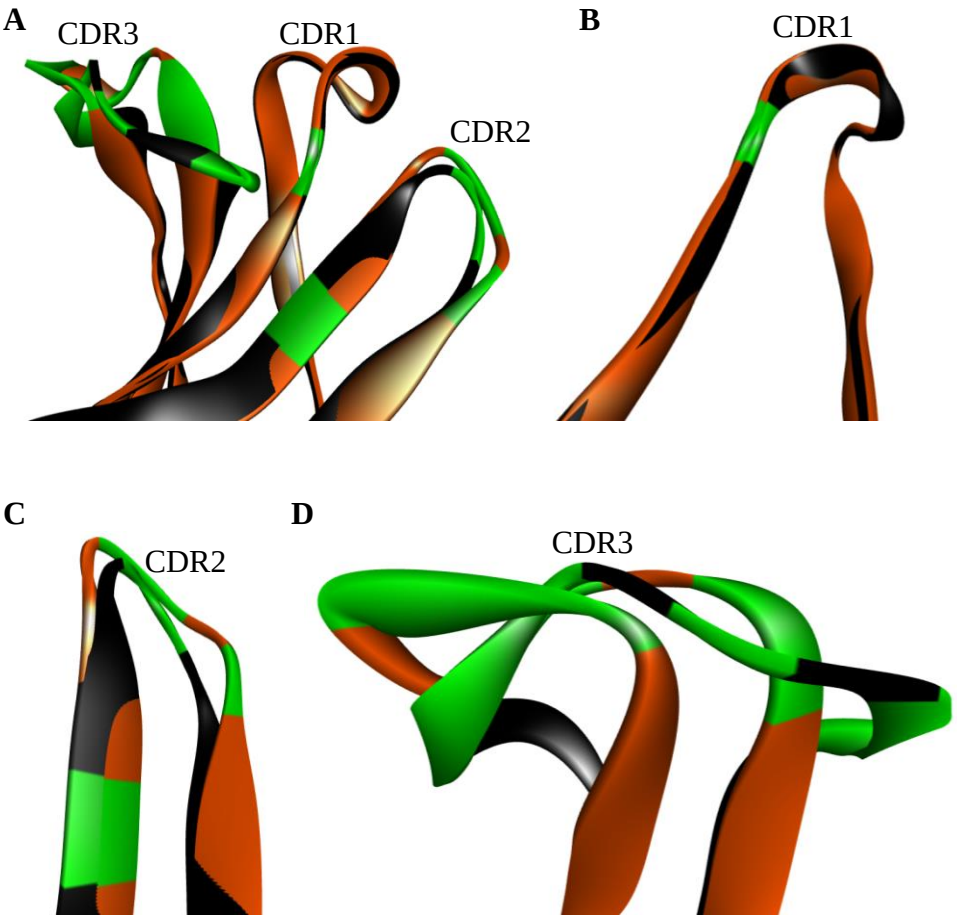

A
CDR3
CDR1
CDR2
B
CDR1
C
CDR2
D
CDR3

Figure 7. Structural alignment of modeled anti-non gal heavy chain antibodies – Structural alignment of modeled xenoantibody heavy chains associated with the immune response induced by GTKO endothelial cells (orange) and GTKO islet cells (black). Residues which are not conserved are highlighted in green on both heavy chains. The high degree of structural similarity suggests conserved amino acids likely make contact with xenoantigen. (A) Complementarity determining regions (CDRs) are depicted together. For emphasis, the (B) CDR1, (C) CDR2, and (D) CDR3 are depicted individually. The CDR1 and CDR2 display a high degree of structural similarity. In contrast, the CDR3 does not display this structural similarity. GTKO islet associated CDR3; GTKO endothelial cell associated CDR3. Alignment is by the α-carbon.

Discussion

Our study has demonstrated that the IGHV genes encoding the restricted xenoantibody response to GTKO/hCD55/hCD59/hHT transgenic islets in the pig-to-baboon model are closely related, but not identical, to IGHV genes encoding the induced xenoantibody response to GTKO endothelial cells without further transgenic modification. A restricted usage of IGHJ4*02 was noted in both models; however, the amino acid sequence of the CDR3 segment was not conserved as shown by both sequence analysis and modeling. The light chain genes encoding xenoantibodies, in contrast, are identical when comparing induced xenoantibodies responding to GTKO pig cells or wild-type pig cells.

The development of GTKO pigs has played an important role in reducing the xenoantibody response after xenograft transplantation [5, 22, 23]. Xenotransplantation using GTKO donors abrogates hyperacute rejection, significantly extending heart survival [22], and resulting in the rapid return of euglycemia [5] after pig heart and islet transplantation in baboons, respectively. The more recent generation of GTKO pigs with additional genetic modifications improves xenograft survival in cardiac models [15] and reduces early islet loss as well [6].

A significant xenoantibody response is induced at 28 days after transplantation of genetically modified GTKO porcine NICC while under immunosuppression. This finding is consistent with recent data from the Kirk laboratory using Gal-deficient pig neonatal islets [5]. Transplantation of Gal-deficient islets improves the rate of achieving insulin independence in diabetic NHP, but rejection is not prevented. Elimination of anti-non-

Gal xenoantibodies may be required to prolong graft survival after porcine islet cell cluster xenotransplantation.

We report here that specific heavy (IGHV3-66) and light chain (IGKV1-12) gene usage was elevated 28% in baboons after transplantation with porcine islet cell clusters. These antibodies preexist in the immune repertoire of baboons, albeit at relatively low levels. Monoclonal antibodies encoded by the IGHV3-66*01 can react to a highly conserved influenza A virus epitope [24], and interestingly, both the IGHV3-21 and IGHV3-66 heavy chain IGHV genes in germline formation can contribute to the production of antibodies which inhibit clotting factor VIII in patients with hemophilia A [25]. It is currently unknown whether the immune response contributes to coagulative dysfunction in xenotransplantation and further investigation is warranted.

Moderate differences that were identified when comparing the IGHV genes encoding antibodies elicited by GTKO/hCD55/hCD59/hHT transgenic islet cells compared to GTKO pig endothelial cells could be due to a difference in the epitopes expressed. It is also possible that the additional genetic modifications may have mildly influenced the surface antigenicity of the transplanted pig cells. Alternatively, subtle differences between rhesus monkey and baboon IGHV genes encoding immune responses could account for these minor differences in IGHV gene usage. Structural analysis indicates that within the heavy chain, the CDR1 sequences of Ig genes encoding anti-non-Gal and anti-Gal xenoantibodies are highly conserved [19, 20, 22, 26]. In contrast, the heavy chain CDR2 segment of anti-non-Gal xenoantibodies demonstrates mild variability, while the CDR3 segment demonstrates no similarity. Those regions which are structurally preserved are more likely to make contact with the relevant non-

Gal xenoantigen(s). Designing reagents which target these regions may be a promising strategy for interfering with antibody–antigen interactions.

In conclusion, this is the first study to identify the immunoglobulin genes encoding the xenoantibody response following transplantation of GTKO/hCD55/hCD59/hHT transgenic pig islets to baboons. Grafts stemming from different cell types, tissues, or organs induce a restricted anti-non-Gal xenoantibody response encoded by a small group of structurally restricted immunoglobulin gene progenitors. Potent immunosuppressive regimens may be substantially reduced in pig islet xenotransplantation by blocking these initial IgM responses in a targeted fashion using anti-idiotypic antibodies or small molecular inhibitors selective for these antibodies.

Acknowledgements

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CHAPTER THREE

RHESUS MONKEYS AND BABOONS DEVELOP CLOTTING FACTOR VIII INHIBITORS IN RESPONSE TO PORCINE ENDOTHELIAL CELLS OR ISLETS

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and M Kearns-Jonker[1]

Xenotransplantation (2014)

TITLE: Rhesus Monkeys and Baboons Develop Clotting Factor VIII Inhibitors in Response to Porcine Endothelial Cells or Islets

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RUNNING HEAD: FVIII inhibitors in Xenotransplantation

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KEYWORDS: Keywords: Xenoantibody, FVIII, FVIII inhibitor, Porcine, Non-Human Primate

Abstract

Background: Xenotransplantation of porcine organs holds promise of solving the human organ donor shortage. The use of α-1,3-galactosyltransferase knockout (GTKO) pig donors mitigates hyperacute rejection, while delayed rejection is currently precipitated by potent immune and hemostatic complications. Previous analysis by our laboratory suggests that clotting factor VIII (FVIII) inhibitors might be elicited by the structurally restricted xenoantibody response which occurs after transplantation of either pig GTKO/hCD55/hCD59/hHT transgenic neonatal islet cell clusters or GTKO endothelial cells.

Methods: A recombinant xenoantibody was generated using sequences from baboons demonstrating an active xenoantibody response at day 28 after GTKO/hCD55/hCD59/hHT transgenic pig neonatal islet cell cluster transplantation. Rhesus monkeys were immunized with GTKO pig endothelial cells to stimulate an anti-non-Gal xenoantibody response. Serum was collected at days 0 and 7 after immunization. A two-stage chromogenic assay was used to measure FVIII cofactor activity and identify antibodies which inhibit FVIII function. Molecular modeling and molecular dynamics simulations were used to predict antibody structure and the residues which contribute to antibody-FVIII interactions. Competition ELISA was used to verify predictions at the domain structural level.

Results: Antibodies that inhibit recombinant human FVIII function are elicited after non-human primates are transplanted with either GTKO pig neonatal islet cell clusters or endothelial cells. There is an apparent increase in inhibitor titer by 15 Bethesda units (Bu) after transplant, where an increase greater than 5 Bu can indicate

pathology in humans. Furthermore, competition ELISA verifies the computer modeled prediction that the recombinant xenoantibody, H66K12, binds the C1 domain of FVIII.

Conclusions: The development of FVIII inhibitors is a novel illustration of the potential impact the humoral immune response can have on coagulative dysfunction in xenotransplantation. However, the contribution of these antibodies to rejection pathology requires further evaluation because "normal" coagulation parameters after successful xenotransplantation are not fully understood.

Introduction

Delayed xenograft rejection of genetically modified porcine organs is a complicated process characterized by vascular antibody deposition, complement activation, hemostatic dysregulation, and potent innate and adaptive immune responses [1-4]. It is currently unknown whether coagulative dysfunction is, at least in part, dependent on the antigraft immune response. The specific importance of the humoral immune system in delayed xenograft rejection is illustrated by the prolongation of median graft survival by 63 days with the addition of B cell depleting (anti-CD20) therapy in baboons transplanted with hearts from genetically modified pigs [5]. Further reducing the immunogenicity of porcine grafts or selectively inhibiting xenotransplant reactive antibodies, also known as xenoantibodies, should prolong xenograft survival while preserving remaining humoral immune surveillance. Unfortunately, the identity of the antigen(s) relevant to rejection of α-1,3-galactosyltransferase knockout (GTKO) porcine organs is still an issue of debate [6-8].

Clotting factor VIII (FVIII) is a feasible non-Gal-α-1,3-Gal (non-Gal) antigen given the recent discovery that endothelial cells synthesize and release FVIII not only in hepatic but also in pulmonary, cardiac, intestinal, and dermal microvascular beds [9, 10]. Generation of FVIII inhibitors has long been known to be a common side effect for patients with hemophilia A receiving FVIII replacement therapy [11] and has even, on occasion, been traced to the mutation of a single amino acid of FVIII [12, 13]. There is only 76–90% amino acid homology between porcine and human FVIII for those domains commonly inhibited [14]. The development of inhibitory anti-FVIII antibodies in a xenotransplantation setting is therefore plausible.

Our laboratory has identified and characterized the immunoglobulin germline genes that encode anti-GTKO xenoantibodies in multiple settings [15, 16]. The induced immunoglobulin heavy chain variable (IGHV) genes, VH3-21 and VH3-66, are 92% similar and were in germline formation. Antibodies utilizing both these IGHV genes in germline configuration have been identified to inhibit clotting factor VIII (FVIII) in humans [17]. In this report, we use a two-step chromogenic assay to identify xenoantibody FVIII inhibitors developed in response to GTKO/hCD55/hCD59/hHT transgenic porcine neonatal islet cell cluster (NICC) transplantation in immune suppressed baboons as well as in non-immunosuppressed rhesus monkeys immunized with GTKO endothelial cells. Furthermore, we use a combination of homology modeling, *in silico* epitope prediction, competitive ELISA, and *in silico* polyalanine scanning to explore FVIII-xenoantibody interactions. The goal of our study was to characterize xenoantibody structure and xenoantibody–antigen interactions that may participate in antibody-mediated injury after xenotransplantation of genetically modified porcine organs, so that this information can be used to rationally design selective immunosuppressive interventions directed at mitigating humoral rejection.

Materials and Methods

Construction of an anti-non-Gal single-chain xenoantibody

Representative cloned IgM cDNA sequences, previously isolated from baboons demonstrating an active xenoantibody response at day 28 after transplantation with GTKO/hCD55/hCD59/hHT porcine NICC xenografts [16], most closely related to the human heavy and light chain variable genes, IGHV3-66 and IGKV1D-12, were inserted

into a pHEN2 phagemid [Center for Protein Engineering, Medical Research Council Center (MRC) Cambridge, UK] [18]. These baboons had developed a xenoantibody response despite treatment with a typical immunosuppressive protocol; including a combination of induction with ATG and ongoing treatment with mycophenolate mofetil and tacrolimus. This single-chain variable fragment (scFv) construct was named H66K12. The primers used to clone the IGHV gene were LD3 and VH3BackSFI for the first reaction and JH4XHOI and VH3BackSFI for the second reaction. The light chain primers were ApaL1.K1D12 and IGJK12NotI. All reactions included 30 cycles; each cycle was 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 1 min. The construct was inserted in frame as determined by sequencing (Beckman Research Institute at the City of Hope, Duarte, CA, USA) using pHEN-SEQ and For_LinkSeq primers. Primer sequences were as follows: LD3 5′ TCT GGG GGA GGC TTG GTC 3′; VH3BackSFI 5′ GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG GTT GAG TCT GGT CG 3′; JH4XHOI 5′ TCG ACC TCG AGC TGA GGA GAC GGT GAC CAG GAC TCC CTG GCC CCA GTA GTC CAC CAC TAT AGT AAA AAC ACC CCC TCT CGC 3′; ApaL1.K1D12 5′ GTC CTC GCA ACT GCG TGC ACA GGA CAT CCA GAT GAC CCA GTC TCC ATC TTC CGT GTC TGC ATC TGT AGG AGA CAA AGT C 3′; IGJK12NotI 5′ TCG ACG CGG CCG CTT TGA TCT CCA CTT TGG TCC CCT GGC CAA AAC TGT ACG GGT AAC TAC TAC CCT GTC GAC AGT AAT AA 3′; pHEN-SEQ 5′ CTA TGC GGC CCC ATT CA 3′; FOR_LinkSeq 5′ GCC TTT TCT GTA TGA GG 3′.

Expression and Purification of Single-Chain Antibody

Chemically competent Escherichia coli strain HB2151 were transfected with the single-chain pHEN2 DNA construct. A 1 : 100 dilution of a bacterial overnight growth was used to seed 2xTY media (1% glucose and 1% ampicillin). Bacteria were grown, shaking, at 37 °C and 225 rpm until an optical density of 0.8–0.9 at 600 nM. Isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 1 mM. After 20–24 h shaking at 225 rpm and 30 °C, bacteria were cleared by centrifugation at 1800 g at 4 °C.

Protein in the bacterial supernatant was concentrated by ammonium sulfate precipitation at 80% saturation (4 °C). Precipitated protein was pelleted by centrifugation for 15 min at 10 000 g and 4 °C and resuspended to 1/50 initial volume in cold phosphate-buffered saline (PBS; pH 7.4). Concentrated protein was dialyzed at 4 °C to remove remaining ammonium sulfate. Protein was purified using Ni-NTA agarose resin according to manufacturer's instructions, with the exception of using 10 mm imidazole wash buffer (Qiagen, Carlsbad, CA, USA). Flow through, washes, and elutions were saved for analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis and visualized. The band at 28 KDa was quantified using Imperial Protein Stain (Thermo Scientific, Rockford, IL, USA) and carbonic anhydrase (Sigma, St. Louis, MO, USA) standards. Total protein in Ni-NTA purified scFv preparations was determined by micro BCA assay (Thermo Scientific).

Animals

Four juvenile rhesus macaques (Macaca mulatta) from the California National Primate Research Center, University of California, Davis, CA, USA, were housed in

accordance with The American Association for Accreditation of Laboratory Animal Care standards. All procedures were in accordance with the requirements of the Animal Welfare Act, and protocols were approved prior to implementation by the University of California, Davis Institutional Animal Care and Use Committee.

Induction of Anti-Non-Gal Xenoantibodies After Immunization with Porcine GTKO Endothelial Cells

The porcine GTKO endothelial cells (PEGKO42) were kindly provided by Dr. David Sachs at Massachusetts General Hospital. Four (2.1–2.4 kg) age-matched (~9 months of age) rhesus monkeys were selected for low levels of preexisting anti-non-Gal xenoantibodies by ELISA using PEGKO42 as a target. Animals were immunized with 60 $\times 10^6$ GTKO endothelial cells by intravenous injection (peripheral vessel). These animals were not provided any immunosuppression. Serum samples were collected at day 0 and at day 7 post-immunization.

Flow Cytometry

The presence of anti-non-Gal reactive IgM antibodies in sera was confirmed by comparison of day 0 and day 7 IgM reactivity to GTKO endothelial cells. Heat-inactivated serum samples were diluted 1/40 in sterile PBS and incubated with GTKO endothelial cells at room temperature (RT) for 60 min. Cells were washed with cold PBS and incubated with FITC-conjugated goat (Fab') anti-human IgM (1/50; Jackson Immunoresearch, West Grove, PA, USA). Cells were then washed, resuspended in cold PBS, and analyzed using a MACSQuant flow cytometer (Becton Dickinson, San Diego,

CA, USA) and FlowJo software (Tree stars, Ashland, OR, USA). Incubation with the secondary antibody alone was used to measure background.

Detection of FVIII Inhibitors

Inhibition of FVIII cofactor activity by xenoantibodies was determined using a Technochrom two-stage chromogenic assay for FVIII activity (Diapharma Group, Inc., West Chester, OH, USA). In this assay, the amount of FXa generated from FX is dependent on the FVIII cofactor activity of the sample of interest. Thus, FVIII activity is actually calculated from the absorbance of a chromophore produced by cleavage of a chromogen by FXa. Advate, recombinant human, von Willebrand factor (vWF) free, FVIII (Baxter Healthcare Corporation, Westlake Village, CA, USA) was used in place of plasma-derived FVIII. FVIII was mixed with recombinant antibody/control, or serum as indicated (in the results) in the provided FVIII dilution buffer. Prepared solutions were treated as plasma samples and the remainder of the assay was performed according to the manufacturer's instructions using a microtiter plate scheme and the endpoint determination method. As indicated, readings of blank wells were used as no measurable FVIII activity. All experiments utilized a minimum of two technical replicates.

Computational Modeling

All *in silico* work was performed using the Discovery Studio 3.5 suite (Accelrys, San Diego, CA, USA) optimized for structural analysis of protein. All crystal structures were extracted from the Protein Data Base (RCSB.org) and are listed with their PDB ID number.

Homology Modeling

Homology modeling makes use of amino acid sequence similarity to deposited crystal structures to generate a three-dimensional protein model which serves as the basis for further refinements. Representative amino acid sequences were derived from cloned heavy and light chain cDNA sequences of IgM xenoantibodies induced in baboons at 28 days after transplantation of GTKO/hCD55/hCD59/hHT transgenic porcine NICC [16]. The variable fragment (Fv) model of H66K12 was prepared by homology modeling using a modeller algorithm optimized for antibody modeling. The antibody framework and complementarity determining regions (CDRs) were modeled independently. Framework modeling utilized one template so as to optimize the relative positions of each chain. A combination of three crystal structures was used to model each separate complementarity determining region. Structural refinement of the heavy chain CDR3 was performed using a loop refinement protocol, and the lowest energy model from the best scoring cluster was chosen for refinement using an implicit solvent-based molecular dynamics simulation in CHARM.

In silico Epitope Prediction

A computer-based protocol for predicting the epitope bound by anti-FVIII antibody was first validated by accurately predicting the inhibitory FVIII epitope of Bo2C11 (PDB ID 1iqd) using the crystal structure of human FVIII (PDB ID 3CDZ). Epitope prediction utilized the shape-based ZDOCK fast Fourier docking protocol. ZRANK was used to evaluate docking results. The top-ranked binding orientations (poses) from the twenty largest pose clusters were further refined using the RDOCK

algorithm which incorporates electrostatic interactions. The average RMSD for the top twenty poses was calculated based on the α-carbon distances.

Competition ELISA

The protocol for a FVIII competition ELISA was adapted from Healey et al. [19]. Nunc Maxisorp 96-well plates (Thermo Scientific) were coated with either Ni-NTA purified recombinant single-chain xenoantibody, H66K12, or protein purified from E. coli transfected with an empty vector matched by total protein (132 μg/ml); 50 μg/ml of recombinant scFv, H66K12, was used to coat each experimental well. Wells were coated overnight at 4 °C. FVIII was diluted to 0.1 μg/ml in Hepes-buffered saline (pH 7.4) with 2 mm CaCl2, 0.05% Tween 20, and 2% BSA. The coated plate was washed and incubated with diluted FVIII for 60 min at RT. Plates were washed and incubated for an hour at RT with 3 μg/ml of either GMA8010 or GMA8011 (Green Mountain Antibodies, Burlington, VA, USA). After washing, plates were incubated with horseradish peroxidase-conjugated anti-mouse IgG (1/1000; KPL, Gaithersburg, MD, USA) for an hour at RT. After a final wash, ABST substrate (KPL) was added and allowed to develop for 15 min before reading. All experiments utilized a minimum of three technical replicates.

In Silico Polyalanine Scanning

The most energetically favorable Fv-FVIII complex generated during epitope prediction was used to further assess the contribution of individual amino acid residues to binding. The “calculate mutation energy” protocol in Discovery Studio was used to

mutate individual residues of either FVIII, or the bound Fv, to alanine, and to determine the relative contribution of each residue to the energetics of Fv-FVIII interaction. Mutations were categorized as stabilizing or destabilizing based on the predicted change in affinity.

Statistics

All statistical analyses were performed using Excel and data represented as mean ± standard error of the mean. Statistical significance ($P < 0.05$) compared with an untreated control was established using either a Student's t-test or ANOVA.

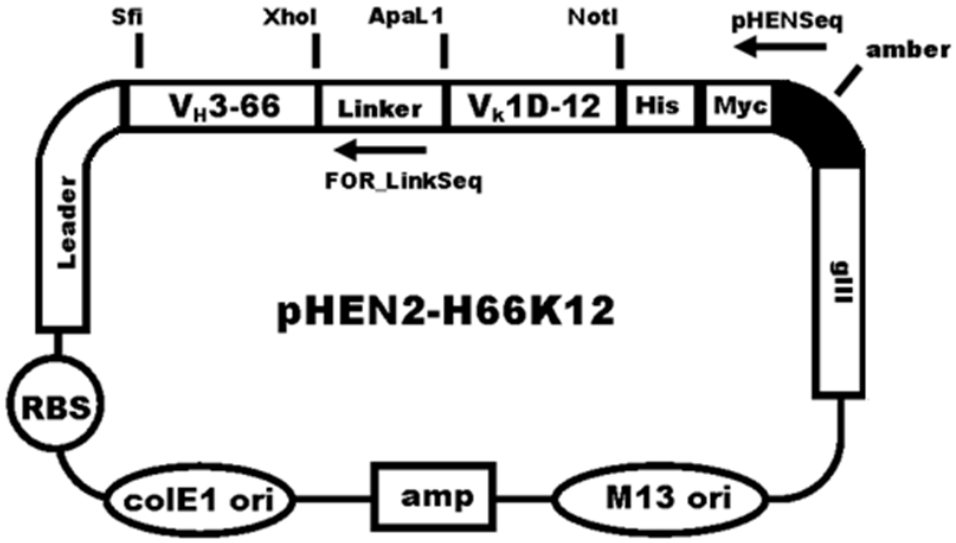
Results

The Recombinant Xenoantibody, H66K12, Inhibits FVIII Activity in Vitro

Our initial investigation utilized recombinant FVIII and a recombinant monoclonal scFv xenoantibody, H66K12, because of the ease with which they could be experimentally manipulated. H66K12 was constructed from representative cloned cDNA IgM sequences derived from baboons demonstrating an active xenoantibody response at day 28 after transplantation with 10,000 IEQ/Kg GTKO/hCD55/hCD59/hHT transgenic porcine NICC (Fig. 1A,B). These baboons had developed a xenoantibody response despite treatment with a typical immunosuppressive protocol, including a combination of induction with ATG and ongoing treatment with mycophenolate mofetil and tacrolimus. FVIII was diluted to 150 IU/dl, 100 IU/dl, or 50 IU/dl with FVIII dilution buffer alone or in combination with either H66K12 or a control matched by total protein (50 μg/ml). The final concentration of 28 KDa xenoantibody was 7.75 μg/ml (275 nM). While this

antibody concentration is well below the normal physiologic concentration of total antibody (630–1830 nM IgM + IgG), it is well above the physiologic concentration of xenoantibody (0.3–1.2 nM IgM + IgG) [20]. However, this experiment was meant to indicate, convincingly, that recombinant xenoantibody is capable of effectively inhibiting the cofactor activity of FVIII. Compared with control, H66K12 significantly inhibited FVIII cofactor activity at every concentration of FVIII ($P < 0.001$; Fig. 1C).

A



B

<---IGHV------------------------------
FW1 CDR1 FW2 CDR2
EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYYMSWVRQAPGKALEWVSYISSGGSTYYADSVKGRFTISRDNSKNTL

-----------------------------------> Linker <--------------------
FW3 CDR3 FW1
SLQMNSLRAEDTAVYYCARGGVFTIVVDYWGQGVLVTVSSSSGGGGSGGGGSGGSAQDIQMTQSPSSVSASVGDKVTI

----------------------IGKV--
CDR1 FW2 CDR2 FW3 CDR3
TCHASQGISSWLAWYQQKPGKAPKLLIYKASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCRQGSSYPYSFG

---------> 6xHis <---Myc--->

QGTKVEIKRAAAAHHHHHHGAAEQKLISEEDLN

C

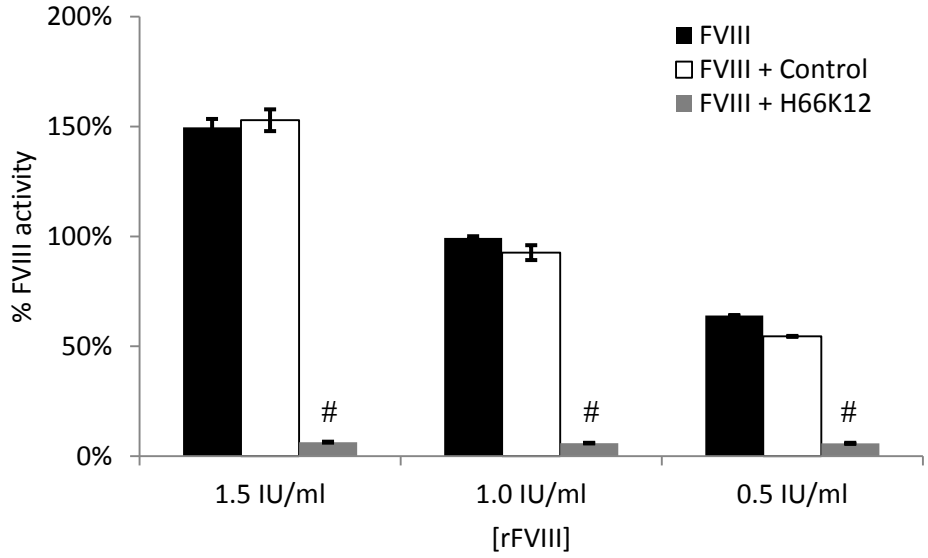


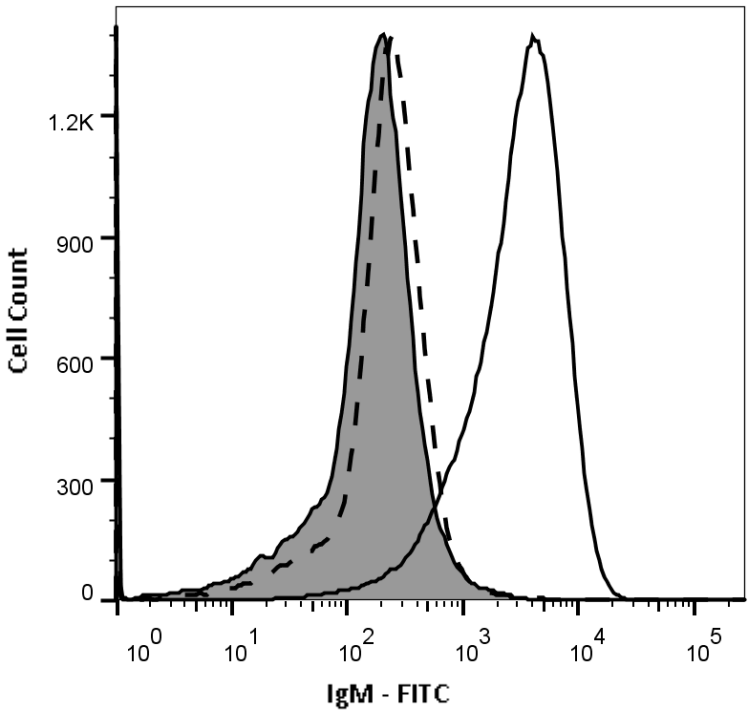
Figure 1. Recombinant single chain xenoantibody (H66K12) inhibits FVIII cofactor activity. A recombinant single chain xenoantibody, H66K12, was constructed from representative cloned cDNA IgM sequences derived from baboons demonstrating an active xenoantibody response at day 28 after transplantation with GTKO/hCD55/hCD59/hHT transgenic porcine NICC. (A) Schematic of pHEN phagemid containing the H66K12 single chain antibody encoded by the IGHV3-66 and IGKV1-12 germline genes. (B) Amino acid translation of the recombinant anti-nonGal single chain antibody H66K12. Complementarity determining regions are labeled according to the Kabat annotation system. (C) Recombinant FVIII was diluted to the final concentration indicated. FVIII alone or FVIII with 50 ug/ml of protein (control) was used for comparison. H66K12 was added to a final concentration of 7.75 ug/ml (275 nM) and matched to the control by total protein. A two-stage chromogenic assay was used to determine resulting FVIII cofactor activity. H66K12 significantly inhibited FVIII cofactor activity. (# indicates $p < 0.001$). Ampicillin resistance gene (amp), origin of replication (ori), Ribosomal binding site (RBS), Heavy chain (IGHV), Light chain (IGKV), FWR (framework region), CDR (complementarity determining region), Recombinant xenoantibody (H66K12) - rXAb.

Serum Derived Xenoantibody Inhibits FVIII Activity in Vitro

We have previously used intravenous injection of GTKO porcine endothelial cells to initiate a humoral immune response in rhesus monkeys to study the DNA and amino acid sequences of the induced xenoantibodies [15]. These animals were not provided any form of immunosuppression. In the current study, we used samples from these experiments to assay for an escalation of xenoantibodies which inhibit FVIII cofactor activity. Flow cytometry was used to measure serum IgM binding to GTKO porcine endothelial cells. At day 7, binding was 71.6% compared with 0.66% at day 0, confirming the initiation of an anti-non-Gal immune response (Fig. 2A).

Clotting factor VIII was diluted with FVIII dilution buffer alone or in combination with either human or monkey serum (1/20 final serum dilution). FVIII concentrations were maintained within what is considered the normal human physiologic range. Inhibition of FVIII activity by pre-transplant serum may reflect structural differences between human and rhesus monkey FVIII. Data collected using serum from four animals demonstrated significantly increased inhibition of FVIII cofactor activity after immunization at FVIII concentrations 150 IU/dl ($P < 0.05$), 100 IU/dl ($P < 0.05$), and 50 IU/dl ($P < 0.001$; Fig. 2B). At 150 IU/dl and 100 IU/dl FVIII concentration, activity was reduced by 60% when comparing inhibition by pre- and post-transplant serum. This is consistent with an increase in inhibitor titer by approximately 16 Bethesda units (Bu); where an increase over 5 Bu in humans can indicate pathology.

A


1.2K
900
600
300
0
Cell Count
10^0
10^1
10^2
10^3
10^4
10^5
IgM - FITC

B

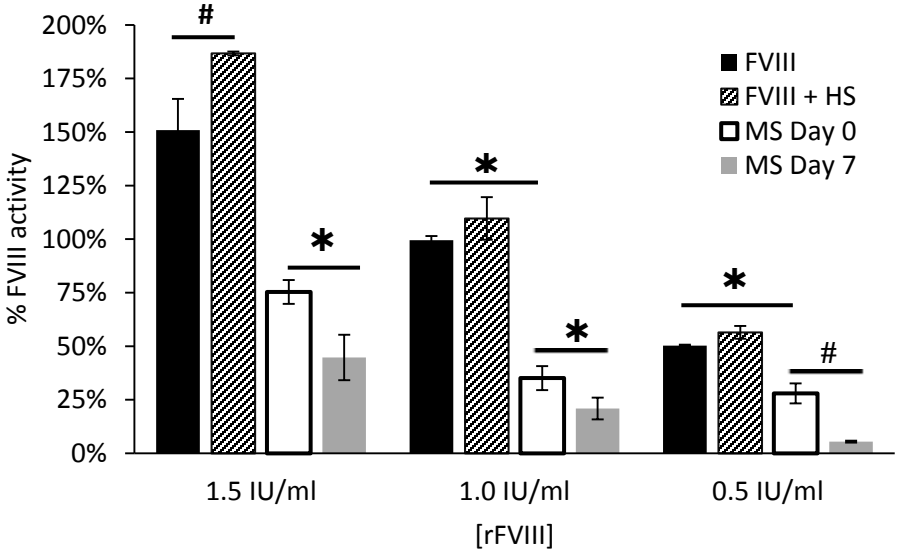

200%
175%
150%
125%
100%
75%
50%
25%
0%
% FVIII activity
#
*
*
*
*
#
FVIII
FVIII + HS
MS Day 0
MS Day 7
1.5 IU/ml
1.0 IU/ml
0.5 IU/ml
[rFVIII]

Figure 2. Serum antibody from rhesus monkeys immunized with GTKO porcine endothelial cells inhibits FVIII cofactor activity. (A) Rhesus monkeys were immunized with GTKO porcine endothelial cells to stimulate an anti-nonGal humoral immune response. Flow cytometry was used to measure serum IgM binding to GTKO endothelial cells. The dashed and solid curves with no shading represent binding at days 0 and 7, respectively. The shaded curve represents background determined by incubation with secondary antibody alone. (B) A two-stage chromogenic assay was used to measure FVIII activity. Recombinant FVIII was diluted to the final concentrations indicated. FVIII alone or FVIII with human serum diluted 1/20 was used as controls. The extent to which FVIII cofactor activity was inhibited by xenoantibody at day 0 and 7 was compared in four animals. There was a significant decrease in FVIII cofactor activity with the addition of post-immunization serum at day 7, compared to day 0, at every FVIII dilution. (* indicates $p < 0.05$; # indicates $p < 0.001$) Human serum – HS; Monkey serum (MS).

Computer Modeling Predicts Induced Xenoantibody Binds the FVIII C1 Domain

Computer modeling was used to address the question of which FVIII domain was bound by H66K12. Using homology, we generated an Fv structural model. All templates used are listed in Table 1. Figure 3 depicts a Ramachandran plot demonstrating the favorable energetics of our final model. A computer-based protocol for predicting the relevant epitope was first validated by accurately predicting the inhibitory FVIII epitope of Bo2C11 (1iqd) using the crystal structure of human FVIII (3CDZ). This protocol was able to accurately predict 14 of 18 FVIII residues which come within 5 Å of Bo2C11 (Fig. 4). The A1 domain was excluded because those FVIII inhibitors characterized to date are known to predominantly bind the A2, A3, C1, and C2 domains, but not the A1 domain [19, 21]. The ZDOCK docking algorithm was used to search for initial complementarity between FVIII and H66K12 based on the shape. The top-ranked binding orientations (poses) from the twenty largest clusters of poses were further refined using the RDOCK algorithm which incorporates electrostatic interactions. All of the top twenty final binding orientations were nearly identical (average RMSD 9.99) and bound the C1 domain. The top scoring pose bound to FVIII is illustrated in Fig. 5.

Table 1. Templates used for homology modeling

Region	PDB	Similarity	Identity	Resolution	Organism
HFR	3bn9	98.9%	90.1%	2.173	Homo sapiens
LFR	3bn9	94.5%	94.4%	2.173	Homo sapiens
HCDR1	1fh5	100.0%	100.0%	2.9	Homo sapiens
	2h1p	100.0%	87.5%	2.4	Mus musculus
	2v17	87.5%	75.0%	1.65	Mus musculus
HCDR2	3ls5	85.7%	85.7%	1.9	Mus musculus
	3cfb	85.7%	85.7%	1.6	Mus musculus
	3utz	85.7%	85.7%	2.18	Escherichia coli
HCDR3	3l95	66.7%	58.3%	2.19	Escherichia coli
	3r1g	58.3%	50.0%	2.8	Homo sapiens
	3eyo	75.0%	41.7%	2.5	Homo sapiens
LCDR1	3hmw	100.0%	100.0%	3	Homo sapiens
	3bn9	100.0%	83.3%	2.173	Homo sapiens
	2x7l	83.3%	83.3%	3.17	Escherichia coli
LCDR2	1dfb	100.0%	100.0%	2.7	Homo sapiens
	2cmr	100.0%	100.0%	2	Homo sapiens
	3ncj	66.7%	66.7%	1.6	Homo sapiens
LCDR3	1yjd	88.9%	55.6%	2.7	Mus musculus
	3ncj	77.8%	44.4%	1.6	Homo sapiens
	1iai	77.8%	55.6%	2.9	Mus musculus

H- heavy; L- light; FR- framework region;
CDR- complementarity determining region

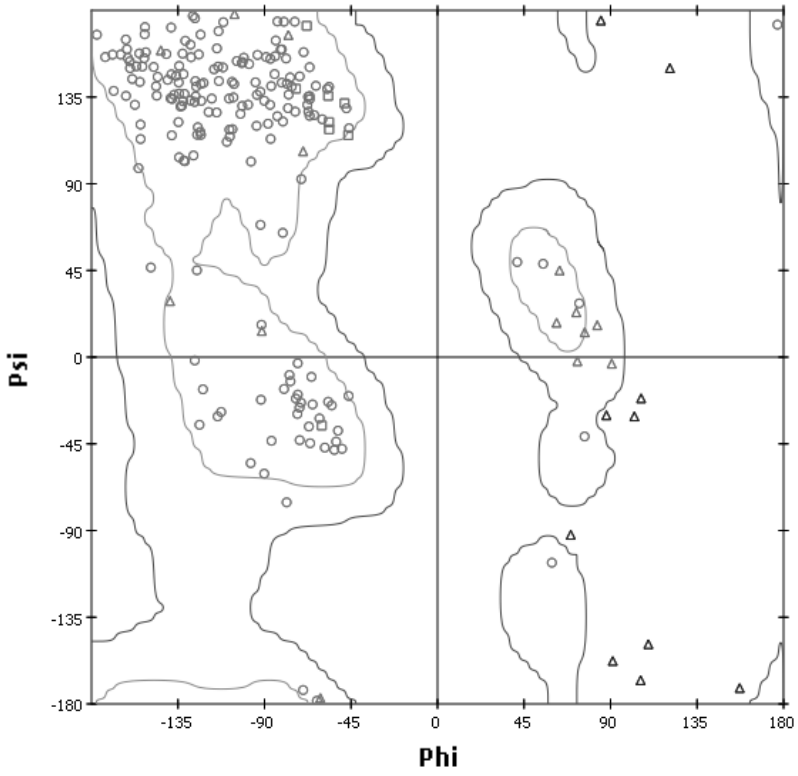

Psi
Phi
135
90
45
0
-45
-90
-135
-180
-135
-90
-45
0
45
90
135
180

Figure 3. Ramachandran plot of the refined model of H66K12. Ramachandran plot of the refined model of the xenoantibody derived from representative cloned cDNA IgM sequences from baboons demonstrating an active xenoantibody response at day 28 after transplantation with GalKO/hCD55/hCD59/hHT transgenic porcine islets. Triangles represent glycine residues, squares represent proline residues, while circles represent all other amino acids.

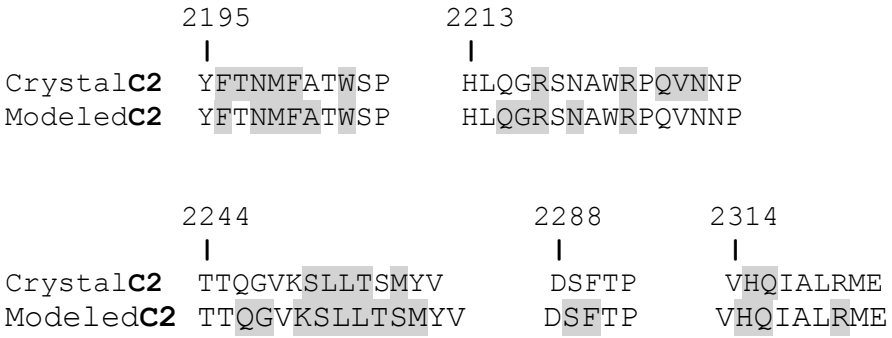

2195
2213
CrystalC2 YFTNMFATWSP HLQGRSNAWRPQVNNP
ModeledC2 YFTNMFATWSP HLQGRSNAWRPQVNNP
2244
2288
2314
CrystalC2 TTQGVKSLLTSMYV DSFTP VHQIALRME
ModeledC2 TTQGVKSLLTSMYV DSFTP VHQIALRME

Figure 4. Molecular modeling accurately predicts 14/18 FVIII C2 domain amino acid residues that make contact with Bo2C11. A computer-based protocol for predicting the epitope bound by a particular anti-FVIII antibody was first validated by accurately predicting the domain bound by Bo2C11. Of 18 FVIII residues which come within 5 Å of Bo2C11 in the deposited crystal structure (PDB ID 1iqd), 14 residues are accurately predicted using molecular modeling. Highlighted residues come within 5 Å of Bo2C11; Modeled C2 indicates residues predicted using computer modeling.

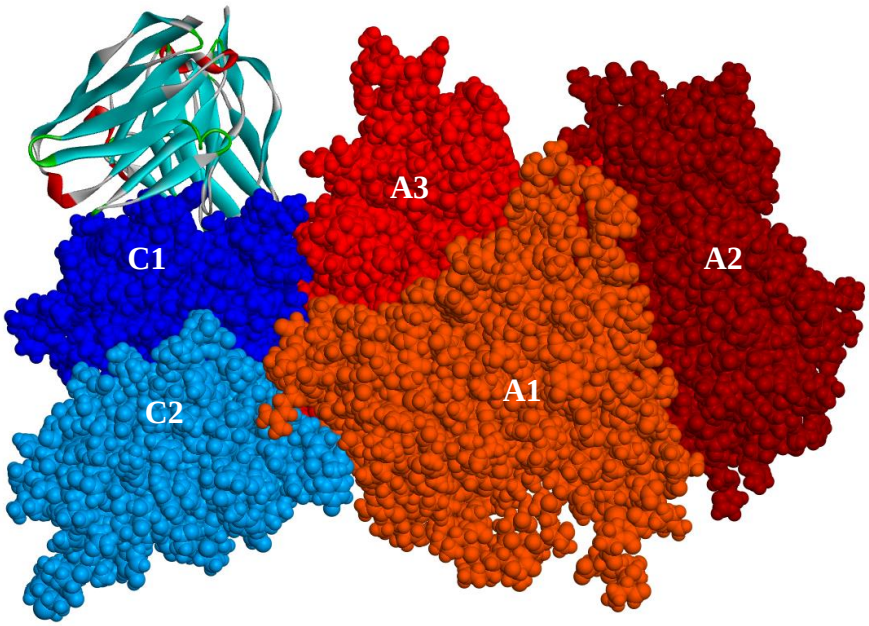

A3
C1
A2
A1
C2

Figure 5. *In silico* protein-docking algorithms predict xenoantibody binds the FVIII C1 domain. Representative cloned IgM cDNA sequences from baboons actively responding to transplantation of GTKO/hCD55/hCD59/hHT porcine NICC were used to model the variable fragment of H66K12 by homology. Docking this antibody model with the crystal structure of human FVIII (PDB ID 3CDZ) using shape complementarity, then refinement with electrostatics, predicted binding to the C1 domain of FVIII. FVIII is depicted as a space filling model and domains are labeled. The best binding motif of H66K12 is depicted as a ribbon model.

Competition ELISA Confirms Induced Xenoantibody Binds the FVIII C1 Domain

We used a competition ELISA to determine the accuracy of the *in silico* epitope prediction. We reasoned that if we captured FVIII using the recombinant xenoantibody H66K12, we should be able to detect it with an antibody which binds elsewhere on the light chain (GMA-8010), but not an inhibitory antibody which binds the C1 domain (GMA-8011). This is because H66K12 would mask the epitope recognized by GMA-8011, but not by GMA-8010. Our results illustrated in Fig. 6 demonstrate that the in silico prediction was correct. FVIII captured by H66K12 is detectable with GMA-8011, but not by GMA-8010. Furthermore, this effect is not present if a control protein suspension generated from an empty vector is used to "capture" FVIII.

In Silico Binding Site Mutation Analysis

To determine which residues of both H66K12 and FVIII contribute the most to binding, polyalanine scanning was performed *in silico*. Table 2 provides a list of residues which, when mutated, stabilize or destabilize the protein–protein interaction. Those residues in the xenoantibody are of particular interest. The heavy chain amino acid sequence induced in baboons in response to transplantation of GTKO/hCD55/hCD59/hHT transgenic porcine NICC and in rhesus monkeys immunized with GTKO porcine endothelial cells are 92% similar [16]. Furthermore, the light chain germline variable genes utilized in response to wild-type porcine organs and GTKO/hCD55/hCD59/hHT are the same [16]. Here, we report that the most important amino acids of both the heavy and light chains are conserved in each case; Tyr33 in the

heavy chain and Trp32 in the light chain. Thus, rational design of xenoantibody inhibitors would target these residues.

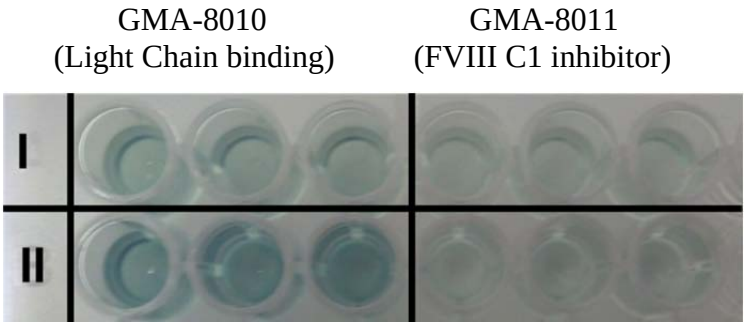
Alignment of human and porcine amino acid sequences of FVIII (Fig. 7A) demonstrates that most amino acid residues, which are predicted, to contribute to this interaction are conserved. Interestingly, Phe2068, present only in human FVIII, is predicted to be the most important individual amino acid. Those FVIII residues which are predicted to interact favorably with xenoantibody (the most destabilizing mutations) are illustrated in Fig. 7B.

Table 2. *In silico* polyalanine scanning to predict amino acid contributions to FVIII-Fv interaction

FVIII - C1	Kcal/mol*	Fv - LC	Kcal/mol*	Fv - HC	Kcal/mol*
Arg2150	+1.72	Lys50	-2.45	Try100c	+1.29
Met2104	+1.82	Ser30	-1.92	Tyr33	+2.58
Trp2046	+2.07	Ser31	-1.84		
Trp2070	+2.42	Leu33	-1.52		
Phe2068	+4.43	Ser53	-1.26		
		Ser52	-1.22		
		Gly91	+1.20		
		Try95e	+1.74		
		Trp32	+5.18		

Fv- variable fragment; LC- light chain; HC- heavy chain; *predicted

A


GMA-8010
(Light Chain binding)
GMA-8011
(FVIII C1 inhibitor)
I
II

B

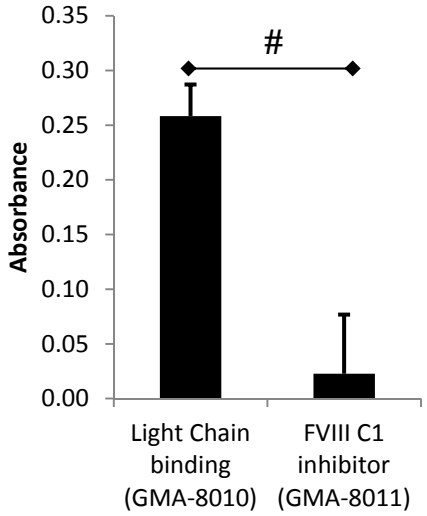

0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Absorbance
#
Light Chain binding (GMA-8010)
FVIII C1 inhibitor (GMA-8011)

Figure 6. Competition ELISA confirms xenoantibody binds to the FVIII C1 domain. Protein generated from a control construct (I) or H66K12 (50 ug/ml) (II) was used to capture FVIII. Capture solutions were matched for total protein content of 132 ug/ml. FVIII was detected with either a FVIII C1 domain-binding inhibitory antibody (GMA-8011) or an antibody which binds elsewhere on the FVIII light chain (GMA-8010). The results are illustrated both as a picture (A) and as a graph (B). # indicates $p < 0.01$; Light chain – LC; A – control capture solution; B – Capture with recombinant xenoantibody.

A

```
           2010      2030      2040      2050      2060      2070
            |         |         |         |         |         |
HSFVIII-C1  KCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYSGSINAWSTKEPFSW
SSFVIII-C1  E--AP------R---------------------------------D-H--

                   2080      2090      2100      2110      2120
                    |         |         |         |         |
HSFVIII-C1  IKVDLLAPMIIHGIKTQGARQKFSSLYISQFIIMYSLDGKKWQTYRGNSTG
SSFVIII-C1  --------------M-----------------------RN--S-------

                  2130      2140      2150      2160      2170
                   |         |         |         |         |
HSFVIII-C1  TLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIRSTLRMELMGCDLN
SSFVIII-C1  ----------A------------V---------------------------
```

B

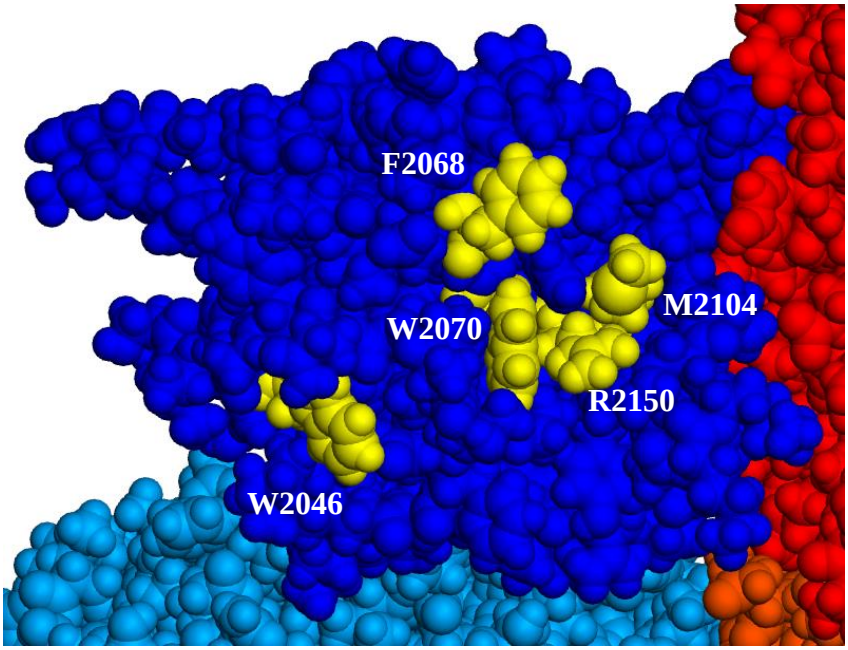


Figure 7. Four of five FVIII residues important for xenoantibody binding are homologous with porcine FVIII. (A) Alignment of human and porcine FVIII amino acid sequences with residues thought to contribute to binding recombinant xenoantibody highlighted and in bold. (B) The structure of FVIII C1 domain (blue) with predicted binding sites labeled and highlighted in yellow. (Sus scrofa accession NM_214167.1; Homo sapiens extracted from crystal structure PDB ID 3cdz)

Discussion

The impact of the immune system on coagulation dysregulation during xenotransplantation and xenograft rejection remains to be fully elucidated. We report the generation of antibodies, which inhibit human FVIII cofactor activity after transplantation of immunosuppressed baboons with GTKO/hCD55/hCD59/hHT transgenic porcine NICC and immunization of rhesus monkeys with GTKO porcine endothelial cells. While a prothrombotic response, such as consumptive coagulopathy, is predominant during acute xenograft rejection within hours to days, development of FVIII inhibitors is likely to impact long-term xenotransplantation. The recombinant xenoantibody, H66K12, generated from post-NICC xenotransplantation IgM xenoantibody sequences, binds to the C1 domain of FVIII. Targeting the xenoantibody pocket, which makes contact with FVIII, may be a promising strategy for selectively countering these inhibitors. However, it is unknown whether other FVIII domains are targeted as well. Nevertheless, FVIII inhibitors developed in response to transplantation with GTKO cells represent a novel pathologic effect on recipient physiology.

Clotting factor VIII is synthesized as a 330-KDa precursor protein with an A1-α1-A2-α2-B-α3-A3-C1-C2 domain structure [21]. Inhibitory antibodies against FVIII are known to bind several domains including the A2, A3, C1, and C2 domains in addition to the acidic α1 region between the A1 and A2 domains. In humans, inhibitors against the C2 domain have been primarily determined to belong to the VH1 family [22]. However, these antibodies are often extensively modified by hypersomatic mutation and have atypically long CDR3 loops (20–23 amino acids). In contrast, inhibitors that bind the A2 and A3 domains display more variation. Anti-A2 antibodies have been reported from

VH1, VH3, VH5, and VH6 families while anti-A3 antibodies are derived from VH1 and VH3 families [23, 24]. By comparison, the VH genes that generate inhibitors of the C1 domain have not been previously characterized. However, analysis of the deposited sequence of KM33, which binds the C1 residues Lys2092 and Phe2093 [25], demonstrates 87.8% identity to IgHV3-30 providing an additional C1 inhibitor which utilizes a member of the VH3 family. The light chain antibodies that contribute to FVIII inhibitors have not been so extensively characterized. However, similar to H66K12, KM33 also utilizes a VK1 family member and demonstrates 86.2% identity to IgKV1-6.

Clotting factor VIII inhibitors that bind the C1 domain can affect interactions with either vWF [26] or phosphatidylserine [27, 28]. Given that the FVIII preparation utilized in this study is vWF free [29], inhibition of membrane binding is the more relevant mechanism *in vitro* for the induced xenoantibody. However, human antibodies that bind to FVIII Arg2150 inhibit FVIII binding to vWF. As H66K12 is predicted to interact with Arg2150, it likely inhibits this interaction and would reduce FVIII half-life *in vivo*. The C1 domain is known to contribute to membrane binding [27] via Lys2092 and Phe2093 [28]; however, these residues are relatively distant from the predicted binding site. Recent experiments using fluorescence energy resonance transfer have determined the angle at which FVIII interacts with phospholipid membranes [30]. Given the distance from Lys2092 and Phe2093, H66K12 might bind residues which interact directly with phospholipid or act in a steric fashion. At least one amino acid predicted to interact with H66K12, Trp2046, is only one residue away from a large, positively charged region of the FVIII C1 domain predicted to interact with negatively charged phosphatidylserine. Binding of FVIII to vWF significantly increases its half-life while interaction with

phospholipid is essential for cofactor function [21]. *In vivo*, it is likely that both these mechanisms can contribute to diminished FVIII function. However, the specific mechanism(s) by which induced xenoantibody inhibits FVIII function will require further investigation.

In this study, we used human FVIII as a source of primate FVIII assuming the sequences are largely similar; the sequences of rhesus monkey and baboon FVIII have not been characterized. In the context of xenotransplantation, it is reasonable to speculate that FVIII inhibitors are initiated by porcine FVIII. Humans can "break" tolerance when they develop acquired hemophilia A [31] or when provided an exogenous source of FVIII via FVIII replacement therapy [11-13]. Thus, xenotransplantation may trigger a lapse in tolerance to the FVIII autoantigen by providing a source of exogenous FVIII. Four of five FVIII residues predicted to interact most favorably with H66K12 are conserved between pigs and humans. However, a single amino acid difference can contribute to the development of inhibitors [12, 13], and humanization of porcine FVIII C1 may preempt formation of antibodies directed at this domain. Of note, there is evidence that anti-CD20 therapy can mitigate the acquisition of FVIII inhibitors in both acquired and inherited hemophilia A [31]. However, in the context of xenotransplantation, anti-CD20 therapy represents a significant addition to an already cumbersome immunosuppressive burden.

The prevalence of induced anti-FVIII antibodies in other xenotransplantation settings is currently unknown. However, FVIII has recently been demonstrated to be expressed in the microvasculature of multiple sites including the liver but also lung, heart, intestine, and dermis [9, 10]. Each model of xenotransplantation has variations in pathology and time to rejection with typical immunosuppressive regimens [1, 32-36].

Current reports in xenotransplantation provide clues as to where antibodies against FVIII might contribute to pathology.

The level of FVIII in the porcine serum is significantly higher than that of baboons [37]. When the anti-fibrinolytic agent Amicar is provided before xenograft reperfusion to prevent thrombocytopenia, it does not overcome spontaneous bleeding. Initial investigations provide normal coagulation parameters for genetically modified pigs or typical primate species utilized in xenotransplantation [38]. However, the "normal" coagulation parameters expected after xenotransplantation are currently under investigation [4, 37]. Our results indicate pre-transplant serum antibody can also affect FVIII activity and further obscure the practicality of traditional assays using human clotting factors. To thoroughly dissect coagulation dysregulation in xenotransplantation, partial thromboplastin time and international normalized ratio assessment should be used in combination with ELISA for clotting factors and chromogenic assays to assess specific factor activity levels and the impact of acquired inhibitors.

Mohiuddin et al. [5] reported extended survival times during cardiac xenotransplantation with the addition of B cell depleting therapy. However, the presence of remaining IgM, but primarily IgG, deposition at explant provides evidence of remaining humoral impact. Interestingly, three of those animals with extended survival times were noted to have died from abdominal bleeding due to unknown causes.

Our co-authors have recently performed thromboelastographic analysis of cynomolgus monkeys after transplantation of porcine GTKO kidneys with various transgenes. They demonstrate progressive prolongation of clotting time via the intrinsic clotting cascade which could be accounted for by either severe deficiencies of the

intrinsic clotting cascade or alternatively, FVIII inhibitors [4]. However, the expression of FVIII in the renal microvasculature has never been investigated.

The specific impact of anti-FVIII antibodies on delayed humoral xenograft rejection in each model of xenotransplantation will require further investigation. Whether FVIII inhibitors precipitate graft rejection or exacerbate more prevalent xenograft pathology will need to be determined. Those grafts that survive long enough to be subject to an adaptive humoral response are more likely to generate FVIII inhibitors. Future experiments should incorporate monitoring of anti-FVIII antibodies. The development of FVIII inhibitors in xenotransplantation is, however, an illustration of the functional impact the humoral immune response can illicit. The expansion of xenoantibodies, which are capable of binding both human and non-human primate proteins, is not likely to be an isolated incident, and xenoantibodies may have other functional effects which alter the physiology of both host and graft.

Acknowledgements

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CHAPTER 4

CLONIDINE INHIBITS ANTI-NON-GAL IGM XENOANTIBODY ELICITED IN MULTIPLE PIG-TO-PRIMATE MODELS

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TITLE: Clonidine inhibits anti-non-Gal IgM xenoantibody elicited in multiple pig-to-primate models

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RUNNING HEAD: Small molecules inhibit xenoantibody

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Abstract

Background: Survival of vascularized xenografts is dependent on preemptive inhibition of the xenoantibody response against galactosyltransferase knockout (GTKO) porcine antigens. Our analysis in multiple GTKO pig-to-primate models of xenotransplantation has demonstrated that the anti-non-gal-α-1,3-gal (anti-non-Gal) xenoantibody response displays limited structural diversity. This allowed our group to identify an experimental compound which selectively inhibited induced anti-non-Gal IgM xenoantibodies. However, because this compound had an unknown safety profile we extended this line of research to include screening small molecules with known safety profiles allowing rapid advancement to large animal models.

Methods: The NIH clinical collections of small molecules were screened by ELISA for their ability to inhibit xenoantibody binding to GTKO pig endothelial cells. Serum collected from non-immunosuppressed rhesus monkeys at day 14 after injection with GTKO pig endothelial cells was utilized as a source of elicited xenoantibody for initial screening. Virtual small molecule screening based on xenoantibody structure was used to assess the likelihood that the identified small molecules bound xenoantibody directly. As a proxy for selectivity, ELISAs against tetanus toxoid and the natural antigens laminin, thyroglobulin, and single stranded DNA (ssDNA) were utilized to assess the ability of the identified reagents to inhibit additional antibody responses. The identified inhibitory small molecules were further tested for their ability to inhibit xenoantibody elicited in multiple settings including: rhesus monkeys pre-treated with an anti-non-Gal selective anti-idiotypic antibody, non-immunosuppressed rhesus monkeys

immunized with wild type fetal pig islet-like cell clusters, and non-immunosuppressed baboons transplanted with GTKO multiple transgenic pig kidneys.

Results: Four clinically relevant small molecules inhibited anti-non-Gal IgM binding to GTKO pig endothelial cells *in vitro*. Three of these drugs displayed a limited region of structural similarity suggesting they may inhibit xenoantibody by a similar mechanism. One of these, the anti-hypertensive agent clonidine, displayed only minimal inhibition of antibodies elicited by vaccination against tetanus toxoid or pre-existing natural antibodies against laminin, thyroglobulin, or ssDNA. Furthermore, clonidine inhibited elicited anti-non-Gal IgM from all animals that demonstrated a xenoantibody response in each experimental setting.

Conclusions: Clinically relevant small molecule drugs with known safety profiles can inhibit xenoantibody elicited against non-Gal antigens in diverse experimental xenotransplantation settings. These molecules are ready to be tested in large animal models. However, it will first be necessary to optimize the timing and dosing required to inhibit xenoantibodies *in vivo*.

Introduction

Xenotransplantation of genetically modified porcine organs and cells is approaching clinical relevance (1). Multiple laboratories have demonstrated xenoislet survival of over one year using non-human primate recipients (2-4) and transplantation of encapsulated pig pancreatic islets is currently in clinical trials (5). More critically, in the extremely onerous heart transplant model, Mohiuddin et al. achieved a median survival time of over 200 days with a maximum survival time of approximately 600 days at the time of publication [reported in supplementary discussion in print edition], albeit in a non-life supporting pig-to-primate model (6). Preemptively inhibiting the xenoantibody response against non-α-1,3-gal terminal disaccharide (non-Gal) antigens present on pig xenografts is important for long term survival of vascularized xenografts (6-8) and thus translation to the clinic. To this end, perioperative B cell depletion with anti-CD20 dramatically prolongs survival of cardiac xenografts (8). However, in the context of transplantation, B cell depletion is known to result in a greater risk of infection and infection-related death (9-11). Targeted inhibition of the anti-non-Gal humoral immune response both perioperatively, and long-term postoperatively, could enhance xenograft survival while preserving the greater portion of B cell-mediated adaptive immunity to ward off infection.

Our group has previously demonstrated that the elicited anti-non-Gal xenoantibody response displays limited structural diversity in multiple galactosyltransferase knockout (GTKO) pig-to-primate models of xenotransplantation (12, 13). This enabled us to identify an anti-non-Gal selective anti-idiotypic single chain antibody and an experimental small molecule capable of selectively inhibiting induced

anti-non-Gal IgM xenoantibodies (14). Although *in vitro* this small molecule could inhibit the binding of residual IgM xenoantibody, in animals pre-treated with anti-idiotypic antibody, it had an unknown safety profile. We therefore extended this line of research to include screening of the NIH clinical collections, which consist almost entirely of small molecules with a history of use in clinical trials. We report here the identification of small molecule drugs with known safety profiles which selectively inhibit anti-non-Gal xenoantibody, allowing rapid translation to experiments in large animal models.

Materials and Methods

Animals

Fifteen juvenile and two adult rhesus monkeys (*Macaca mulatta*) from the California National Primate Research Center, University of California, Davis, CA were utilized in this study. All procedures met the requirements of the Animal Welfare Act. Protocols were approved prior to implementation by the University of California, Davis Institutional Animal Care and Use Committee.

Six baboons (*Papio hamadryas*) were supplied by the NH&MRC Australian National Baboon Colony, Sydney, Australia. All procedures were approved by Local Area Health Service Animal Ethics Committees and conducted in compliance with State Government legislation and NH&MRC Animal Research Guidelines.

Models of Xenotransplantation

Immunization with GTKO pig aortic endothelial cells

Fifteen juvenile rhesus monkeys were screened for low levels of non-Gal-reactive xenoantibody by ELISA as previously reported (14). Five animals with the lowest levels of pre-existing anti-nonGal IgM xenoantibody were selected for further study. Two non-immunosuppressed animals (NIS.A and NIS.B) were intravenously injected with GTKO pig aortic endothelial cells (PAECs) in the absence of immunosuppression. This allowed for the induction of an anti-nonGal xenoantibody response without the procedural complications of islet or solid organ xenotransplantation. Three additional animals (AIA.A-C) were pre-treated with a novel anti-non-Gal selective anti-idiotypic single chain antibody (B4N190). This anti-idiotypic antibody was generated in our laboratory using a sequence and structure based approach to select a reagent specific for induced anti-non-Gal antibody. The anti-idiotypic antibody was administered once per week for three weeks before immunization with GTKO PAECs.

Islet Xenotransplantation

Two non-immunosuppressed adult rhesus monkeys (ISLET.1-2) were immunized with wild type fetal porcine islet-like cell clusters in order to elicit a xenoantibody response, as previously reported (15). Briefly, fetal porcine islet-like cell clusters (15 x 10^6 cells) were prepared by culturing collagenase-digested pancreata from fetuses at 66 days of gestation (term ~114 days). Cells were cultured for 1-3 weeks before intraperitoneal injection on experimental day 0. Serum samples from the current set of studies were collected before immunization on day 0 and on day 8 after immunization.

Kidney Xenotransplantation

Genetically modified pigs (*Sus scrofa*), which were GTKO and co-expressed human CD55, CD59, and α 1,2-fucosyltransferase (H-transferase, HT) were generated by Cowan et al. (16, 17). Six non-immunosuppressed baboons (KIDNEY.1-6) were transplanted with GTKO/hCD55/hCD59/hHT pig kidneys. Additional details regarding the conditions of transplant will be published separately by our collaborators. The grafts from two animals (KIDNEY.1 and KIDNEY.2) survived over 100 hours. This was a sufficient length of time for the anti-non-Gal xenoantibody response to be detectable in peripheral blood. Serum samples from the current studies were collected from these two animals on day 0 and at the time of rejection.

Small Molecule Library

The NIH Clinical Collections 1 and 2 composed of 446 and 281 compounds respectively consist of drugs selected for their history of use in clinical trials, purity, solubility, and availability from commercial sources. The NIH Clinical Collections were provided through the National Institutes of Health Molecular Libraries Roadmap Initiative. Fifty microliters of each drug was provided in a 96-well plate format diluted to 10 mM in DMSO. Furthermore, these drugs were dispensed into 96-well plates in an anoxic environment and stored at ≤-70°C to ensure minimal degradation during shipping and storage before screening.

In Vitro Small Molecule Screening

The xenoantibody response and inhibition by small molecule drugs was measured

by ELISA as previously described (18) but using GTKO/hCD55 PAECs as targets. GTKO/hCD55 PAECs (NSRRC:0009) were purchased from the National Swine Research and Resource Center (Columbia, MO). Initial screening for drugs with the ability to inhibit xenoantibody responses was performed to identify promising candidates. These candidates were then tested in triplicate with at least two independent repeated experiments. Individual plates of drugs were allowed to thaw at room temperature before drugs were diluted to 60 μM in blocking solution for screening. Blocking solution was composed of 1x phosphate buffered saline (pH 7.4) with 0.05% tween-20 and 2% bovine serum albumin. This was then mixed 1+1 with serum (1:50 in blocking solution) to obtain a solution with a final drug concentration of 30 μM and serum 1:100 dilution. This solution was allowed to equilibrate at room temperature for 15-30 minutes before incubation with GTKO/hCD55 PAECs at room temperature for one hour. Anti-human IgM HRP (1:1000) (Jackson Immunoresearch, West Grove, PA) was used as a secondary antibody. Each plate of NIH Clinical Collection drugs was re-frozen at ≤-70°C after each study and utilized a maximum of five times. After initial screening each drug identified to inhibit anti-non-Gal IgM xenoantibody was purchased from a commercial source.

In Silico Small Molecule Screening

The anti-non-Gal IgM xenoantibody response is known to display a high degree of sequence variation in the heavy and light chain third complementarity determining regions (CDRs) (12, 13). Thus, inclusion of multiple xenoantibody structural models was required to refine our previous virtual screening process and account for this inherent structural variation. All three xenoantibody models were encoded by representative

xenoantibody sequences, which were highly similar within the CDR1 and CDR2 regions. Consistent with our previous analysis, each heavy chain utilized the IGHV3-66 and IGHJ4 germline genes. However, the heavy chain CDR3 amino acid translation displayed a high degree of variation. All three xenoantibody models utilized in our refined small molecule screening protocol were generated using the ROSIE antibody-modeling server (19), which has been demonstrated to have comparable accuracy to discovery studio (20) which we previously utilized. The combination of autodock vina (21) and PyRx (22) were used to perform virtual screening over the entire antigen-binding region of each model. The entirety of the NIH clinical collections was screened against each xenoantibody model. The results of each independent screen were ranked by predicted affinity. Those molecules from the NIH clinical collection which were predicted to be in the top 30% using all three xenoantibody structural models, and inhibited xenoantibody *in vitro*, were considered to be the most likely to bind directly to anti-non-Gal xenoantibody.

Natural Antibody ELISA

Natural antibody ELISAs were performed as previously described (14). Briefly, single stranded DNA (ssDNA) (Sigma, St. Louis, MO), laminin (Sigma, St. Louis, MO), and thyroglobulin (Sigma) were pre-coated on 96 96-well plates at a concentration of 20 µg/well. Serum (1:100) with, or without drug (30 µM), in blocking solution was incubated one hour at room temperature with the antigen of interest. Anti-human IgM HRP (1:1000) (Jackson Immunoresearch, West Grove, PA) was used as a secondary antibody. After 10 minutes incubation at room temperature with 100 µl of OPD solution

(Pierce Biotechnology, Rockford, IL) development was stopped using 50 µl of 2.5 M sulfuric acid and read at 490 nm.

Tetanus Toxoid IgG ELISA

Tetanus toxoid IgG titer ELISA was performed according to the manufacturer's instructions (Genway biotech, San Diego, CA) with one exception. Either a stock small molecule suspension or an equal volume of vehicle was added as necessary to the serum diluent of the experimental and control conditions respectively. Serum was diluted 1:101 as per the manufacturer's instructions while the final concentration of clonidine was 30 µM. A standard curve calibrated against the World Health Organization reference preparation enabled us to quantify anti-tetanus toxoid IgG in international units per milliliter (IU/ml).

Statistical Analysis

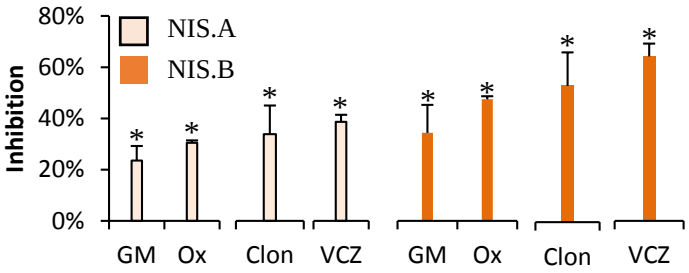
All statistical analyses were performed using Excel. Data is represented as mean ± standard error of the mean (SEM) of at least three technical replicates. Statistical significance (P-value < 0.05) was established using a 2-tailed Student's t-test or analysis of variance as appropriate.

Results

Screening for Clinically Relevant Small Molecule Drugs Capable of Inhibiting Anti-Non-Gal Xenoantibody

Serum collected from non-immunosuppressed animals 14 days after injection with GTKO PAECs was used as a source of anti-non-Gal IgM xenoantibody for screening. Drugs were initially screened for the ability to inhibit induced xenoantibodies from one monkey (NIS.A), because this animal had a larger anti-non-Gal xenoantibody response allowing more sensitive detection of inhibition. This also minimized the quantity of serum required in early stage screening experiments. However, promising candidates were retested for the ability to inhibit binding of xenoantibodies elicited in both NIS.A and NIS.B. Over the course of screening all 727 molecules, four compounds were identified to inhibit 23-64% of IgM xenoantibody elicited in non-immunosuppressed rhesus monkeys (Fig. 1). Gabexate mesilate, a protease inhibitor utilized in treatment of acute pancreatitis (23) was able to inhibit 24-34% of induced IgM xenoantibody. Oxiconazole, an azole anti-fungal agent (24) was able to inhibit 31-48% of induced IgM xenoantibody. Clonidine, an α_2 adrenergic receptor agonist most commonly used to treat hypertension (25) was able to inhibit 30-48% of induced IgM xenoantibody. Voriconazole, a triazole anti-fungal agent (24) was able to inhibit 39-64% of induced IgM xenoantibody.

A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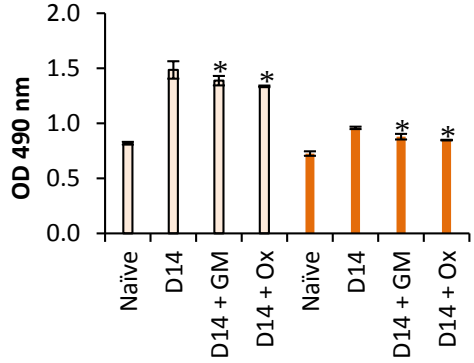
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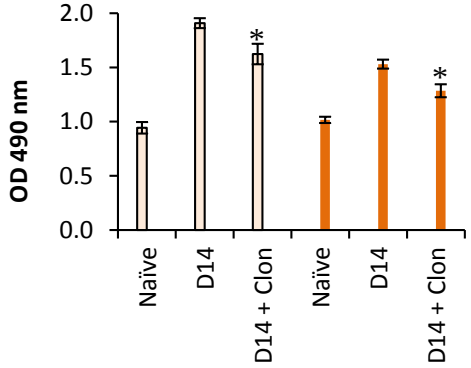
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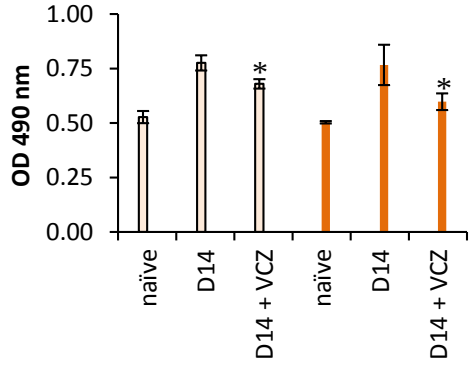
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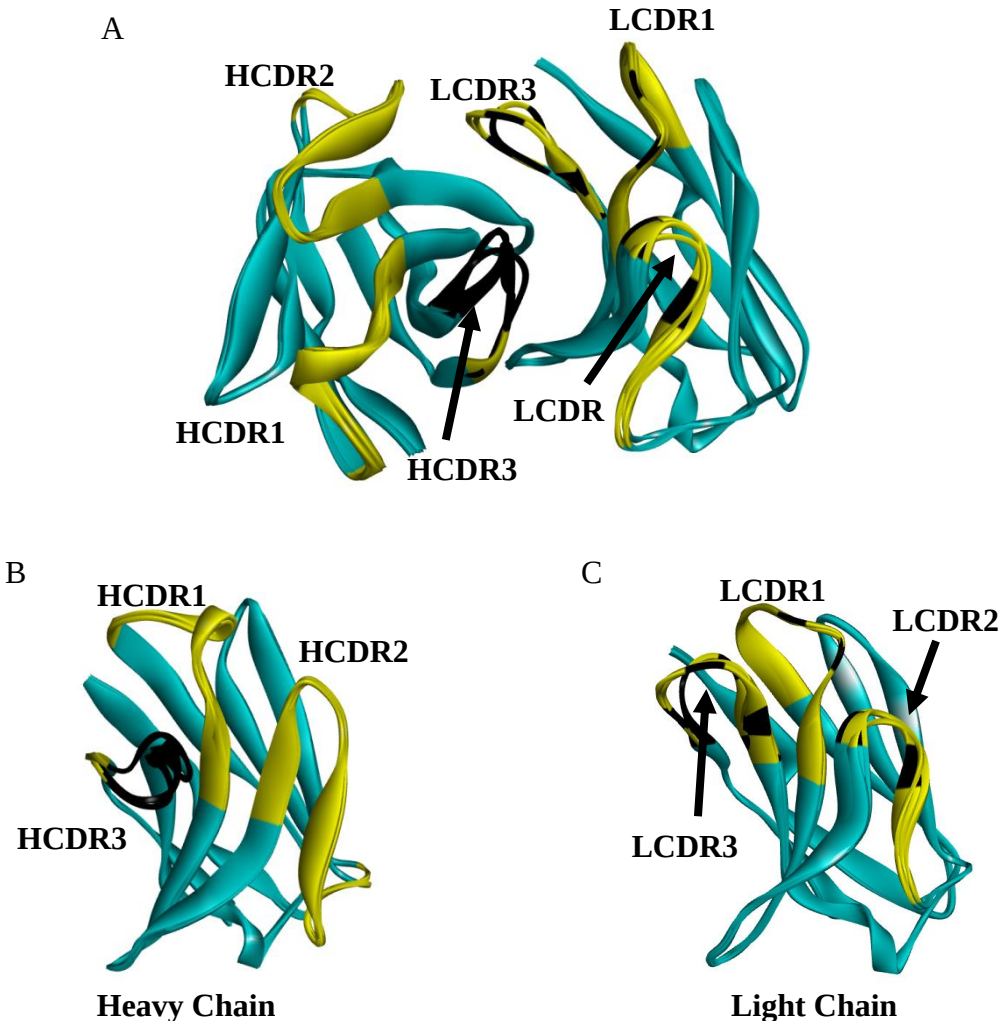
Figure 1. The NIH clinical collections of small molecule drugs were screened by ELISA against GTKO/hCD55 pig aortic endothelial cells. Serum collected from two non-immunosuppressed rhesus monkeys (NIS.A and NIS.B) at day 14 (D14) after immunization with GTKO pig endothelial cells was utilized as a source of elicited xenoantibody. Gabexate mesilate (GM), oxiconazole (OX), clonidine (Clon), and voriconazole (VCZ) were identified to inhibit elicited anti-non-Gal IgM. These drugs were able to inhibit 23.5-38.6% of the anti-non-Gal IgM xenoantibody elicited by NIS.A and 34.4-64.1% elicited by NIS.B. Values are reported as both the (A) % inhibition ± the standard error of the mean (SEM) and (B-D) the optical density (OD) ± SEM. % inhibition = 100% x [D14OD – (D14 with drug)OD]/(D14OD – D0OD); * indicates $P < 0.05$ compared to D14 without drug.

In Silico Small Molecule Screening Suggests Azole Antifungals Bind Directly to Xenoantibody

All xenoantibody sequences utilized in antibody modeling were representative of those elicited in baboons responding to xenotransplantation with GTKO/hCD55/hCD59/hHT neonatal islet-like cell clusters (13). We sought to refine our previous *in silico* screening process by more accurately representing the diversity of xenoantibody CDR3 regions. The ROSIE antibody-modeling server (19) was utilized to generate three xenoantibody models. Variations in xenoantibody CDR3 amino acid sequences resulted in notable differences in the predicted structure of the heavy chain CDR3 and to a lesser degree the light chain CDR3 (Fig. 2). However, these differences had little impact on the structures and relative positioning of the heavy and light chain CDR1 and CDR2 loops.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HCDR2
LCDR3
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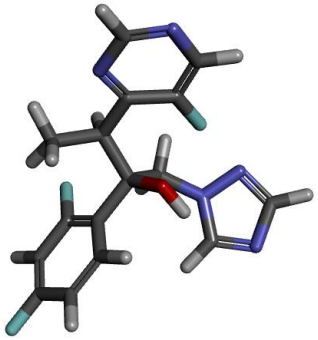
HCDR1
HCDR3
B
HCDR1
HCDR2
HCDR3
Heavy Chain
C
LCDR1
LCDR2
LCDR3
Light Chain

Figure 2. Three structural models of xenoantibody encoded by the germline genes IGHV3-66 and IGKV1D-12 are aligned. All xenoantibody sequences utilized for antibody modeling were derived from sequences elicited in baboons against GTKO/hCD55/hCD59/hHT neonatal islet-like cell cluster transplantation (13). (A) Computer modeling predicts that structure of the heavy chain complementarity determining region (CDR) 3 displays a greater degree of structural variation than the light chain CDR3. In contrast, the structures of the CDR loops 1 and 2 of both the (B) heavy and (C) light chain display minimal variation. CDR loops are highlighted in yellow for emphasis. Yellow – homologous region of complementarity determining region (CDR); black – non-homologous region of CDR; teal – Framework regions; HCDR – heavy CDR; LCDR – light chain CDR.

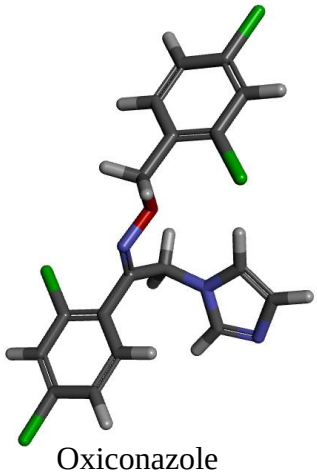
Neither gabexate mesilate nor clonidine were included in the list of molecules predicted to bind xenoantibody. However, four structurally related azole anti-fungal agents were predicted to bind all three xenoantibody structural models with high affinity including ketoconazole, voriconazole, clotrimazole, and oxiconazole. Given that both voriconazole and oxiconazole were identified to inhibit xenoantibody by screening *in vitro,* we were prompted to retest all azole antifungals included in the NIH clinical collection. Unfortunately, no additional xenoantibody inhibitors were identified. Interestingly, both oxiconazole and voriconazole are the most structurally similar pair of these four drugs. Furthermore, they share a limited region of structural similarity with clonidine (Fig. 3), suggesting all three molecules may share a similar mechanism of inhibition. Of note, both voriconazole and oxiconazole likely bind directly to anti-non-Gal xenoantibody given that they were both predicted *in silico* and identified *in vitro*. However, oxiconazole is only suitable for topical use and thus would not be appropriate for application as a xenoantibody inhibitor *in vivo*. Thus, further experiments did not include oxiconazole.

A



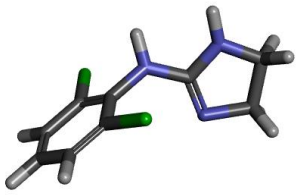
Voriconazole

B



Oxiconazole

C



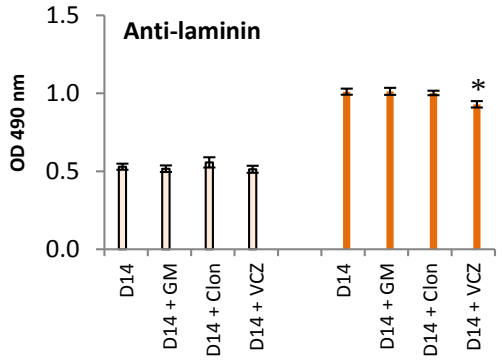
Clonidine

Figure 3. Voriconazole (A), oxiconazole (B), and clonidine (C), which were identified to inhibit anti-non-Gal IgM xenoantibody share a limited region of structural similarity suggesting they may inhibit xenoantibody utilizing a common mechanism. Molecules are depicted in three dimensions so as to highlight structural similarities. Grey – Carbon, white – hydrogen, red – oxygen, blue – nitrogen, green – chlorine, teal – fluorine.

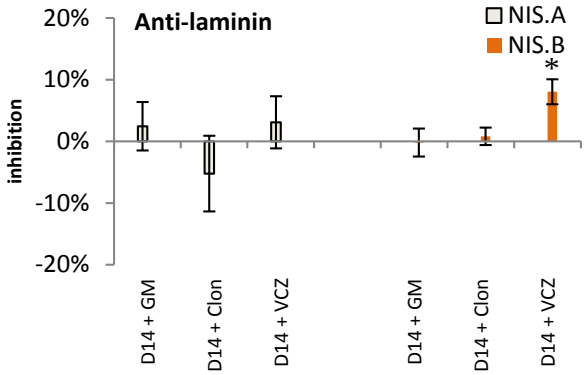
Natural IgM Antibodies are Minimally Affected by Clonidine and Moderately Affected by Gabexate Mesilate and Voriconazole

In order to determine whether the identified compounds could inhibit other antibody responses we tested their ability to interfere with natural IgM antibodies. Serum collected at day 14 after injection of NIS.A and NIS.B with GTKO PAECs was used as a source of antibody. This was essential because the identified compounds were known to inhibit IgM xenoantibody present at this time point. Ideally these compounds would not interact with natural antibodies which would suggest a high level of selectivity for anti-non-Gal xenoantibodies. Gabexate mesilate and voriconazole displayed a moderate level of selectivity while clonidine demonstrated a high level of selectivity for anti-non-Gal IgM xenoantibody (Fig. 4). Gabexate mesilate and voriconazole displayed minimal interference of binding to laminin (Fig 4A-B), however both inhibited a proportion of natural antibodies that bound to thyroglobulin (16-38%; Fig. 4C-D) and ssDNA (13-50%; Fig. 4E-F). In contrast, clonidine inhibited a relatively small proportion of anti-ssDNA antibody binding in only one animal (19 ± 1 % in NIS.B; Fig. 4E-F), and had no significant effect on other types of natural antibodies examined. Therefore, in this context, clonidine displayed the highest level of selectivity for anti-non-Gal IgM xenoantibody.

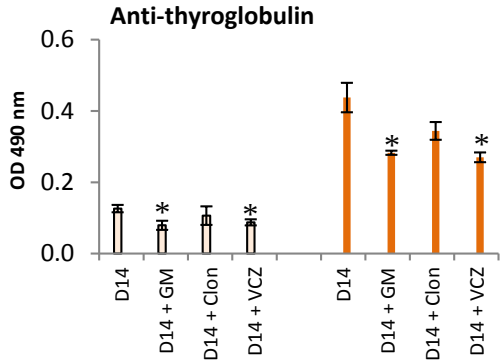
A


Anti-laminin
OD 490 nm
1.5
1.0
0.5
0.0
D14
D14 + GM
D14 + Clon
D14 + VCZ
D14
D14 + GM
D14 + Clon
D14 + VCZ
*

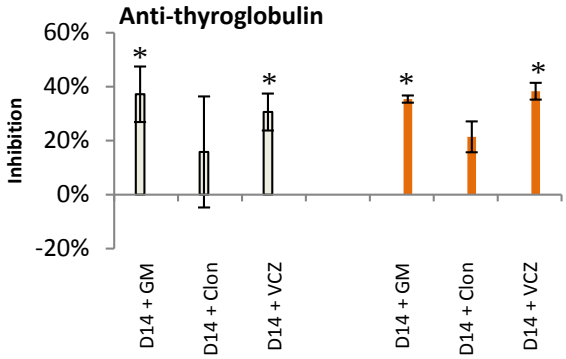
B


Anti-laminin
NIS.A
NIS.B
inhibition
20%
10%
0%
-10%
-20%
D14 + GM
D14 + Clon
D14 + VCZ
D14 + GM
D14 + Clon
D14 + VCZ
*

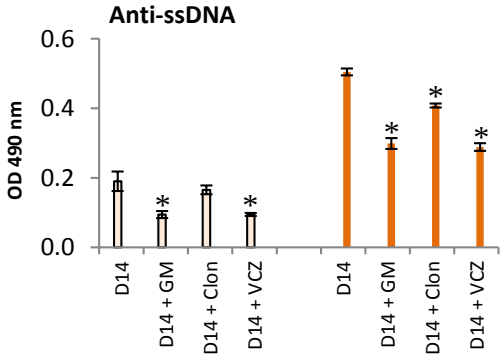
C


Anti-thyroglobulin
OD 490 nm
0.6
0.4
0.2
0.0
D14
D14 + GM
D14 + Clon
D14 + VCZ
D14
D14 + GM
D14 + Clon
D14 + VCZ
*

D


Anti-thyroglobulin
Inhibition
60%
40%
20%
0%
-20%
D14 + GM
D14 + Clon
D14 + VCZ
D14 + GM
D14 + Clon
D14 + VCZ
*

E


Anti-ssDNA
OD 490 nm
0.6
0.4
0.2
0.0
D14
D14 + GM
D14 + Clon
D14 + VCZ
D14
D14 + GM
D14 + Clon
D14 + VCZ
*

F

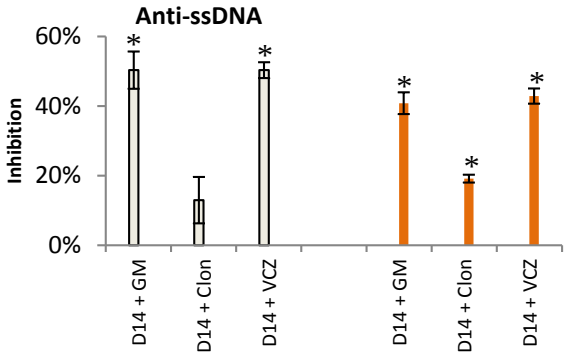
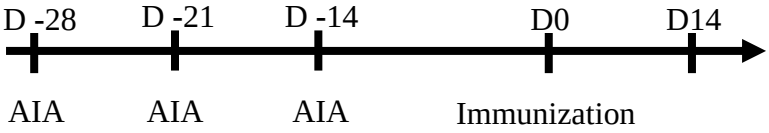

Anti-ssDNA
Inhibition
60%
40%
20%
0%
D14 + GM
D14 + Clon
D14 + VCZ
D14 + GM
D14 + Clon
D14 + VCZ
*

Figure 4. Clonidine has minimal impact on natural IgM antibodies from non-immunosuppressed rhesus monkeys. Gabexate mesilate (GM), clonidine (Clon), and voriconazole (VCZ) were tested for their ability to inhibit natural antibodies against (A-B) laminin, (C-D) thyroglobulin, and (E-F) single stranded DNA (ssDNA). These drugs minimally interfere with anti-laminin IgM natural antibodies (8 ± 2% inhibition by VCZ). However, both gabexate and voriconazole inhibit natural IgM antibodies against thyroglobulin (16-38%) and ssDNA (13-50%). In contrast, clonidine only inhibited anti-ssDNA antibodies from one of the two non-immunosuppressed animals (19 ± 1%). Values are reported as mean optical density (OD) and % inhibition ± the standard error of the mean; * indicates P <0.05 compared to day 14 without drug; Non-immunosuppressed animals – NIS.A and NIS.B; % inhibition = 100% x [D14OD – (D14 with drug)OD]/(D14OD).

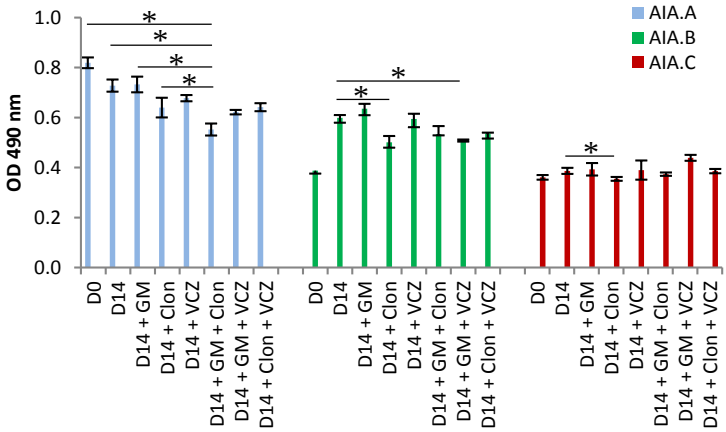
Clinically Relevant Small Molecule Drugs can Provide Added Benefit In Vitro in Combination with Anti-Idiotypic Antibody In Vivo

While these drugs can inhibit xenoantibody when utilized in isolation, it is more likely that they will be used in combination with other immunosuppressive reagents *in vivo*. We sought to test whether they could provide additional benefit in the context of pre-treatment with an anti-non-Gal selective anti-idiotypic antibody. Three rhesus monkeys (AIA.A-C) were pre-treated with anti-idiotypic antibody intravenously on experimental days -28, -21, and -14 before intravenous injections with GTKO PAECs on experimental day 0 (Fig. 5A). Serum collected from these animals on experimental day 14 was utilized as a source of induced xenoantibody for this experiment. Clonidine was able to reduce anti-non-Gal IgM detectable in serum samples from all animals tested (Fig. 5B). For one animal (AIA.A), the combination of clonidine and gabexate mesilate inhibited anti-non-Gal IgM to a greater extent than either drug alone. Additionally, the combination of gabexate mesilate and voriconazole inhibited detection of anti-non-Gal IgM binding in serum samples from two of three animals (AIA.A and AIA.B). It should be noted that the levels of anti-non-Gal IgM detected after combination treatment [anti-idiotypic antibody + small molecule(s)] were comparable to, or lower than that of the matched naïve animal (Fig. 5C) with only one exception (AIA.A + gabexate mesilate).

A


D -28
D -21
D -14
D0
D14
AIA
AIA
AIA
Immunization

B


AIA.A
AIA.B
AIA.C
OD 490 nm
1.0
0.8
0.6
0.4
0.2
0.0
D0
D14
D14 + GM
D14 + Clon
D14 + VCZ
D14 + GM + Clon
D14 + GM + VCZ
D14 + Clon + VCZ

C

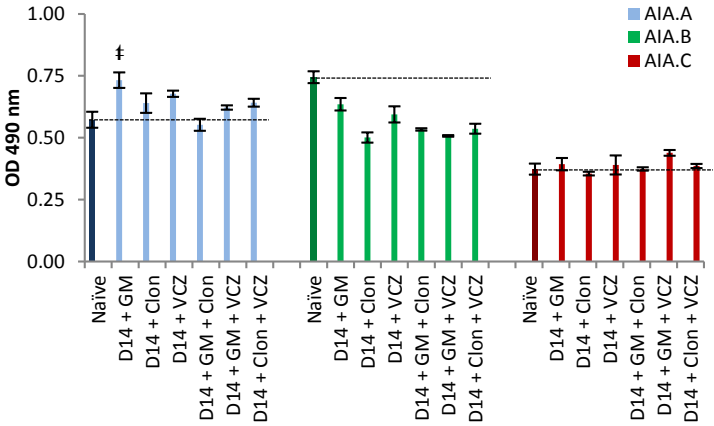

AIA.A
AIA.B
AIA.C
OD 490 nm
1.00
0.75
0.50
0.25
0.00
Naïve
D14 + GM
D14 + Clon
D14 + VCZ
D14 + GM + Clon
D14 + GM + VCZ
D14 + Clon + VCZ

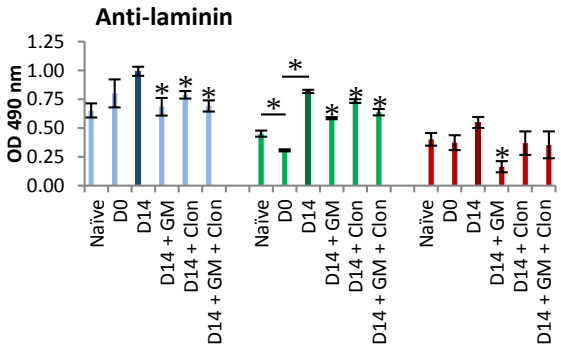
Figure 5. In all animals tested, clonidine (Clon) is able to inhibit anti-non-Gal IgM detectable by ELISA at day 14 after immunization of anti-idiotypic antibody pre-treated rhesus monkeys (AIA.A, AIA.B, and AIA.C). (A) The *in vivo* experimental time line. (B) In one animal (AIA.A), the combination of clonidine and gabexate (GM) was significantly more effective than either drug alone. In contrast, the only other effective treatment in this setting was the combination of gabexate and voriconazole (VCZ), which was able to inhibit xenoantibody in two of the three animals tested. (C) After pre-treatment with anti-idiotypic antibody and inhibition of residual xenoantibody only AIA.A + GM displays significant binding to non-Gal antigen above that of the naïve animal (dashed line/dark bar). Values are reported as mean optical density (OD) ± the standard error of the mean; * indicates P <0.05 compared to day 14 without drug. Ŧ indicates $p < 0.05$ that the detectable antibody is above naïve levels.

Anti-Idiotypic Antibody and Clonidine have Marginal Impact on Natural Antibodies

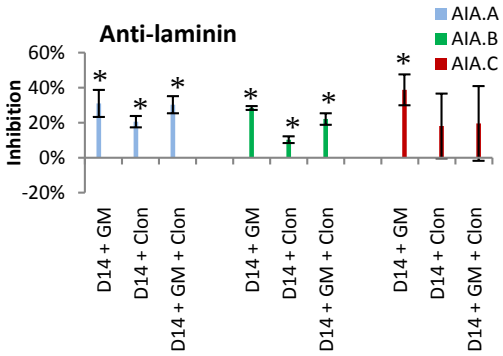
Because it was effective in all animals tested, further experimentation in the context of pre-treatment with anti-idiotypic antibody utilized clonidine alone or in combination with gabexate mesilate. In order to investigate the impact of anti-idiotypic antibody and small molecule drugs on other antibody responses, we tested their ability to interfere with natural IgM antibodies (Fig. 6). Three animals were tested for the impact on natural antibodies against laminin, thyroglobulin, and ssDNA. Of the nine possible combinations [animals x (natural antibody)], only two demonstrated a significant change due to anti-idiotypic antibody. AIA.B demonstrated a 32 ± 2% reduction of anti-laminin antibody levels (Fig. 6A-B) but no change in anti-thyroglobulin antibodies (Fig. 6C-D) while AIA.A demonstrated a 32 ± 1% reduction of anti-ssDNA antibodies (Fig. 6E-F). Thus, pre-treatment with the anti-idiotypic antibody was associated with only marginal changes in natural antibody levels. In this context, clonidine displayed minimal inhibition of the natural antibodies present at experimental day 14 (Fig. 6). Clonidine by itself displayed only marginal inhibition (10-21%) of natural anti-laminin IgM antibodies in 2 of 3 animals but did not impact any other classes of natural antibodies (Fig. 6A-B). This suggests clonidine is highly selective for anti-non-Gal IgM xenoantibody. In contrast, gabexate mesilate, alone or in combination with clonidine inhibited natural antibodies against laminin, thyroglobulin, and ssDNA. Gabexate mesilate inhibited anti-laminin antibodies from each animal (28-70%), anti-ssDNA antibodies from two animals (AIA.A and AIA.C; 45-56%), and anti-thyroglobulin antibodies from one animal (AIA.A; 42%). Thus, gabexate mesilate appears to have little specificity for anti-non-Gal IgM xenoantibody in this context.

Figure 6

A



B



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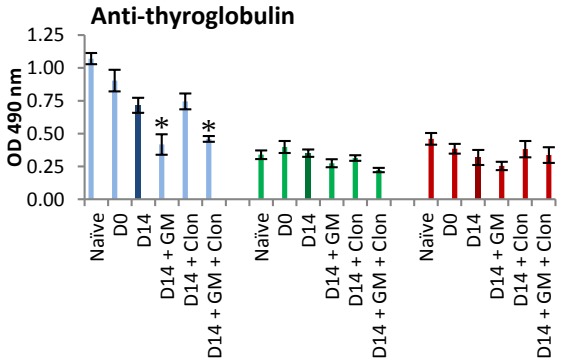
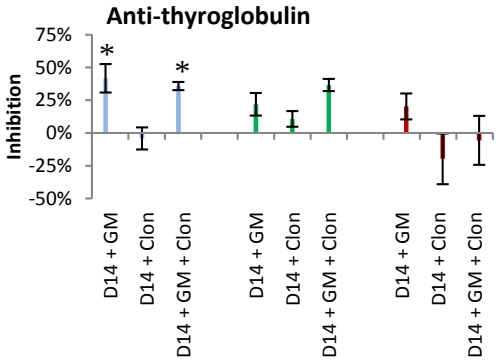
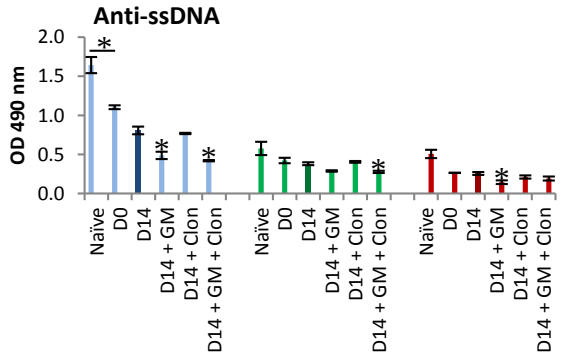


D



E



F

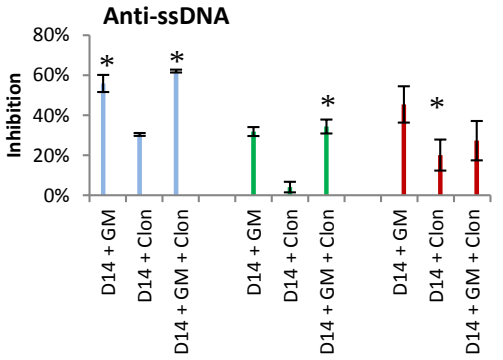
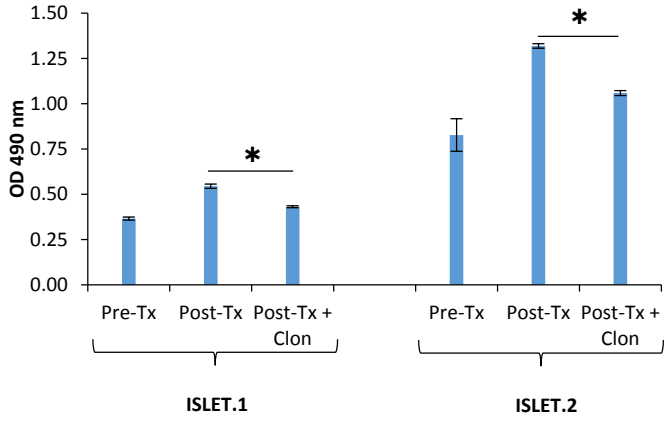


Figure 6. Clonidine (Clon) has minimal impact on natural IgM antibodies after pre-treatment with anti-idiotypic antibody and immunization with GTKO pig endothelial cells. (A-B) Clonidine did inhibit some natural antibodies against laminin (10-21%). However, clonidine did not interfere with natural IgM antibodies against (C-D) thyroglobulin or (E-F) single stranded DNA (ssDNA) in this context. In contrast, gabexate frequently inhibited natural antibodies against laminin (28-70%), ssDNA (34-56%), and thyroglobulin (42 ± 11%). Values are reported as mean optical density (OD) ± the standard error of the mean; * indicates $P < 0.05$ compared to day 14 without drug (dark bar) unless otherwise indicated; % inhibition = 100% x [D14OD – (D14 with drug)OD]/(D14OD).

Clonidine Inhibits Anti-Non-Gal IgM Xenoantibody Elicited in Rhesus Monkeys after Transplantation with Fetal Pig Islet-Like Cell Clusters

Clonidine was further tested for the ability to inhibit IgM xenoantibody elicited in rhesus monkeys in response to transplantation with fetal pig islet-like cell clusters. Two adult rhesus monkeys were transplanted with porcine islet-like cell clusters isolated from wild type fetal pigs at day 66 of gestation (15). Although we have studied the anti-gal carbohydrate antibody response of these animals, they also demonstrated a notable anti-non-Gal IgM xenoantibody response (Fig. 7). Serum collected at day 8 after xenotransplantation was used as a source of elicited anti-non-Gal xenoantibodies. As determined by ELISA, clonidine was able to inhibit over 50% of the anti-non-Gal IgM xenoantibodies elicited in both monkeys.

A


1.50
1.25
1.00
0.75
0.50
0.25
0.00
OD 490 nm
*
*
Pre-Tx
Post-Tx
Post-Tx + Clon
Pre-Tx
Post-Tx
Post-Tx + Clon
ISLET.1
ISLET.2

B

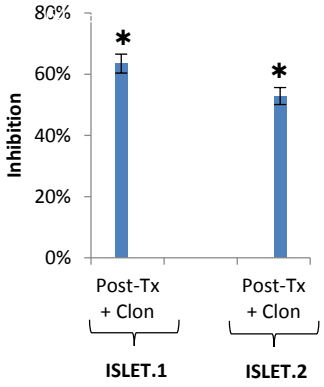

80%
60%
40%
20%
0%
Inhibition
*
*
Post-Tx + Clon
Post-Tx + Clon
ISLET.1
ISLET.2

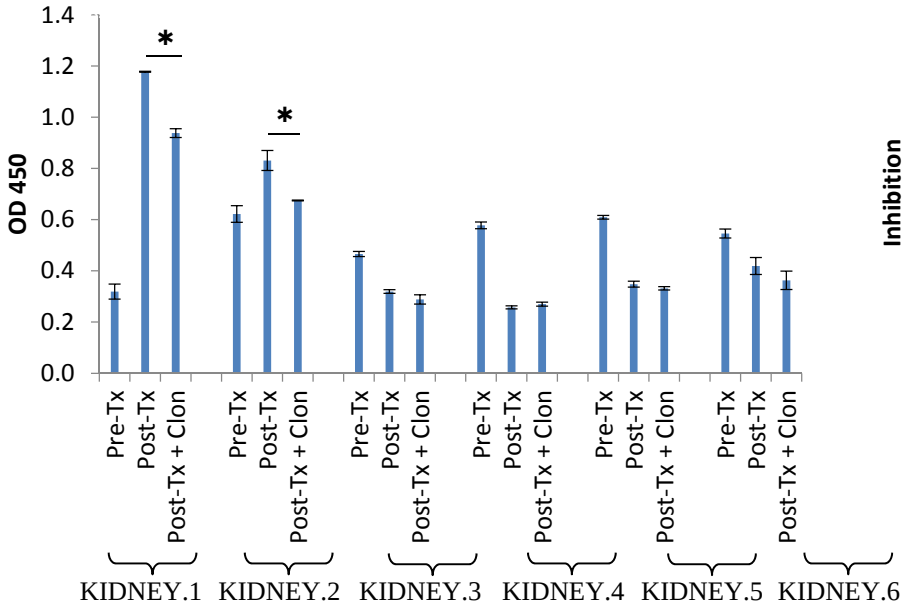
Figure 7. Clonidine (Clon) is able to inhibit anti-non-Gal IgM elicited in rhesus monkeys (ISELT.1-2) after transplantation with fetal islet-like cell clusters (15 x 10^6). Although we previously studied the anti-Gal carbohydrate xenoantibody response of these animals (15) they also demonstrated an anti-non-Gal IgM xenoantibody response. Serum used in this study was collected just before transplantation (Pre-Tx) and on post-operative day 8 (Post-Tx). Clonidine was able to inhibit 64 ± 3% of anti-non-Gal IgM elicited in ISLET.1 and 53 ± 3% elicited in ISLET.2. Values are reported as mean optical density (OD) ± the standard error of the mean; * indicates $P < 0.05$; % inhibition = 100% x [D14OD – (D14 with drug)OD]/(D14OD – D0OD).

Clonidine Selectively Inhibits Anti-Non-Gal IgM Xenoantibody Elicited in Baboons after GTKO Pig Kidney Transplantation

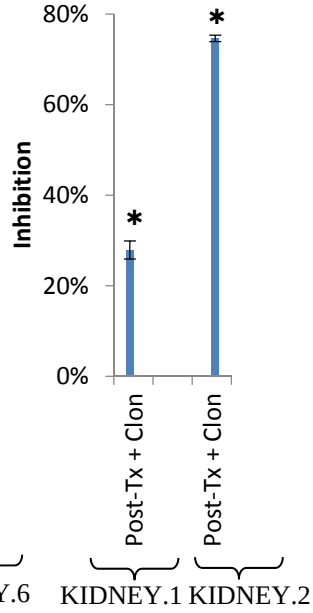
IgM xenoantibodies elicited by kidney xenotransplantation in the absence of additional immunosuppression were measured with or without the addition of clonidine *in vitro*. Circulating anti-non-Gal xenoantibody is known to be dramatically reduced by absorption onto the graft in the first few days after transplant (26). Thus, there were only detectable xenoantibody responses in two of the six baboons whose GTKO multiple transgenic kidney xenografts survived over 100 hours (KIDNEY.1 and KIDNEY.2; Fig. 8A-B). Serum samples utilized in this study were collected just before transplant and at the time of rejection. As determined by ELISA, clonidine was able to inhibit 28 ± 2% and 75 ± 1% of the elicited anti-non-Gal IgM xenoantibody response of these two animals (Fig. 8A-B).

All six baboons were previously immunized and routinely boosted every 5 years for tetanus. This allowed us to determine the impact of clonidine on the levels of a clinically relevant set of protective antibodies elicited by vaccination. Anti-tetanus toxoid IgG antibodies were unaffected by clonidine in five of six animals (Fig. 8C). In the other animal (KIDNEY.1), clonidine significantly reduced the detectable anti-tetanus toxoid IgG in post-transplant serum by 0.084 IU/ml. However, the remaining antibody (0.19 IU/ml) was still well above 0.10 IU/ml which is known to provide substantial protection against tetanus (27, 28). Furthermore, because clonidine was able to inhibit an equal or greater percentage of anti-non-Gal xenoantibodies from both animals that demonstrated a xenoantibody response, we considered this outcome to show a high degree of selectivity in this experimental setting.

A


OD 450
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Pre-Tx
Post-Tx
Post-Tx + Clon
KIDNEY.1
KIDNEY.2
KIDNEY.3
KIDNEY.4
KIDNEY.5
KIDNEY.6

B


Inhibition
80%
60%
40%
20%
0%
*
*
Post-Tx + Clon
KIDNEY.1
KIDNEY.2

C

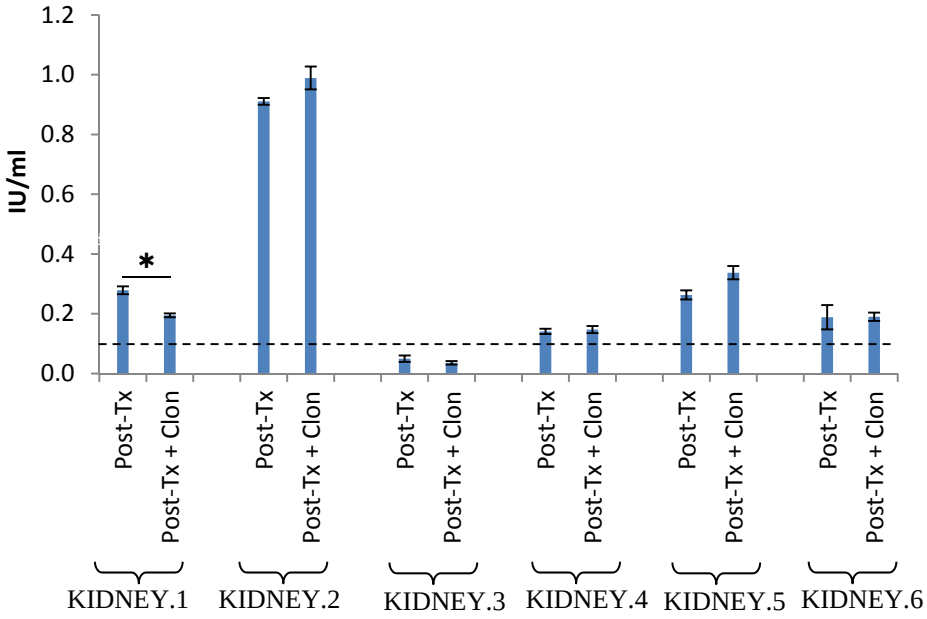

IU/ml
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Post-Tx
Post-Tx + Clon
KIDNEY.1
KIDNEY.2
KIDNEY.3
KIDNEY.4
KIDNEY.5
KIDNEY.6

Figure 8. Clonidine (Clon) is able to selectively inhibit the anti-non-Gal IgM response elicited in baboons in response to GTKO multitransgenic kidney xenotransplantation. Serum was collected before transplantation (pre-Tx) and at rejection (post-Tx) from six baboons (KIDNEY.1-6). (A-B) Xenografts from two animals (KIDNEY.1-2) were rejected late enough (> 100 hours) to detect an anti-non-Gal IgM xenoantibody response. Clonidine was able to inhibit 28 ± 2% of IgM xenoantibody elicited in KIDNEY.1 and 75 ± 1% elicited in KIDNEY.2. Values are reported as mean optical density (OD) or % inhibition ± the standard error of the mean; % inhibition = 100% x [D14OD – (D14 with drug)OD]/(D14OD – D0OD) (C) Clonidine only reduced detectable anti-tetanus toxoid IgG from one of six animals. However, clonidine inhibits anti-non-Gal IgM in all animals, which demonstrated a detectable xenoantibody response suggesting it is highly selective. Anti-tetanus toxoid IgG is known to be protective at 0.1 IU/ml (dashed line). Values are reported as IU/ml ± the standard error of the mean. * indicates $P < 0.05$.

Discussion

In this study, we identified three clinically relevant small molecule drugs with the ability to selectively inhibit anti-non-Gal IgM xenoantibody. Clonidine maintained this capability when utilized against xenoantibodies elicited against pig endothelial cells as well as islet and kidney xenografts. Furthermore, clonidine was effective against elicited xenoantibody from all animals that demonstrated a xenoantibody response, including baboons and rhesus monkeys. Given that clonidine is consistently effective in such diverse experimental models of xenotransplantation it is a highly promising candidate for *in vivo* studies.

The small molecule drugs identified in our current study may also have beneficial effects beyond selective inhibition of anti-non-Gal xenoantibody. For instance, clonidine has been used clinically in multiple settings for decades (25, 29-35) and has well documented effects on the immune system. It has recently been found to prevent neutrophil extravasation by stabilizing endothelial cell expression of adherens junctional molecules (36). Clonidine can also reduce serum concentrations of inflammatory cytokines such as IL-1β and IL-6 in healthy young adults (37) and mitigate the post-operative rise of serum TNF-α in response to lower-extremity revascularization (38). Furthermore, voriconazole can be an effective antifungal prophylactic treatment (39) while gabexate mesilate's anticoagulant effect has been demonstrated to improve islet engraftment in a syngeneic rat model of islet transplantation (40). Xenoislet engraftment could therefore be enhanced by inhibition of the instant blood mediated inflammatory reaction as well as by inhibition of anti-non-Gal xenoantibody.

In our previous work we identified an experimental small molecule, JMS022, from the NCI Diversity Set III that was capable of inhibiting xenoantibody elicited by GTKO PAECs (14). While this library was large enough and sufficiently structurally diverse to enhance the likelihood of successful *in silico* screening, it was composed of small molecules with unknown safety profiles. The NIH Clinical Collections were selected for the current study because they consist almost entirely of drugs that have been in phase I-III clinical trials. Additionally, all compounds are available commercially. These two qualities dramatically enhanced the likelihood that any small molecule inhibitors of anti-non-Gal xenoantibody could be advanced rapidly and tested *in vivo* for efficacy in large animals. Furthermore, screening all 727 drugs *in vitro* ensured that we would not miss compounds that may not come up in virtual screening.

It is of particular interest that clonidine was able to effectively inhibit post-transplant/immunization xenoantibody from all animals that demonstrated a xenoantibody response. Furthermore, clonidine was effective against anti-non-Gal IgM xenoantibody elicited by endothelial cells, islet-like cell clusters, and kidney xenotransplantation. This is consistent with our previous analysis suggesting that the majority of the anti-non-Gal IgM antibody response is structurally restricted in multiple settings (12, 13). However, the previously identified experimental compound JMS022 was only capable of inhibiting the IgM xenoantibody response of 3 of 5 rhesus monkeys tested (NIS.A, AIA.A, and AIA.C) (14). This suggested some degree of structural variation at the site to which JMS022 binds. We speculate that this may be due to previously noted variation in the structures of the CDR3 loops of the antibody heavy and light chains (13).

Our refined *in silico* screening methodology incorporates the structural variation of the CDR3 loops. Using this modified algorithm, oxiconazole and voriconazole, but not clonidine or gabexate, were both predicted to bind anti-non-Gal IgM xenoantibody with high affinity. This suggests that voriconazole and oxiconazole were capable of inhibiting xenoantibody directly. However, clonidine shares a limited region of structural similarity suggesting all three molecules may inhibit xenoantibody via a common mechanism. A recent analysis of 227 antigen-antibody complexes suggests that tyrosine and tryptophan residues in the hypervariable region almost exclusively interact with the corresponding target antigen (41). Furthermore, these side chains are generally responsible for a large proportion of the binding free energy. Thus, hypervariable region tyrosine and tryptophan residues which are conserved in all three xenoantibody structural models (i.e. W32 in the light chain CDR1, Y32, and Y33 in the heavy chain CDR1, and Y50, Y58, and Y59 in the heavy chain CDR2; (13)) are highly likely to mediate both xenoantibody-xenoantigen interactions and interact with small molecule inhibitors. The region of limited structural similarity shared between clonidine, voriconazole, and oxiconazole is likely to interact with these side chains via pi-pi interactions and halogen bonding. Unfortunately, neither of these mechanisms are accurately represented by the most accessible virtual screening or molecular docking programs since these programs utilize atomic point-charge based calculations (42-45). Therefore, further refinement is necessary to enable us to use computational modeling to more accurately predict which molecules will selectively inhibit xenoantibody.

In the current work we utilized inhibition of natural antibodies against laminin, thyroglobulin, and ssDNA as a proxy for the ability of small molecule drugs to interfere

with non-xenoantibody responses. The source of natural antibodies in primates is controversial (46, 47). However, natural antibodies are thought to contribute not only to immune pathologies (48) but also to normal physiologic processes (49) including immune surveillance (50). Gabexate mesilate and voriconazole reduced natural antibody binding in the majority of animals tested, whereas clonidine did not. This suggests clonidine is a highly selective inhibitor of anti-non-Gal IgM xenoantibody.

Interference with the anti-tetanus antibody response elicited by vaccination represents a more clinically relevant measure of the ability of small molecules to affect non-xenoantibody responses. Clonidine only inhibited this protective induced category of antibodies in one of six animals tested. Furthermore, the detectable anti-tetanus toxoid IgG in this animal remained significantly above the lower limits of what is known to be protective in spite of inhibition by clonidine. Importantly, clonidine still displayed greater inhibition of anti-non-Gal IgM xenoantibody. Thus, clonidine displayed clinically relevant selectivity for anti-non-Gal xenoantibody while demonstrating efficacy in all animals that elicited a xenoantibody response.

It should be emphasized that no treatment in isolation, neither anti-idiotypic antibody nor small molecule, was capable of fully inhibiting the anti-non-Gal IgM xenoantibody response in all animals. It was only when both reagents were utilized in combination that the xenoantibody detected at experimental day 14 was comparable to or below those levels found in the naïve animals. Thus, these small molecule inhibitors of anti-non-Gal antibody are likely to be the most effective when utilized in combination or in the context of additional reagents.

We have demonstrated that clonidine is effective in a diversity of settings and can inhibit anti-non-Gal xenoantibodies elicited not only by kidney or fetal islet-like cell cluster xenotransplantation, but also by pig endothelial cells. Together, these results indicate that it is feasible to block the initial anti-non-Gal IgM response in a targeted fashion, and also that the elicited xenoantibody responses are structurally related. Further scientific inquiry in these models should be focused *in vivo*. However, selective inhibitors are likely to be beneficial in these and related settings. For instance, selective inhibitors may be able to supplement or replace B cell-depletion which is currently required to extend GTKO cardiac xenotransplantation (6, 8). Thus, reagents capable of inhibiting anti-non-Gal xenoantibodies in a targeted fashion are a fruitful area of inquiry, and are likely to enhance survival and/or function in multiple xenotransplantation settings.

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CHAPTER FIVE

CONCLUSIONS

Discussion

The purpose of this dissertation has been to 1) provide further molecular characterization of the elicited antibodies which mediate damage and rejection of porcine xenografts, and 2) to identify clinically applicable small molecules capable of selectively inhibiting these antibodies. Before this body of work there was only preliminary evidence, provided by our lab (1), that the majority of the anti-non-Gal antibody response was structurally related. However, the works described in chapters 2-4 now provide strong support for this theory. Perhaps most importantly, this perspective led us to identify a clinically relevant small molecule drug capable of inhibiting antibody elicited in multiple xenotransplantation settings. However, along the way, and quite unexpectedly, our initial molecular analysis of anti-non-Gal antibody response led us to challenge longstanding theories with regard to the nature of xenoantibodies.

Even before the generation of GTKO pigs, the dogma has been that antibodies binding to the cell surface initiate xenograft damage. As such, previously utilized methods of identifying novel xenoantigens have focused on either membrane bound carbohydrates (2-4) or membrane bound proteins (5-7). It is therefore notable that FVIII is the first, and currently the only, xenoantigen identified to date that is secreted rather than membrane bound. However, FVIII is unlikely to be the only soluble porcine antigen with functional consequences. In addition to further defining the role of FVIII in

xenograft damage, studies seeking to identify novel soluble pig antigens are likely to provide valuable insight into hidden aspects of xenograft rejection.

In chapter three, we found that all four rhesus monkeys tested had pre-existing antibodies capable of inhibiting recombinant human clotting factor VIII (FVIII). This is likely due to amino acid differences between human and rhesus FVIII. In a similar fashion, 17% of apparently healthy individuals with FVIII polymorphisms have FVIII inhibitors which inhibit donor FVIII, but not autologous FVIII (8).

The apparent rise in FVIII inhibitor titer after xenotransplantation is also likely due to the low level of amino acid homology shared between pig and primate FVIII. As previously discussed in chapter three, this is likely to contribute to pathology in multiple pre-clinical models of xenotransplantation. More importantly, it is also likely to impact pig-to-human xenotransplantation. Patients with hemophilia A treated with porcine FVIII have been shown to have elevated anti-human and anti-porcine FVIII titers in spite of immunosuppression (9-11). Furthermore, the elicited inhibitors can act on FVIII from one or both species.

At this time the functional impact on coagulation parameters in the context of xenotransplantation is still speculative. Even though experiments in non-human primates are the gold standard in this field, they are unlikely to fully reflect the clinical implications of elicited clotting factor inhibitors. GTKO pigs, humans, and non-human primates are recognized as having different coagulation parameters (12, 13). Non-human primates are hyper-coagulable, compared to humans, while GTKO pigs are relatively similar. However, GTKO pigs have higher levels of clotting factors V, VIII, IX, and XI and lower levels of II, VII, and X (14).

Even in successful xenotransplantation the resulting hemostatic profile is likely to depend on what type of organ is being transplanted with differences dependent on the varying characteristics of each organ's vasculature. The liver is considered to be the major source of clotting factors, including FVIII. However, in humans clotting FVIII is also known to be synthesized and released by endothelial cells in the vascular beds of the dermis, aorta, intestine, kidney, and lung (15-17). Thus, FVIII inhibitors are likely to develop if any these tissues and organs undergo xenotransplantation.

In chapter three, we demonstrated that antibodies which inhibit human FVIII were elicited after aortic endothelial cell or pancreatic islet xenotransplantation. While expression of FVIII in pig aortic endothelial cells is well known (18), expression of FVIII in pancreatic islet endothelial cells has not been investigated. Endothelial cells do make up the majority of non-endocrine cells within an intact islet however, after isolation and culture of mouse islets for only 4 days endothelial cells amount to only 5% of their initial quantity (19). Those utilized in our study were only cultured for six days (20), making it plausible that FVIII expression in these isolated islets could have elicited an antibody response. However, the expression of FVIII should be investigated in freshly isolated and cultured porcine islets. If culturing isolated islets for longer than six days can reduce immunogenicity, it would make for an easy way to reduce antibody-mediated islet damage. However, mitigating damage to fully vascular organs will be more challenging.

Importantly, in chapter 4, we were able to identify a small molecule drug capable of inhibiting a significant portion of post-transplant anti-non-Gal antibody in multiple xenotransplantation models. This suggests that each of these settings share common immunogens. It is particularly interesting that this drug (clonidine) was able to inhibit

post-transplant anti-non-Gal antibody from all animals tested. This reinforced our previous analysis in chapter two which suggested that the humoral response to xenotransplantation is primarily composed of structurally related antibodies. However, one reagent utilized in isolation has never been able to inhibit more than 75% of elicited antibody. This could be due to structural variation of the elicited antibodies, or just as likely insufficient affinity of clonidine for elicited anti-non-Gal antibody.

Additionally, the question remains as to whether clonidine is capable of inhibiting anti-non-Gal antibodies elicited in humans. Non-human primates and humans have been noted to have minor sequence variations in their antibodies directed at the Gal antigen (21, 22). In the case of anti-Gal antibodies, amino acids important for contact with the antigen were conserved between species, however this may not be sufficient for anti-non-Gal antibodies to maintain an inhibitory interaction with clonidine.

In humans, typhoid vaccination has been associated with lower pre-existing anti-non-Gal IgG titers while influenza vaccination has been associated with higher pre-existing anti-non-Gal IgG titers (23). This association between anti-non-Gal antibody titer and vaccination suggests that selective inhibition of antibodies directed against GTKO pig xenografts could also impact protective immune responses. In chapter 4 we chose to measure the impact on anti-tetanus titer because these baboons had received regular tetanus boosters. *In vitro* analysis demonstrated clonidine inhibited anti-tetanus antibodies from one animal. In this case, the anti-tetanus titer of this animal was still considered protective after the addition of clonidine, though this may not be the case in all settings.

The immunoglobulin light chain gene identified in chapter 2 can encode antibodies against tetanus toxoid (24). This likely explains why clonidine was able to inhibit a portion of anti-tetanus antibodies from one animal in chapter 4. Additionally, the immunoglobulin heavy chain genes identified to encode anti-non-Gal antibodies, IGHV3-66 and IGHV3-21, can both encode antibodies against influenza (25-27). Therefore, it is feasible that clonidine, and/or other selective inhibitors, may impact susceptibility to the flu. However, it is notable that, unlike the anti-non-Gal response, the antibody responses against tetanus and influenza are encoded by a diversity of antibody germline gene progenitors. Therefore, as we saw in chapter 4, after treatment with selectively inhibitory reagents, most individuals should still demonstrate protective antibody titers. Even so, early experimentation with clonidine, and other selective reagents, in xenotransplantation should include monitoring of protective antibody titers. It is notable though, that clonidine has been utilized in clinical medicine since 1966 (28) and as of yet has not been linked to any immune vulnerability.

Future Directions

While the efficacy of clonidine needs to be assessed *in vivo* using large animals in the context of the most commonly utilized immunosuppressive regimens, it also will be important to test in a variety of other settings. Our lab has provided preliminary evidence suggesting that a combination of reagents which selectively inhibit anti-non-Gal antibodies would be more beneficial than either reagent alone [see chapter 4 and (29)]. At least two selective inhibitors were required to reduce the post-transplant anti-non-Gal antibody to levels below or comparable to those present before transplantation. As of yet, this remains to be tested when both reagents are administered *in vivo*.

Not all clinically relevant drugs are included in the NIH clinical collection. Therefore, we could still feasibly identify more inhibitory reagents that would be immediately useful in large animal studies. Initial screening for additional inhibitory small molecules should focus on clinically relevant drugs with chemical structures similar to clonidine. However, there are only a limited number of drugs with immediate clinical relevance. Therefore, it will also be important to screen small molecule libraries containing experimental compounds with chemical features which are related to, and build off of, a clonidine (or imidizoline aryl halide) molecular scaffold. This would allow refinement of clonidine affinity and selectivity. Additionally, screening of experimental compounds should include small molecules structurally unrelated to clonidine. However, due to the sheer number of small molecules available for testing this would be more feasible after more detailed analysis of the xenograft-rejection antibody response and refinement of computational modeling.

More accurate computational modeling of molecular interactions would enhance our *in silico* prediction, including analysis of antigen-antibody and small molecule-antibody interactions. As previously mentioned in chapter 4, until recently the most accessible virtual screening and/or molecular docking programs utilized atomic point-charge based calculations (30-33). Thus, these programs could not accurately represent all intermolecular interactions (halogen bonding; pi-pi stacking) likely to be important for interaction with clonidine. Notably, the latest version of Schrödinger's Glide molecular docking software can now take these into account and will be important for future molecular analysis and prediction.

Much of what we have already proposed can, with some finesse, be applied to pre-existing anti-non-Gal antibodies. Most people have levels too low to precipitate delayed humoral xenograft rejection without an adaptive response, which can be negated. However, it is still likely that pre-existing antibodies will affect long term damage of cardiac xenografts (34). Furthermore, recent experiments have provided evidence which suggests that when the adaptive response is suppressed, pre-existing antibodies still contribute to graft dysfunction in kidney xenotransplantation (35). However, the molecular diversity of these pre-existing antibodies is virtually unknown. Therefore, molecular characterization and selective inhibition of pre-existing anti-non-Gal xenoantibodies is likely to be a fruitful area of inquiry. As was the case with anti-Gal antibodies, non-Gal xenoantigens characterized to date can be utilized to capture and purify pre-existing anti-non-Gal antibodies. Additionally, recent advances in mass spectroscopy make it possible to identify the amino acid sequences of heterogeneous antibodies isolated from serum (36). If these antibodies display limited structural diversity it may be possible to selectively inhibit them as well.

Additionally, our lab has previously demonstrated that it is possible to induce tolerance to a specific antigenic structure in non-human primates (37). Following ablation of bone marrow using sublethal irradiation, rhesus monkeys were transplanted with autologous bone marrow transduced to express galactosyltransferase (GGTA1) using a lentiviral vector. This mitigated the antibody response against Gal, and expression was maintained in B cells and macrophages for as long as 355 days. In a similar vein, transducing autologous bone marrow to express the porcine FVIII C1 domain, or other xenoantigens, should induce tolerance and preempt rejection mechanisms initiated by

elicited antibody. Notably, there is already a large body of evidence, directed at patients with hemophilia A, to suggest this approach is feasible (38-40). Currently, the level of FVIII produced by the bone marrow in these settings can only cure mice with hemophilia A in some models. However, more universally, they have demonstrated induction of low level chimerism and tolerance. It is therefore feasible, that our lab could utilize these methods, along with our previous work in non-human primates, to induce long-term tolerance to pig FVIII.

In conclusion, the results of this dissertation challenge long-standing presumptions with regard to the nature of xenoantigens and their role in antibody-mediated xenograft rejection. Furthermore, these studies provide valuable insight into the nature of elicited antibodies, their interactions with FVIII, and their potential to be selectively inhibited by small molecule drugs.

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