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An Investigation of SLA Class II as a Xenoantigen

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ABSTRACT

Xenotransplantation, using genetically-modified pigs as organ donors, is a potential solution to the growing transplant organ shortage. Avoiding antibody-mediated rejection (AMR) is the remaining hurdle to widespread application. The development of a triple glycan knockout pig reduced antibody binding to clinically acceptable levels, allowing for the screening of the history of antibodies to the major histocompatibility complex (MHC). In allotransplantation, the MHC is a group of proteins divided into two classes and anti-MHC antibodies possess the ability to cause both acute and chronic AMR. The pig MHC, the swine leukocyte antigen (SLA), is a hypothesized target of antibodies.

This dissertation developed several models to screen human sera for anti-SLA class II antibodies. The findings suggest that SLA class II is a target of antibodies, and these antibodies are specific to certain SLA class II proteins, cytotoxic, and bind conserved amino acid epitopes found in the pig and human MHC. Genetic engineering of these epitopes is potential strategy to decrease human sera antibody binding without compromising pig immunity. This approach would provide transplant patients highly sensitized to the human MHC with a non-immunoreactive pig organ donor.

Key words: major histocompatibility complex, swine leukocyte antigen, xenoantibody, xenoantigen

DEDICATION

To Alicia - whose unending love, support, and selflessness continue to surprise me. I would not have the life I have now without you

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TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

LIST OF TABLES

Table *Page*

EXAMINING THE BIOSYNTHESIS AND XENOANTIGENICITY OF CLASS II SLA PROTEINS

LIST OF FIGURES

Figure *Page*

INTRODUCTION

SLA CLASS II IS A XENOANTIGEN

EXAMINING THE BIOSYNTHESIS AND XENOANTIGENICITY OF CLASS II SLA PROTEINS

LIST OF FIGURES (Continued)

Figure *Page*

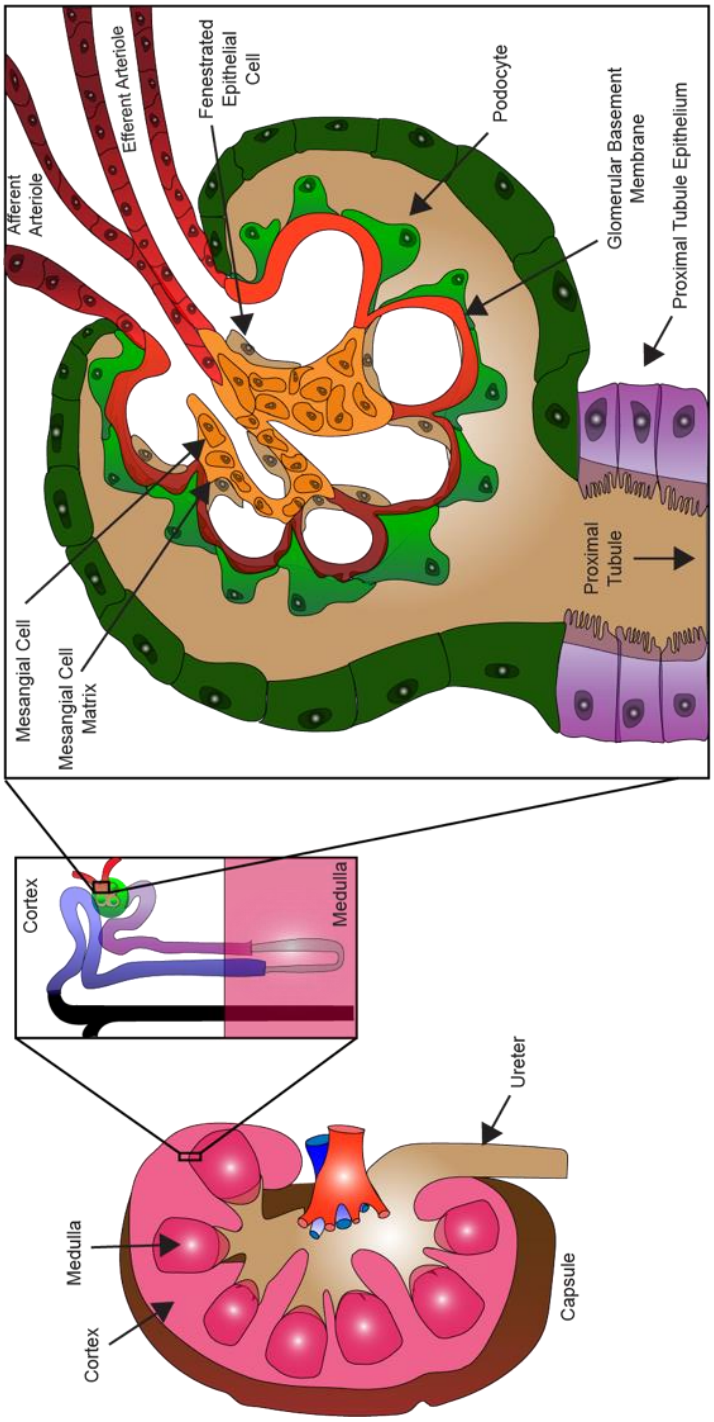
INTRODUCTION

Xenotransplantation as a Solution to the Organ Shortage

The first successful long-term, life-sustaining organ transplant in 1954 ushered in a new era of medicine: an opportunity for end-stage organ failure patients to lead a normal life. The development of transplantation as a medical procedure is attributed in part to the research of a few individuals: Alexis Carrel for the development of the vascular anastomoses, Sir Peter Medawar for the discovery of the mechanisms rejection, and Thomas Starzl for the refinement of immunosuppressive regimens. The work of these scientists, and many others, resulted in successful heart, lung, kidney, pancreas, and liver transplants performed routinely.

Despite this impressive achievement, the supply of organs for transplant is outpaced dramatically by the those in need of life-saving organ transplant (Figure 1). In 2017, over 120,000 Americans remain on the transplant waiting list but fewer than 30,000 will receive a transplant each year. This does not include individuals who would benefit from a transplant but are excluded from the waitlist due to age, malignancy, weight, social structure, and financial resources. The inclusion of these "extended criteria" patients would dramatically increase the wait list. Of note, in the United States of the ~468,000 individuals with kidney failure, only ~98,000 are currently on the transplant wait list.

Figure 1: Anatomy of the Kidney. A representation of the anatomy of the human kidney. Particular focus is paid to the kidney glomerulus and associated endothelial cells, a common site of graft rejection.



Xenotransplantation, the use of genetically-modified pigs as organ sources, is a potential strategy to address the organ shortage. Compared to non-human primates (NHPs), pigs produce larger litters with a shorter gestational period, cost less to raise, possess a physiologically compatible size, and carry fewer ethical concerns. Given the evolutionary distance between pigs and humans, pigs also carry a lower zoonotic risk than NHPs. These advantages make the pig an ideal source of rapidly generated, human compatible, and safe donor organs.

The Humoral Theory of Allograft Rejection

Sir Peter Medawar demonstrated that an immunological reaction of the host leukocytes, eventually determined to be T cells, cause the rejection of foreign skin allografts in rabbits (1,2). These observations were reaffirmed in whole-organ allotransplant by Simonsen et al using a dog model (3). It was realized that sufficient immunosuppression greatly increased the survival of skin and whole-organ allografts (4-7). The resulting immunosuppressive regimens efficiently suppressed the cellular immune system, but the humoral system causing antibody-mediated rejection (AMR) of the allograft remained.

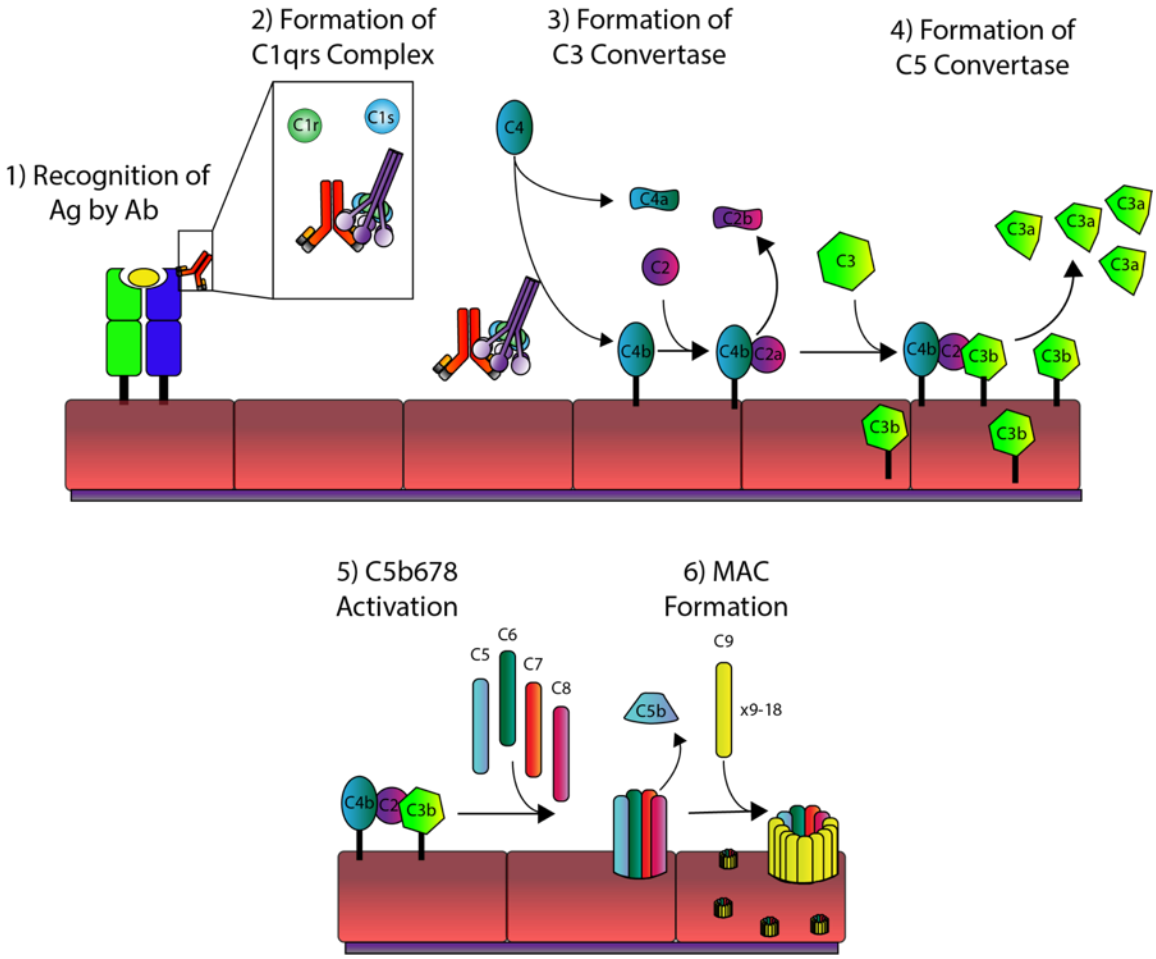
Mechanism of Antibody-Mediated Rejection

When an antibody binds an antigen, specifically IgM, IgG1, and IgG3, it initiates the classical pathway of complement cascade (Figure 2 Upper Panel**)**. C1q, a protein with six globular heads at the end of 6 collagen-like stalks that fasten the molecule into a single unit, binds the Fc portion of the antibody with a minimum of 2 globular heads (8).

The affinity of C1q for a single IgG is low (*Kd* ~100 μM) but when multiple IgGs are cell-bound in close proximity, the affinity dramatically increases (*Kd* ~10nM) (9,10). The tetrameric complex of C1r2s2, with a centrally-located X-shaped C1r2 and distally bound C1s2, wraps around the C1q base and brings the distal C1s catalytic domain into contact with those of the central C1r (8). The binding of C1r2s2 to the collagenous arms of C1q activates the zymogen. C1r autoactivates through the cleavage of an Arg-Ile bond in the C1r catalytic region (11). Activated C1r then cleaves an Arg-Ile bond in C1s, converting it to an active serine-protease (12). The activation of C1r2s2 allows the tetramer to unfold so the C1s serine-protease can interact with substrates C4 and C2 (13).

C4, composed of three polypeptide chains (α, β, and γ) is cleaved by C1s between peptide bonds 74-75 of the α chain, producing C4a and C4b. The newly formed C4b contains an exposed thioester bond between a cysteine and glutamate residue that is susceptible to nucleophilic attacks by amine and hydroxyl. The thioester bond can react with water or factor I to produce a transient inactive iC4b (14-16). In a Mg2+-dependent reaction, C2 binds to the cell-bound C4b for cleavage by C1s, producing C2a and C2b. The C2a associates with the C4b to form C4bC2a, the C3 convertase(17). C3 convertase cleaves C3 into C3a and C3b, the latter which binds the cell surface. As the C3 convertase regenerates itself and continues to produce C3b, one C3b molecule binds the C4bC2 complex and is stabilized by a thioester bond forming with Ser 1217 of C4b. This C4bC2aC3b complex is known as the C5 convertase and generates C5a and C5b from complement component C5 (18), critical for formation of the membrane attack complex (MAC).

Figure 2: The Complement Cascade. An overview of the complement cascade demonstrating 1) the ability of antibody to recognize antigen, 2) become fixed by C1q, r, and s to form the C1qrs complex, 3) the conversion of C4 to C4b and C2 to C2a which results in the C4bC2a C3 convertase, 4) the binding of C3 and lysis to C3b, resulting in C4bC2aC3b C5 convertase, 5) the deposit of C5, C6, C7, C8, and multiple C9 to form the MAC complex.



Formation of the MAC

The C5 convertase initiates a series of events that result in the formation of the amphipathic MAC complex, composed of (in order of assembly) C5, C6, C7, C8, and C9 (Figure 2 Lower Panel). None of these proteins possess enzymatic activity, but in aggregate result in cellular death. C5, a two-chain α/β plasma protein, is bound by C3b and the α-chain is cleaved at a single site by the adjacent C2a. The cleavage of C5

exposes a surface membrane binding domain and a C6 binding site on the larger C5b fragment and releases C5a, a potent anaphylotoxin. C5b remains bound to the C5 convertase and binds C6, a single-chain plasma protein. The C5b-C6 interaction stabilizes the membrane binding domain in C5b and exposes a C7 binding domain. The single-chain C7 binds C5b6, resulting in a conformational shift that releases the C5b67 complex from the C5 convertase, and exposes a transient membrane binding domain. If the membrane binding domain of C5b67 encounters a plasma membrane, the C5b67 complex will bind but not yet disrupt the lipid bilayer.

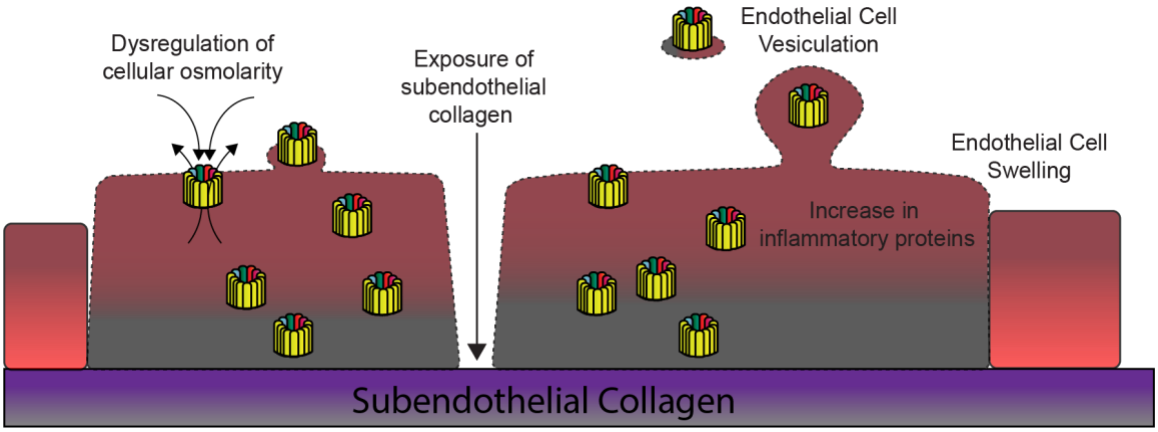
The C5b67 complex possess a binding site for C8, a three-chained (α, β, and γ) plasma protein. The α and β chains are homologous with each other and C6 and C7, while the C8γ displays no homology with other complement components. The C8β chain binds the C7 in the C5b67 complex and the resulting structure, C5b-8, burrows more deeply in the plasma membrane. The final component, the single-chained C9, is homologous to C6, C7, C8α, and C8β. The first C9 protein encounters the C5b-8 complex and binds C8α, releasing from its native globular, hydrophilic form into an elongated, amphipathic protein and exposes additional C9 binding domains for multiple C9 molecules to bind, release, and insert into the plasma membrane. The final result is a poly C9 pore, up to 18 C9 per complex, that increases the initial membrane leakiness caused by C5b-8 burrowing (19,20).

Consequences of MAC Formation

MAC with multiple C9s can be visualized by electron microscopy as ring-like structures surrounding a pore (19). MAC insertion into the endothelial cell plasma

membrane results in increased cytosolic calcium, release of von Willebrand factor from intracellular storage granules, increase in prothrombinase activity, and vesiculation of plasma membrane producing vesicles containing C5b-9 (21) (Figure 3). The vesiculation and eventual lysis of endothelium exposes platelets to the subendothelial collagen layer, providing fertile ground for platelet adhesion, aggregation and activation (22,23). The result is a thrombus formation that, depending on the size and severity, can result in a thrombotic microangiopathy (TMA).

Figure 3: Consequences of Membrane Attack Complex (MAC) Deposition. The endothelial cell response to MAC deposition is vesiculation of the complex, exposing the subendothelial collagen for platelet aggregation and thrombus formation. The MAC complex exposes the cell to the extracellular compartment, resulting in dysregulation of the cellular osmolarity, endothelial cell swelling, and increase in intracellular inflammatory proteins.



Diagnosing Antibody-Mediated Graft Rejection

AMR is implicated in approximately 50% of renal allograft failures and 60% of cases of late allograft dysfunction (24)**.** Prior to transplant, the specificity of the recipient's antibodies is measured through the flow cytometry crossmatch (FCXM), where recipient serum is incubated with donor cells and antibody binding is measured with anti-human immunoglobulin fluorescent antibodies by flow cytometry. The quantity

and specificity of DSAs can be detected by flow cytometry to donor cells, a representative cellular panel, or a panel of antigens coupled to microbeads. A negative pre-transplant but positive post-transplant FCXM is indicative of donor specific antibody (DSA) formation. The ability of the DSAs to kill donor cells is measured in a complement-dependent cytotoxicity (CDC) crossmatch: recipient serum is incubated with cells in the presence of rabbit complement, and the cellular killing is measured by a live/dead flow cytometry stain (25).

Diagnosis of AMR, at the time of this dissertation, is categorized by time course: 1) hyperacute resulting in graft failure minutes to hours after reperfusion, 2) delayed rejection occurring in days to weeks, and 3) chronic being a slow, progressive loss of graft in months to years. Much of the diagnosis is reliant on recognition of antibody binding and subsequent complement deposition in the graft. A byproduct of iC4b degradation, C4d, was initially proposed by Feucht et al as a marker for early renal graft loss (26). C4d is formed by further factor I cleavage of iC4b into soluble C4c and membrane-bound C4d. C4d is an advantageous marker for complement activation because it binds close to the site of activation and one molecule of C1 can cleave up to 25 C4 molecules before inactivation by inhibitory molecules. This amplification gives C4d an increased sensitivity relative to an upstream complement component (16).

Hyperacute Rejection

Hyperacute rejection, a rare event in allotransplantation, is presumed to be a consequence of transplant in the setting of high titer preformed antibodies. This disease

process is characterized by rapid endothelial damage and capillary thrombosis (27). The etiology of these antibodies is thought to be prior sensitization to donor glycans (28,29).

Delayed Rejection

According to the 2013 Banff criteria, diagnosis of delayed rejection requires three findings: histological evidence, evidence of antibody interaction with vascular endothelium, and detection of DSAs. Typical histology shows glomerulitis, peritubular capillaritis, intimal and/or transmural arteritis, and acute TMA. C4d staining is used as evidence of antibody and complement deposition on the vasculature, with microvascular inflammation and expression of genes indicative of endothelial injury supporting the stain(30,31). Recipient preformed and *de novo* DSA directed against the donor major histocompatibility complex (MHC) class I are thought to be the cause of delayed rejection (32,33). The incidence of delayed rejection in kidney allograft recipients has decreased as immunosuppressive and HLA matching technologies have evolved (34,35).

Chronic Rejection

The timeline and diagnostic criteria between delayed and chronic overlap significantly with no clear time point separating the two and episodes of delayed rejection are risk factors for chronic rejection (34). The required categories of findings for diagnosis are identical to delayed rejection. Histological findings with chronic tissue injury include transplant glomerulopathy (TG) in the absence of chronic TMA, electron microscopy findings of severe peritubular capillary basement membrane multilayering, and new-onset arterial intimal fibrosis with unknown etiology (32,33). TG is a disease

state marked by immunological damage to the glomerulus due to transplant antigens resulting in mesangial cell proliferation, interposition, and matrix expansion accompanied by duplication of the glomerular basement membrane, cellular crescents, and proteinuria (27,35). Histology of antibody interaction with vascular endothelium is marked by C4d deposition, microvascular inflammation, and endothelial inflammatory gene upregulation (32,33,36). DSAs are measured by flow cytometry. Chronic rejection is associated with *de novo* antibody production, especially to the MHC class II antibodies, as a result of misaligned or non-matched MHC groups (37-40).

Antibodies to Allografts

Sir Peter Medawar was among the first to show that exposure to an antigen sensitizes an individual, resulting in antibody formation and a rapid immunologic response upon second exposure (1,41). The sensitization occurs when the recipient does not possess the identical antigen to the donor. In transplantation, these antibodies are directed against glycans and the MHC. Humans possess anti-glycan antibodies as a result of bacteria colonizing the gastrointestinal tract possessing non-host glycans (42). Anti-MHC antibodies occur due to exposure of non-donor MHC in previous transplants, blood transfusions, or placental crossing of fetal tissue (39).

Glycan Antibodies

The ABO blood group is the predominant sugar dissimilarity between humans. With no histocompatibility testing, ABO-incompatible transplants resulted in hyperacute

rejection (28,43). Despite being considered a contraindication to transplant, the paucity of cadaveric donors made overcoming the ABO-incompatibility a necessity.

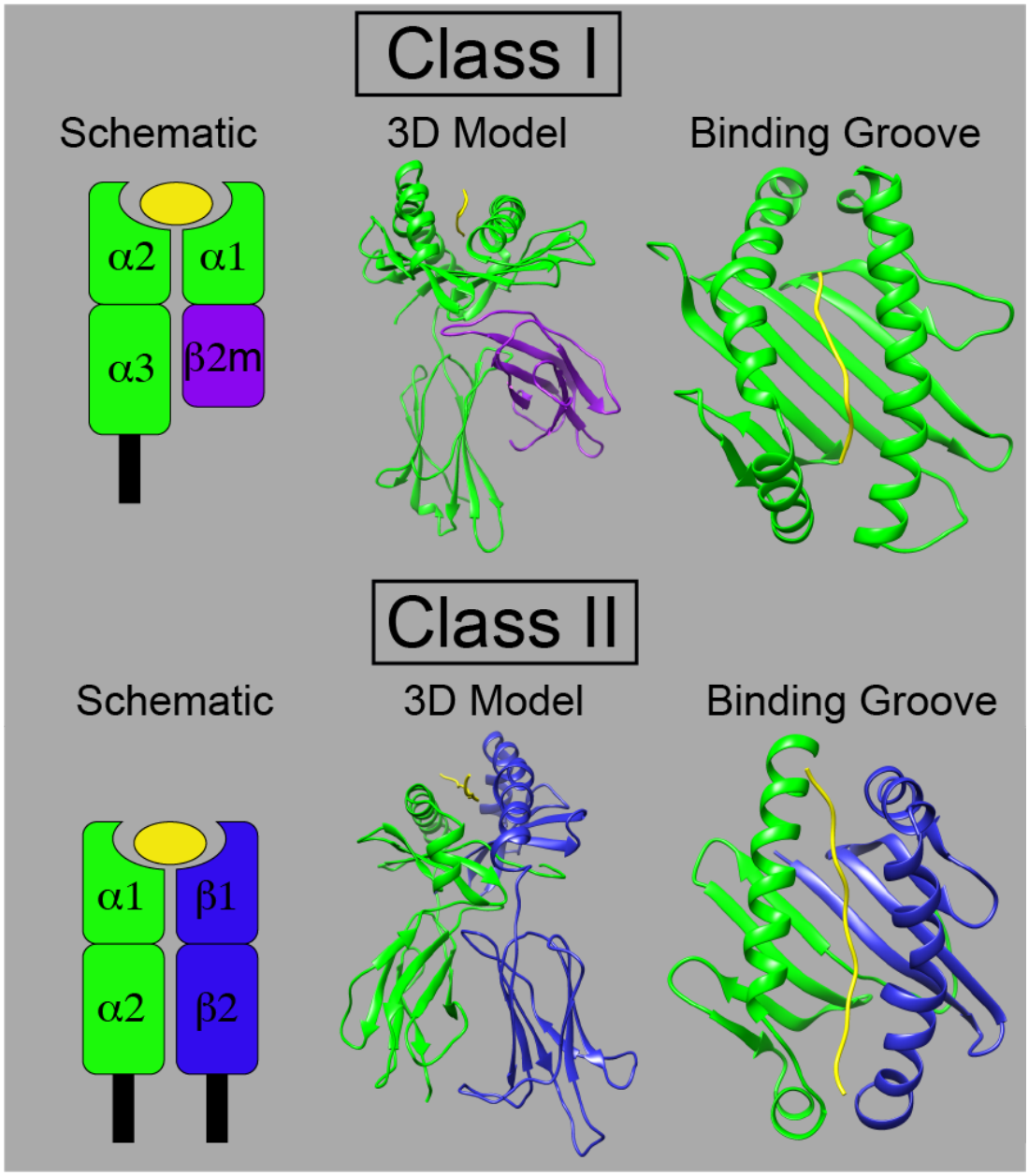
By the late 1990s, results with ABO-incompatible transplants began to approach the success of ABO-compatible transplants with a few caveats: many of the incompatible studies required A2 donors because this antigen was believed to be expressed at a lower level than A1, and as a result less rejection would occur compared to A1 equivalent organs. Additionally, it seemed that low pretransplant antibody titer recipients required no antibody-reducing regimen but in the event of high titers, plasmapheresis was necessary (44,45). These studies revealed that anti-glycan antibodies can be overcome with a combination of pretransplant antibody-reducing regimens, a low antibody titer, and a low antigen density.

MHC Antibodies

The MHC is a group of proteins, subdivided into two classes relevant to transplant (class I and II), on the cell surface responsible for presentation of peptides to T cells. MHC class I is a heterodimer of a single heavy chain non-covalently bound to the β2-microglobulin and subdivided into class I-A, -B, and –C (Figure 4 Upper Panel). MHC class II is a heterodimer of two non-covalently bound heavy chains (α and β), subdivided into –DR, -DQ, and –DP (Figure 4 Lower Panel). In both molecules, a peptide is non-covalently bound to the binding groove for T cell presentation. The structure of class II will be discussed in detail in a following section. The human leukocyte antigen (HLA) is the classification of MHC in humans. HLA class I is found on every nucleated cell while

HLA class II is typically confined to antigen-presenting cells such as macrophages, B cells, and dendritic cells.

Figure 4: Models of Major Histocompatibility Complex (MHC) Class I and II. The MHC class I is composed of a heavy chain bound to the β2-microglobulin. Shown in the upper panel are the schematic model, the 3D front-view and binding groove with peptide of SLA-2*1001. In the lower panel are the schematic model, the 3D front-view and binding groove with peptide of SLA-DQA*0101-DQB*0601. All 3D models were generated in the UCSF Chimera program.



A 1969 report published by Drs. Patel and Terasaki outlined the importance of anti-HLA antibodies and graft outcomes: a prospective study of 248 transplants performed in 225 recipients demonstrated that 43% of kidney transplants failed when anti-HLA antibodies were detected, and 80% failed when DSAs were detected. This is in contrast to the 4% of graft failures in crossmatch negative individuals (46). This research cemented the previous signficiance of HLA-typing developed by the collaboration of Drs. Terasaki and Starzl (46-51). Initial HLA typing was performed based on a six antigen basis of HLA-A, B, and –DR. Mismatches between donor-recipient pairs commonly led to poor outcomes, but research indicated that some incompatibilities could be overcome with immunosuppression (52,53). The field eventually accepted that antibodies bind epitopes on the MHC surface, some of these epitopes are conserved on multiple MHC alleles, and it is possible to avoid these epitopes in sensitized individuals with careful crossmatching (54,55).

HLA class I antibodies. A 1990 study by Halloran et al was one of the first to link anti-class I antibodies with delayed rejection. The study examined 7 patients with pre-transplant high titer HLA class I antibodies, and all 7 underwent an episode of rejection within 7 days of transplant, marked by peritubular capillaritis and vascular lesions, with 6 demonstrating complement deposition as measured by C3 immunoflourescence staining (32). The relationship between class I antibodies and delayed rejection was solidified by further studies (39,56).

HLA class II antibodies. High titer HLA class II antibodies are rare, but can be associated with delayed rejection (57,58). The more common finding is a slower action of class II antibodies, as renal function and biopsies of class II DSA+ recipients is similar to conventional transplants in the short-term (58). In the long-term, class II antibodies are difficult to remove and the *de novo* development of class II antibodies typically precedes chronic rejection (37,59,60).

In a study highlighting the role of *de novo* class II antibodies and chronic rejection, 103 patients with preexisting DSA were compared to 102 with *de novo* DSA. The *de novo* group displayed increased proteinuria, TG lesions, lower GFR, and worse graft survival (63% versus 34% at 8 years after rejection) compared to the preformed group. Within the *de novo* group 75% formed antibodies predominately directed at class II compared to class I ($p = 0.02$), although it was not reported if the remaining 25% also developed antibodies to class II (60).

The relationship between class II antibodies and TG, the histological finding of immunological damage to the glomerulus, has also been elucidated in recent years. A report published by Gloor et al studied 582 renal transplants with a negative pre-transplant CDC crossmatch. TG was diagnosed in 55 patients and the risk of TG was significantly higher in patients with anti-HLA class II antibodies ($p < 0.0001$) but not anti-HLA class I (35). Multiple studies demonstrated similar findings linking class II antibodies to TG (61-63). Of the class II antibodies generated, recent publications suggest that HLA-DQ may be the predominant target (64-66).

It is interesting to postulate why HLA-DQ antibodies appear to be the more problematic group compared to HLA-DR and –DP. The HLA-DQ complex was not

discovered until the 1980s and as a result, there was a paucity of reagents to screen for –DQ antibodies until recently (67). Early donor-recipient transplants were also matched on a 6-point scale (HLA-A, -B, and –DR), excluding –DQ status. The early matching system may have led to routine –DQ mismatches, and the resultant formation of –DQ antibodies causing chronic rejection. However, HLA-DQ could be a more immunogenic molecule given the more number of polymorphic -DQα chains compared to -DRα (35 compared to 2).

The Humoral Theory of Xenograft Rejection

Xenoantibodies, causing the activation of the complement cascade, are the primary cause of graft failure in pig to human or NHP transplants. Early work by Dalmasso et al demonstrated that in rhesus monkeys rejecting pig hearts and kidneys, hyperacute reaction was accompanied by IgM and classical complement and MAC deposition. *In vitro* studies established that depletion of IgM, C2, or C5 from human serum abrogated the cytotoxicity and reconstitution with purified human IgM or corresponding complement component restored the killing (68). Identification and elimination of xenoantigens would increase xenograft survival by avoiding the complement activation associated with graft failure.

Antibodies to Xenografts

Similar to allotransplantation, individuals are sensitized to xenoantigens through prior exposure. In the case of the anti-glycan antibodies, intestinal flora is again the

sensitizing agent (42). For sensitization to protein antigens, it is unknown what the sensitizing agent is but could be due to shared cross reactive epitopes or diet (69,70).

Glycan Antibodies

Galα1-3Galβ1-4GlcNAc-R antibodies. The pig Galα1-3Galβ1-4GlcNAc-R epitope (Gal) is the most important of the glycan xenoantigens and the primary cause of hyperacute rejection (71). The enzyme responsible for the construction of the Gal epitope is α1,3Galactosyltransferase (GT), a pseudogene in Old World monkeys, apes, and humans (72). As a result of this inactivation, humans produce anti-Gal antibodies, constituting up to 1% of circulating immunoglobulins (73). GT synthesizes the Gal epitope by transferring a galactose from UDP-Gal to N-acetyllactosamine residues in the *trans*-Golgi network, producing 10-30 x 10^6 epitopes per pig cell (72).

Despite the prevalence of a high titer antibody and antigen, Cooper et al demonstrated that intravenous infusion with Gal sugar prior to pig-to-baboon cardiac xenotransplantation delayed the onset of hyperacute rejection (74). Additionally, plasmapheresis with depletion of Gal antibodies resulted in delayed rejection although the antibodies typically rebounded by day 6 (75,76). The success of this approach is admirable given the density of Gal antibodies. The generation of live, homozygous GT knockout (GTKO) pigs in 2002 confirmed the necessity of Gal removal in future pigs organ donors (77). In contrast to WT pig organs, which consistently failed within 6 hours, organs derived from GTKO pigs generally lived weeks to months before ultimately rejection (78-82).

N-glycolyneuraminic acid antibodies. Despite the progress achieved with GTKO pig organs, delayed rejection remained an issue (79). One potential xenoantigen suspected to be of relevance were the N-glycolylneuraminic acid (Neu5Gc) epitopes (the Hanganutziu-Deicher antigens), expressed on all mammalian cells except human and New World primates (83,84). Cytidine monophospho-N-acetylneuraminic acid hydroxylase (CMAH) generates Neu5Gc through hydroxylation of the precursor N-acetylneuraminic (Neu5Ac) epitope, but a 92-base pair frameshift deletion in exon 6 of the human CMAH renders the cells incapable of Neu5Gc formation (85).

Neu5Gc possess potent antigen density, present at up to 6.3×10^7 residues per pig aortic endothelial cell (PAEC), three times that of Gal on the same cells, and the antibody is shown to be present in human serum (86-88). Human serum incubated on mice cells deficient in Gal and Neu5GC resulted in significantly reduced antibody binding, approaching background levels (89). In 2013 a pig with mutated CMAH and GT (CMAH/GTKO) was generated. PBMCs and red blood cells (RBCs) from this animal incubated with human serum demonstrated significantly reduced antibody binding and CDC (90-92).

SDa antibodies. Although the CMAH/GTKO pig improved crossmatch results, a significant number of individuals possessed antibodies to another glycan. An expression library was constructed from GTKO PAECs and transfected into a human embryonic kidney (HEK) cell line for antibody screening against 5 NHPs with a previous cardiac xenograft. Of note, a β1,4 N-acetylgalactosaminyl transferase 2 (β4GalNT2) homolog induced antibody binding in 5 of the 5 tested NHPs (93,94). The β4GalNT2 gene controls

the β1-4 linkage of a N-acetylgalactosamine residue to a galactose coupled in an α2,3 linkage to Neu5Ac, resulting in prodcution of the SDa, CAD, and sialyl Lewis X antigens (95). It is not a human pseudogene with ~96% of the human population possessing a functional copy expressed in normal human tissue. The tissue expression and timing of β4GalNT2 varies, demonstrated by the fact that 75% of pregnant women become SDa negative (96). Approximately 50% of SDa negative individuals will form anti- SDa antibodies but this response is largely dependent on SDa antigen density (97,98).

A healthy GT, CMAH, β4GalNT2 knockout (GT/CMAH/β4GalNT2 KO) pig was generated by Estrada et al in 2015 (99). The PBMCs and RBCs from this animal demonstrated decreased antibody binding for baboons and humans (69,99). *In vivo* models of pig kidney transplants into NHPs suggest that in the presence of low-titer antibodies, a GT/β4GalNT2 KO yields superior survival compared to GTKO pigs (69). At the time of this dissertation, cells from a GT/CMAH/β4GalNT2 KO pig possessed the lowest human and NHP antibody binding of all tested genetically-modified pigs.

MHC Antibodies

The swine MHC is referred to as the swine leukocyte antigen (SLA) and bears significant structural homology and composition to their HLA counterparts. SLA class I is found on every nucleated pig cell, but the SLA class II is more broadly expressed than HLA class II and also present on the kidney endothelium (100-102). The MHC–DR/-DQ/-DP subfamilies are more closely related to the –DR/-DQ/-DP of other species than they are to each other (103,104) and as a result the similarity between the human and pig –DRβ is 75-80%, but 70% between HLA-DRβ alleles. This trend holds for -DRα, -DQα,

-DRβ, and -DQβ (pigs lack the -DP genes) (104-109). The significant sequence and structural homology between HLA and SLA suggested that highly sensitized individuals to HLA may possess cross-reactive anti-SLA antibodies.

SLA class I antibodies. Before the development of nuclease-based genetic engineering techniques, the studies exploring the existence and etiology of SLA class I antibodies relied on indirect measures of antibody binding. One difficulty these studies faced was the high and variable glycan antigen background. Using WT and GTKO cells, no correlation was found between HLA sensitization and presence of SLA reactivity (110-112). The studies that found a correlation between HLA sensitization and SLA antibodies reduced the background antibody binding through either elution from tissue or serum absorption on glycan columns or RBCs (113-117). Diaz Varela et al demonstrated that RBCs do not express class I or II but are fully glycosylated and absorption removes glycan antibodies but leaves HLA specific antibodies intact. Unsensitized and sensitized unabsorbed serum reacted highly to pig PBMCs, but RBC absorption removed almost all xenoantibodies except in highly HLA class I sensitized individuals, suggesting cross-reactive HLA antibodies capable of binding SLA class I. Serum absorption on human platelets to remove HLA class I resulted in 82% of the crossmatches becoming negative (117). The structural similarity of SLA class I to HLA was confirmed by crystallography (118).

The advances in genetic engineering techniques to rapidly generate live pig models provided reagents to directly measure SLA class I antibody binding. In 2014, Reyes et al generated a live SLA class I deficient pigs (119). PBMCs from this pig were

utilized by Martens et al to demonstrate that SLA class I is a xenoantigen and reactivity correlates with HLA class I sensitization. A cross-reactivity between HLA and SLA class I antibodies was confirmed by demonstrating that antibodies eluted from HLA class I positive cells bound SLA class I cells, and SLA class I antibodies bound HLA class I cells. Martens et al also bound SLA class I positive serum to a panel of HLA class I single antigen microbeads, reporting that SLA class I antibodies bound HLA class I in an epitope restricted pattern (69). This proposal that public MHC class I epitopes are targets of antibody binding confirmed previous hypotheses (116,117).

SLA class II antibodies. The difficulties of high antibody background extend to SLA class II studies. Additionally, human class II antibodies were initially discovered by comparing T cell (class I positive) with B cell (class I and II positive) antibody binding – but SLA class II cannot be measured in this manner as pig T cells express class II (100).

Two studies were able to find SLA class II antibodies through indirect measures. Taylor et al absorbed highly sensitized human serum on human platelets, finding that in 3 out of 6 cases the FCXM remained elevated, suggesting the presence of SLA class II antibodies (113). Diaz Varela et al performed a similar absorption study, finding 18% of tested HLA sensitized serum to potentially carry SLA class II antibodies (117). No studies have clarified the structure of SL class II, the ability to bind swine invariant chain (Ii), or the size of the class II protein repertoire.

The Biosynthesis of MHC Class II

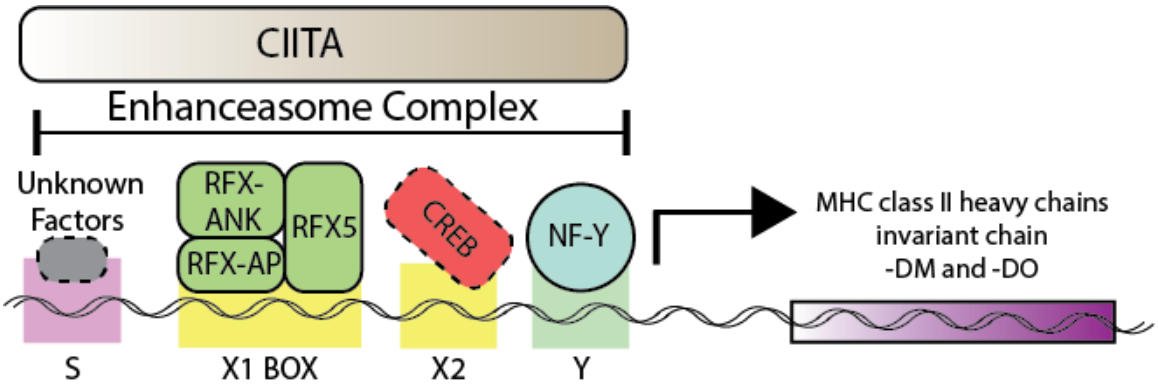
MHC class II, a heterodimer composed of an α and β chain non-covalently bound, is largely responsible for presentation of antigens to CD4+ T cells. The binding groove contains either exogenously derived antigens or class II-associated invariant chain peptide (CLIP), a fragment of Ii, the class II scaffold and regulatory protein. Transcription and expression of the class II associated genes is controlled by the class II transactivator (CITA), a molecular on/off switch (120).

Class II gene regulation. CIITA is controlled by multiple promoters that are selectively activated and lead to inducible or constitutive class II expression depending on cell etiology. CIITA expression can be upregulated by interferon-γ (IFN-γ) in a JAK/STAT pathway, a process relevant in the inflammatory conditions associated with transplantation (121-123). Binding of IFN-γ to the IFN-γ receptor (IFNGR) 1 results in rapid dimerization of the IFNGR1 chains and exposure of a binding site for IFNGR2. The assembly of the IFNGR complex, composed of 2 IFNGR1 and 2 IFNGR2 chains, allows for the closely associated Jak1 and Jak2 kinases to transactivate one another and IFNGR1. The phosphorylated IFNGR1 subunit serves as a binding site for Stat1. The two Stat1 molecules bind the two phosphorylated IFNGR1 chains, becoming phosphorylated and activated by the nearby JAK kinases. Phosphorylated Stat1 can then dissociate from IFNGR1 and form an activated Stat1 homodimer complex, translocate to the nucleus, and upregulates the CIITA enhanceasome complex proteins and CIITA (124,125).

The CIITA enhanceasome complex consists of RFX, RFX-AP, RFX-ANK, the NF-Y complex, and CREB (Figure 5). The RFX family is a group of proteins that bind

the X1 box region, NF-Y factors bind the Y region, and CREB binds the X2 region of the class II promoter (126-129). The enhanceasome creates a scaffold for CIITA binding, resulting in transcription of all class II associated genes including the α and β heavy chains, Ii, class II-DM and –DO (126)**.** Human cell lines transfected with human CIITA driven by an exogenous promoter resulted in HLA class II expression and mutation results in bare lymphocyte syndrome (120). With 85% nucleotide homology between human and pig CIITA, the gene can cross species-barriers and transfection with a dominant-negative human CIITA results in downregulation of SLA class II expression (130,131).

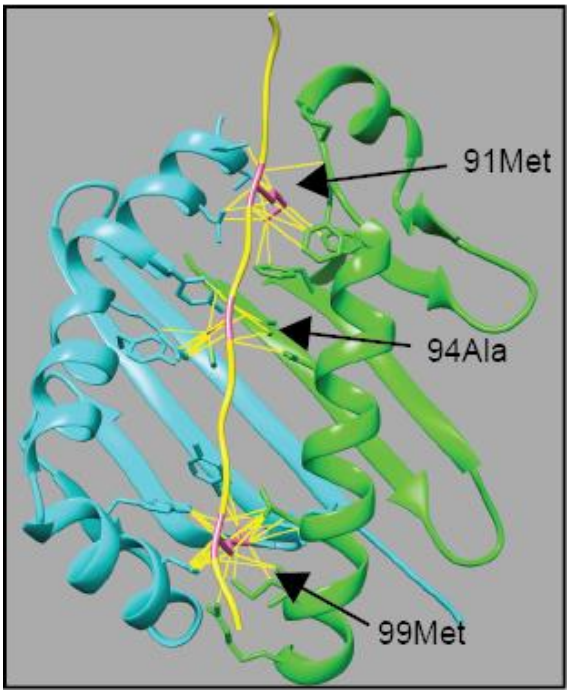
Figure 5: Diagram of the Class II Transactivator (CIITA) Enhanceasome. The CIITA enhanceasome forms a scaffold for the CIITA to bind and upregulate expression of the MHC class II genes. The known components of the enhanceasome include the RFX family that binds the X1 Box region, the CREB molecule that binds X2, and NF-Y that binds the Y region of the DNA. This scaffold then allows CIITA to bind, leading to expression of the class II heavy chains, invariant chain, and –DM and –DO.



Class II protein construction. Within 5 minutes of class II α chain synthesis and folding in the endoplasmic reticulum (ER), the 91Met of the trimeric Ii binds the first pocket of the α chain (Figure 6**)**. Multiple isoforms of Ii exist and distinct class II

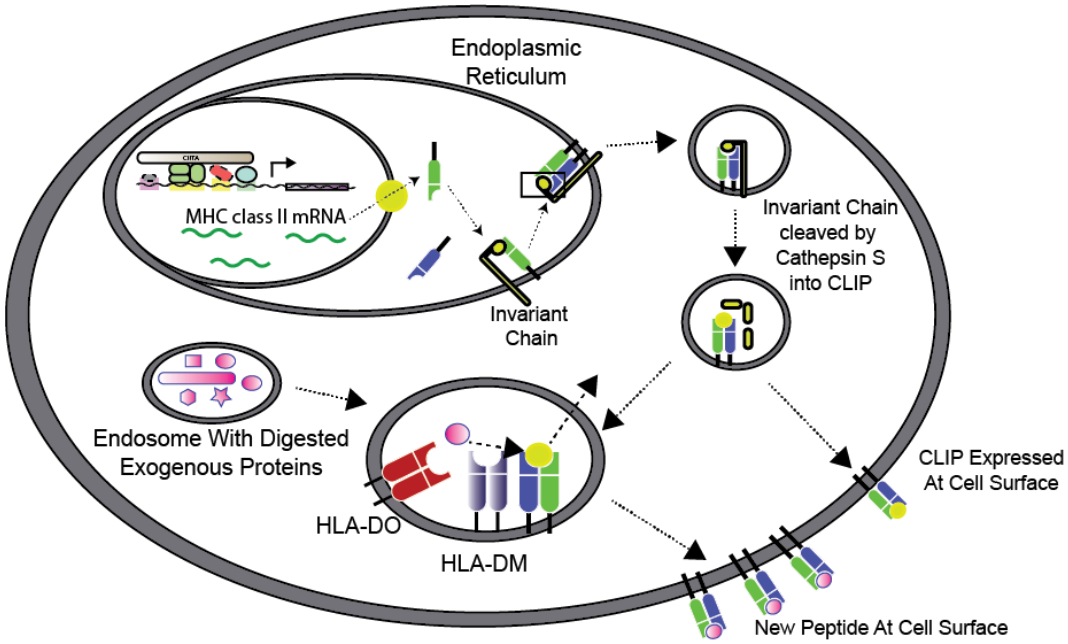
molecules have varying affinities and requirements for Ii (132,133). The αIi complex serves as a scaffold for binding of β chains, with Ii 94Ala and 99Met binding the pockets 4 and 9 of the newly formed MHC class II groove (134,135). Ii bound to the α/β chains prevents endogenous peptides in the ER from binding the class II groove, allowing the class II molecule to bind peptides derived from exogenous antigens that will be encountered in the endocytic pathway (132). The typical class II molecule consists of pairing between -DRα/β genes, -DQα/β genes, and -DPα/β genes in both a *cis* (same chromosome) and *trans* (across chromosomes) manner (136). Instances of hybrid formation, with -DRα pairing with -DQβ, have been shown *in vitro* and *in vivo* although these hybrid molecules are likely poorly expressed (137,138).

Figure 6: Interactions of Invariant Chain (Ii) with HLA-DR3. Ii serves as a scaffold and regulatory protein for MHC class II development and ultimately is cleaved by cathepsin S, leaving a portion of the fragment bound to the class II binding groove known as the class II-associated invariant peptide (CLIP). The 91Met, 94Ala, and 99Met residues of Ii interact with the 1st, 4th, and 9th binding pockets of class II respectively. Shown in yellow are the interactions of CLIP with a molecule of HLA-DR3.



Although not essential for class II formation, Ii serves as a regulator of class II heterodimer expression and trafficking (138-142) **(**Figure 7). The presence of Ii results in increased class II protein and recognition by monoclonal antibodies (139,143). Ii may also cause slight conformational changes to the class II molecule that result in a shift of the presented peptide repertoire (144,145). Ii has at least three trafficking signals, and the creation of the complete Iiαβ complex may obscure the first signal for ER retention, allowing class II to progress through the trans-golgi network (TGN) for glycosylation and proper folding(146).

Figure 7: Diagram of MHC Class II Molecule Construction. Upregulated by CIITA, the mRNA heavy chains of class II are translated into protein. The class II alpha chain is thought to bind Ii, and the beta chain is subsequently added to form an αβIi complex. This complex leaves the endoplasmic reticulum, passing through the trans-golgi network, and the Ii is processed by cathepsin S in the late endosome to yield CLIP bound to the αβ molecule. This CLIP-bound molecule can then exit the cell, presenting CLIP at the cell surface, or join with a phagolysosome to exchange the CLIP fragment for an exogenously derived protein under the control of –DM and –DO.



From the TGN, Iiαβ is targeted to the endocytic pathway, with a minority of Iiαβ molecules escaping directly to the cell surface (147)((148). The endocytic pathway sorting is dependent on the isoform of Ii associated in the Iiαβ complex, as plasma-membrane associated AP-2 adaptors recognize di-leucine motifs on Ii and target it to an endosome directly from the TGN(132,148). Within the late endosome, called the MHC class II compartment (MIIC), the pH drops and proteolytic enzymes such as cathepsin S degrade Ii into CLIP. CLIP is composed of Ii amino acids 81-104 and remains bound to the class II binding groove until replaced by a peptide with suitable affinity (132,149-151).

Class II antigen loading and presentation. The MIIC joins with a phagolysosome containing digested exogenous proteins. The exchange of processed peptide for CLIP is dependent on HLA-DM, a class II-like molecule. The structure of HLA-DM is highly homologous to –DR, -DQ, and –DP with α and β heavy chain subunits. The –DMα/β heavy chains are less polymorphic than classical class II heavy chains and the assembled DMαβ complex lacks an accessible binding groove, more closely resembling a FcRn binding groove than –DR/-DQ/-DP(152). HLA-DM does not interact with Ii and possesses endogenous trafficking signals; a tyrosine motif at position 230 on the DMβ chain directs the molecule to the late endosome for interaction with classical CLIPαβ molecules (153).

In the absence of HLA-DM, class II molecules are expressed at the cell surface loaded with CLIP (154,155). *In vitro* peptide binding to a class II molecule with an empty binding site is very slow (t1/2 = 24 hr) but –DM increases this rate by ~104 (156,157).

HLA-DM binds to the class II complex, induces CLIP dissociation through a conformational change of the class II molecule that disrupts the class II – CLIP hydrogen bonds, stabilizes the empty binding groove intermediate, and dissociates following peptide binding(132,152,158-160). The low pH of the MIIC acts as a stringent control on class II peptide presentation, and only peptides with sufficiently high affinity are capable of withstanding the acidic environment (161,162). The peptide-αβ molecule can then transit to the cell surface for presentation to CD4+ T cells (132).

The function of HLA-DM is regulated by another class II-like molecule, HLA-DO, which bears significant homology to –DM. The DOαβ dimer also lacks a binding groove for Ii and requires a tight association with HLA-DM to escape the ER(163). The binding site on –DM is suggested to be identical for both –DO and the classical class II molecules (164,165), suggesting a competitive inhibition of –DO for the classical class II binding site. The exact biological function of -DO is unknown, studies have shown slight defects in antigen presentation in -DO deficient mice and the ability of -DO to stabilize -DM at low pH. It is suggested that -DO serves as a co-chaperone of -DM, ensuring the survival of short-lived empty binding groove class II molecules, increasing the efficiency of –DM peptide swapping, and optimizing the efficiency of –DM peptide swapping (166,167).

Structure of the class II molecule. The class II molecule is composed of membrane-anchored α and β chains (containing extracellular α1/α1 and β1/β2 subunits respectively). Four strands each from the α1 and β1 domains form an interchain 8-strand antiparallel dimer that forms the flat floor of the class II binding groove. One long uninterrupted helix from each domain then forms the walls of the groove that is

approximately 30 Å long and 12Å wide. In contrast to the binding groove of class I, the class II binding groove is open at each end and capable of binding peptides between 13-25 amino acids(168-170). The regions with the highest polymorphism in class II is clustered around the peptide binding groove (103). The membrane proximal side of the class II molecule is composed of the α2 and β2 domains in a typical immunoglobulin fold of tightly packed β-pleated sheets(168-170).

The class II peptide binding groove of a specific molecule can bind up to thousands of peptides. The binding affinity of a peptide to the binding groove is dependent on hydrogen bonds, salt bridges, and van der Waals contacts made between the charged, polar, and non-polar atoms of the peptide and class II molecule. These required amino acid contacts provide regions of conservation to the class II binding groove. In the HLA-DR1 molecule 62Asn, 69Asn, and 76Arg of the α1 domain and 61Trp, 81His, and 82An of the β1 domain appear to be conserved regions. The binding groove of HLA-DR1 also contains pockets that result in peptide binding motifs, although the open nature of the binding groove results in less restrictive pockets compared to class I. The first pocket is the most stringent and binds hydrophobic and aromatic side chains. The remaining pockets are secondary and less stringent: the fourth pocket prefers nonpolar, hydrophobic side chains, the sixth pocket binds small side chains, the ninth pocket has a weak preference for hydrophobic side chains(171).

T cell recognition of the class II molecule. For T cell receptor (TCR) recognition, three interactions are of importance: the CD4 binding of class II, the TCR recognizing class II, and the TCR recognizing peptide. The TCR is composed of three

TCR complementarity-determining regions (CDRs), each with an α and β subunit. *In vitro* studies have shown that TCR binds with low affinity (~1-100 μM) to the peptide-MHC (pMHC) (172). The binding of CD4 to MHC does not increase the affinity of the TCR-pMHC interaction, but does enhance the TCR signaling(173). One mechanism by which CD4 binds class II is through insertion of 43Phe into the hydrophobic pocket formed between the α2 and β2 domains. As a result, 43Phe of CD4 will interact with 91Val, 92Phe, 178Trp of the α2 and 148Ile and 158Leu of the β2 domains of the class II molecule, stabilized by the CD4 46Lys-44Leu interaction with 144Ser-146Gln of the β2 domain (103,174). The TCR will interact with class II through CDR1 and CDR2 regions recognizing the α1 and β1 helices. The CDR3 region senses the binding groove peptide. A sequence analysis of all HLA class II alleles revealed constant TCR contact residues at 39Lys, 57Gln, 60Leu, 62Asn, 64Ala in the α1 domain and 61Gln, 69Glu, 72Arg, 76Asp, and 81His β1 domain (103).

Similarity of class II proteins. As of the writing of this dissertation, there are 4,700 HLA class II (2 –DRA, 1,751 –DRB, 35 –DQA1, 779 –DQB1, -28 DPA, 658 –DPB proteins) alleles compared to 12,631 HLA class I (2,792 –A, 3,518 –B, 2,497 –C proteins). The majority of the class II variability is found in the β, and the location of polymorphism is common within the β1 domain (103). The structure of class I as a single heavy chain coupled to β2m results in a 1:1 linear correlation between number of known class I proteins and number of functional proteins. The discovery of a novel class II protein results in a near exponential increase in potential protein structures. For example, the existence of *in vivo* hybrid molecules means a single -DRα chain has the potential to

pair with every -DRβ, -DQβ, and -DPβ chain and a single -DRβ could pair with every known -DRα, -DQα, and -DPα. Functional limits exist in terms of the linkage disequilibrium between the MHC genes, but the capacity for class II to create an expanded repertoire exists.

Dissertation Objective

The relationship between anti-HLA class II antibodies and poor allograft outcomes suggest that SLA class II will play a role in xenograft rejection. It is unknown if SLA class II is a xenoantigen and if anti-SLA class II antibodies exist, if they are capable of cytotoxicity, and how they bind class II. It is also unknown what the role of swine invariant chain is on class II expression, antibody recognition, and hybrid formation.

The central hypothesis of my dissertation is that human serum possesses allele-specific anti-SLA class II antibodies capable of cytotoxicity. These preformed antibodies are cross-reactive anti-HLA antibodies binding SLA class II at a conserved amino acid epitope. Mutation of these epitopes is a genetic engineering strategy to decrease antibody binding for highly sensitized individuals.

SLA CLASS II IS A XENOANTIGEN

by

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Format adapted for dissertation

Abstract

Background. Over 130 000 patients in the United States alone are in need of a life-saving organ transplant. Genetically modified porcine organs could resolve the donor organ shortage, but human xenoreactive antibodies destroy pig cells and are the major barrier to clinical application of xenotransplantation. The objective of this study was to determine whether waitlisted patients possess preformed antibodies to swine leukocyte antigen (SLA) class II, homologs of the class II human leukocyte antigens.

Methods. Sera from people currently awaiting solid organ transplant were tested for IgG binding to class II SLA proteins when expressed on mammalian cells. Pig fibroblasts were made positive by transfection with the class II transactivator. As a second expression system, transgenes encoding the alpha and beta chains of class II SLA were transfected into Human embryonic kidney cells.

Results. Human sera containing IgG specific for class II HLA molecules exhibited greater binding to class II SLA positive cells than to SLA negative cells. Sera lacking antibodies against class II HLA showed no change in binding regardless of the presence of class II SLA. These antibodies could recognize either SLA-DR or SLA-DQ complexes.

Conclusions. Class II SLA proteins may behave as xenoantigens for people with humoral immunity towards class II HLA molecules.

Introduction

Xenoreactive antibodies have been a significant barrier to implementation of clinical xenotransplantation (1,2). Recent advances in genetic engineering are making it possible to delete multiple xenoantigens in a single reaction (3,4). The creation of the GGTA1/CMAH/B4GALNT2 (triple knockout [KO]) knockout pig has eliminated the xenoreactive antibody barrier for many but not all waitlisted patients (5).

Major histocompatibility antigens have been recognized targets of humoral rejection in allotransplantation for more than 50 years (6,7). The development of single Human Leukocyte Antigen (HLA) beads has simplified the analysis of a broad-spectrum of HLA antibodies in clinical allotransplantation, and facilitated the detection of donor specific antibodies (DSA) directed against class I and class II HLA proteins (8,9). The sensitivity of single antigen beads also helped determine the importance of class II antibodies on long term graft survival, something that was previously difficult to determine when relying on CDC and flow cytometry (FCM) using donor cells. Previous studies suggested that HLA-specific antibodies cross-react with the homologous class I and class II swine leukocyte antigens (SLA) (10,11).

Our recent work using PBMCs from pigs deficient in SLA class I shows that some class I HLA-specific antibodies cross-react with SLA class I molecules explains the positive crossmatch that some people continue to have against the triple KO pig (12). Whether humans have antibodies to SLA class II is less well established. Class II SLA reactivity was indicated by the inability to fully deplete binding with class I HLA positive/class II HLA negative pooled human platelets (10,11). Insufficient platelet material used for depletion or sera containing HLA specificities not expressed on the

platelets could also explain the appearance of antibodies cross-reacting with class II HLA and SLA. Here we compared human IgG binding a pig cell line made to express a human class II transactivator (CIITA) transgene which drives class II SLA expression (13). We also examined human immunoglobulin binding to a human cell line expressing functional SLA-DR or SLA-DQ molecules. These assays enabled SLA antibody-reactivity to be tested without relying on platelet depletion of the antibodies in question and demonstrated that class II SLA can be xenoantigens.

Materials and Methods

Culture of Parent Cell Line

A SV40 T-Antigen immortalized fibroblast cell line derived from a SLA class I and galactose-α1,3-galactose deficient pig (14) was cultured in minimum essential media (MEM-α) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan UT) and Amphotericin B (Thermo Fisher Scientific, Waltham, MA) in collagen-I-coated plates (Becton Dickinson, Bedford, MA) at 37°C and 5% CO_2. Cells were confirmed to be SLA class II negative by incubation with anti-SLA-DR-FITC Ab or with anti-SLA-DQ-FITC (AbD Serotec, Raleigh, NC) and analyzed using BD Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA).

Creation of Pig Cells Expressing Class II SLA Molecules

Parent cells were grown to 90% confluency in a 10-cm culture plate and transfected with Lipofectamine 2000CD (Invitrogen, Carlsbad, CA) as specified by company protocol. A transgene encoding human class II transactivator was used to drive

SLA class II expression in the parent cell line. The donor plasmid, pCDNA3 myc CIITA was a gift from Matija Peterlin (Addgene plasmid #14650) (15). Three-days post transfection cells were screened on a BD Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA) using anti-SLA class II DR-FITC Ab (AbD Serotec, Raleigh, NC). Cells with high levels of class II DR expression were sorted one cell per well into 96-well plates by the FACS Aria flow cytometer. The cells were placed into selection against Geneticin, G418 (Invitrogen, Carlsbad, CA). Expanded clonal cultures were then analyzed for presence or absence of SLA class II DR using the previously mentioned anti-SLA class II DR antibody. Clones with a high level of SLA class II DR Ab binding were then evaluated for SLA class II DQ **(**AbD Serotec, Raleigh, NC). Finally, two clones were selected, one that demonstrated a stable class II positive (DR+/DQ+) phenotype and another with a class II negative (DR-/DQ-) phenotype, both resistant to G418 selection. These cells contained the following class II SLA genes: DRα(*02102/*w04re01), DRβ (1*1001/ 1*0403), DQα (*0204/*0101), DQβ (1*0601/1*0303) (16).

Creation of Human Cells Expressing Class II SLA Molecules

The expression vector, pBudCE4.1 (Thermo Fisher Scientific, Waltham, MA), was engineered to simultaneously express cDNA encoding SLA-DRA1-DRB1, -DRA1--DRB2, -DRA2-DRB1, -DRA2-DRB2, -DQA1-DQB1, -DQA1-DQB2, -DQA2-DQB1, -DQA2-DQB2. The alpha chains of the alleles were inserted into the CMV promoter site and the beta chains were inserted into the EF-1a site of pBudCE4.1 using restriction enzyme digestion. These plasmids were introduced into HEK 293 cells that had been

made deficient in class I HLA expression by using the gRNA (Forward: 5'-CTACTCTCTCTTTCTGGC-3' and Reverse: 5'-GGCCAGAAAGAGAGAGTAG-3') to disrupt the b2-microglobulin gene which is critical for cell surface expression of class I HLA. Class I HLA-deficient HEK cells were isolated by staining with anti-HLA class I monoclonal antibody (Clone W6/32, Thermo Fisher Scientific, Waltham, MA) and sorting on a BD FCSAria II at the UAB Comprehensive Flow Cytometry Core. Once a HLA class I negative population was obtained, the cells were transfected using a calcium phosphate protocol. Briefly, 1E6 cells were plated into a 6-well dish and transfected by adding a cocktail of 214 uL water, 31 uL 2 M CaCl2, 2.5 ug DNA, and 250 uL of 2x HBS. The cells were grown in MEM-α + 10% FBS + Amphotericin and three days post-transfection were placed in selection with the antibiotic Zeocin (Thermo Fisher Scientific, Waltham, MA). The cells were sorted again at the UAB Comprehensive Flow Cytometry Core on a BD FACSAria II for SLA class II expression as described above.

Sequencing

Polymerase chain reaction (PCR) amplification of the class II alleles was performed using the primers and conditions as described by Reyes et al.10 PCR products were ligated into pCR2.1-TOPO plasmid using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and analyzed by Sanger sequencing (Genewiz Inc., South Plainfield, NJ).

Analysis of Human-Anti-Porcine IgG Binding

One hundred four sera samples were discarded and de-identified material from the University of Alabama-Birmingham Histocompatibility Lab that had been selected based

on the presence or absence of class II HLA-specific antibodies. This activity was determined to meet the definition of non-human-subjects research by the UAB IRB. These samples were heat inactivated at 57°C for 30 minutes and the sera was absorbed for 30 minutes with an equal volume of packed WT pig RBCs to reduce background binding by removing any anti-pig glycan antibodies. Pig use was approved by the IACUC. This protocol removes human anti-pig glycan antibodies to lower the background when evaluating anti-SLA reactivity (12).

25 uL of absorbed sera was incubated for 30 minutes at 4°C with 1 x 10^5 cells in EX-CELL 610-HSF Serum-Free Medium (Sigma, St. Louis, MO, USA) with 0.1% sodium azide on either the class II positive or negative cell lines. Cells were washed three times with EX-CELL + sodium azide and then stained with goat anti-human IgG Alexa Fluor 647 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 30 minutes at 4°C. Cells were washed three times using EX-CELL medium as above and flow cytometric analysis was completed on BD Accuri C6 flow cytometer. Samples were gated on FSC-A by SSC-A.

Statistical Analysis of Antibody Binding

Antibody binding results were reported as means of median fluorescence intensity (MFI) after subtracting out fluorescence values obtained with secondary antibodies alone. Graph and data analyses were completed using Prism 7 for Macintosh (GraphPad Software Inc., La Jolla, CA, USA). The resulting data did not approximate normal distributions even after logarithmic transformation. Therefore, the Kruskal-Wallis test

was used to compare group means with correction by the Dunn's multiple comparison test.

Results

An immortal fibroblast cell line was derived from an engineered knockout pig lacking expression of the GGTA1 and all three classical class I SLA genes (14). Because this line does not express cell surface class II SLA (Figure 1B), a human CIITA transgene was introduced to drive transcription of the swine class II major histocompatibility complex (MHC) genes, SLA-DRα/β and SLA-DQα/β (13). Cells expressing the CIITA transgene were identified by their cell surface display of class II SLA (Figure 1A). Sanger sequence analysis of PCR products generated from the class II loci confirmed expression of two alleles each of the DRα, DRβ, DQα and DQβ genes. (Figure 1C).

Human Antibody Binding to SLA Class II

The target cells described above were incubated with human sera previously absorbed with wild type red blood cells which lack SLA proteins. This absorption eliminates antibodies recognizing the three-known carbohydrate xenoantigens (alpha galactose, Neu5GC, and B4GalNT2-derived glycans) which enhances the detection of additional xenoantigens such as class I SLA proteins (12).

Of the 104 tested sera, 19 lacked antibodies specific for class II HLA proteins. The remaining 85 contained class II HLA-specific IgG. These 85 sera showed elevated binding to cells expressing SLA-DR and -DQ when compared to cells which did not express SLA. Sera containing class II HLA reactive antibodies also exhibited elevated

binding to SLA-DR and –DQ positive cells relative to human sera lacking class II HLA specific antibodies (Figure 2). These data indicate that HLA-specific antibodies may cross-react with class II SLA proteins.

Because the CIITA transcription activator regulates several genes in addition to -DR and –DQ, it is possible that antibody binding may be influenced by antigens other than the SLA proteins (17). Therefore, we developed an additional class II SLA expression system which avoided the use of CIITA. Transgenes encoding SLA-DRα/β and -DQα/β were expressed in immortalized HEK293 human cells. Antibody binding to HLA proteins on these human cells because under the conditions used, these cells also do not express class II HLA, and Class I HLA protein expression was eliminated by CRISPR/Cas9-targeted disruption of the β_2-microglobulin gene (Figure 3A). These transfectants exhibited stable expression of all combinations of SLA-DRα/β and SLA-DQα/β heterodimers (Figure 3B). While sera lacking class II HLA-specific antibodies did not bind these cells (Figure 3C), IgG from sera containing class II HLA reactive immunoglobulin revealed multiple patterns of reactivity. Some did not cross-react with either SLA-DR or –DQ (Figure 3C). Other samples contained antibodies that bound to SLA-DR, SLA-DR and –DQ, or to SLA–DQ only. One sera only bound cells expressing SLA-DQ (Figure 3C) and generated MFI 130-fold over HEK293 cells that lacked any class II SLA proteins. The DQα1 allele appears to be the primary target of the antibodies in this sera as its pairing with either DQβ allele has minimal impact on the level of antigenicity.

Discussion

Preformed antibodies have been recognized as a significant barrier to kidney transplantation since 1964 (18). Allografts and xenografts each initially had glycan antigens to which recipients had antibodies. In the case of the allograft the ABO blood group antigens were a barrier, while in xenotransplantation, α-gal, Neu5Gc, and the B4GalNT2-derived antigen(s) were the barriers (1,2). The longstanding strategy to avoid antibody-mediated rejection (AMR) in clinical allotransplantation has been to refrain from transplanting patients in situations where they had pre-formed antibody to a donor antigen (6,18,19). Preclinical studies in the pig-to-primate model confirm that lowered antibody levels are important in xenotransplantation as well, with gal KO pig kidneys surviving for greater than six months if recipients were pre-screened and selected on the basis of having low xenoreactive antibody levels (20). Genome editing using CRISPR/Cas has facilitated the creation of triple-xenoantigen KO pigs (GGTA1/CMAH/B4GALNT2) that have a favorable crossmatch for xenotransplantation of many allo-unsensitized patients. Examination of the pathology of the long surviving kidneys shows that AMR with thrombotic microangiopathy is the cause of graft failure, highlighting the continued importance of identifying and developing genome engineering strategies to eliminate all xenoantigens. Cross reactivity of human HLA and SLA may also have implications in settings where a patient first receives a xenotransplant followed by an allo-transplant (21).

SLA and HLA proteins have extensive sequence identity and structural identity suggesting that they also may share cross-reactive humoral epitopes. Consequently, anti-HLA antibodies in highly sensitized patients may cross-react with SLA, and pose a

barrier to these patients from participating in initial clinical trials. In support of this concept, we used PBMCs from SLA I deficient pigs to evaluate class I SLA as a xenoantigen, and showed that there were some epitopes shared between class I HLA and class I SLA (12). These antigenic determinants were likely responsible for the anti-HLA binding to the class I SLA proteins.

Alternative approaches to determine the existence of anti-class II SLA antibodies were attempted but presented a few confounding issues. Swine B cell versus T crossmatches cannot be used to provide insight into the presence of class II SLA antibodies in human sera. Pig CD8+ T cells constitutively express class II SLA. Given that pigs contain abundant circulating CD4+/CD8+ double positive T-cells, isolating a specific population of lymphocytes lacking class II MHC poses significant challenges (22,23). Additionally, there are other unknown xenoantigens and a marked variability in xeno-reactivity from serum to serum. A crossmatch of WT RBC absorbed sera on swine PBMCs would remove the unknown anti-glycan xenoantibodies but leave anti-class I SLA antibodies, making interpretation difficult. To overcome these challenges, we developed the assays described in this report, namely the direct antibody binding to cells forced to express class II SLA proteins by the insertion of transgenes.

The described lack of available reagents to examine class II SLA previously forced indirect evaluations of class II HLA antibody cross reactivity with class II SLA. Class II SLA is similar to HLA in that it expresses DR and DQ, but is distinct from HLA in that there is no DP (24,25). The importance of anti-class II antibodies in allotransplantation has been appreciated more recently, as class II antibodies are now recognized as an important contributor to transplant glomerulopathy and graft loss (26).

Our CIITA positive pig cells express both SLA-DR and DQ at the cell surface and demonstrate increased reactivity with human sera containing class II HLA antibodies (Figure 2). CIITA modifies expression of several genes in addition to class II MHC molecles (17). Consequently, it may alter the production of non-SLA xenoantigens, causing human IgG binding to increase independently of –DR and –DQ expression. The fact that human immunoglobulin binding also increases on human cell lines expressing either SLA-DR or SLA-DQ (Figure 3) supports the idea that these molecules are xenoantigens recognized in CIITA positive pig cells. Both SLA-DR and –DQ appear to be xenoantigens. Anti-DQ antibodies are among the most frequent HLA antibodies found in recipients with a failed renal allograft, so it may be important to address the SLA DQ in xenotransplantation (27). Genome editing provides several potential strategies, which include either deleting DQ from the pig using CRISPR/Cas, or altering the sequence to eliminate epitopes present in our pig's DQ genes. We are performing broader analyses to gain insight into the epitopes and frequency of –DR and –DQ related cross-reactive antibodies.

In summary, in highly sensitized patients the xenoreactive antibody barrier to clinical application of xenotransplantation is still significant. The studies described in this paper show the presence of anti-SLA class II antibodies that bind to SLA DR and/or DQ. Further studies are required to determine if this response is due to SLA specific or cross-reactive anti-HLA class II antibodies. Genome editing with CRISPR/Cas can be used to eliminate either the class II antigen or its epitope in the donor pig. The prospects of creating donor pigs that have a negative crossmatch for every patient with renal failure regardless of their degree of HLA sensitization are bright.

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Figure 1. Class II SLA Phenotype of Pig Fibroblasts Expressing a Human CIITA Transgene. A) Cells cloned after transfection with the CIITA transgene were stained with monoclonal antibodies specific for either SLA-DR or SLA-DQ. Grey histograms represent cells with cell surface class II SLA. Open histograms represent a cloned cell that contains the expression vector based on its resistance to the selection marker G418, but fails to express class II SLA. These negative cells expressed no class II SLA protein as shown by the identical fluorescence intensities when stained with monoclonal antibodies specific for SLA-DR or –DQ (grey histograms, panel B) and isotype control antibodies, (unfilled histograms, panel B). Panel C shows an RT-PCR analysis of the class II SLA positive cells with primers specific for SLA-DRα (lane A), DRβ (lane B), DQα (lane C) and DQβ (lane D) genes.

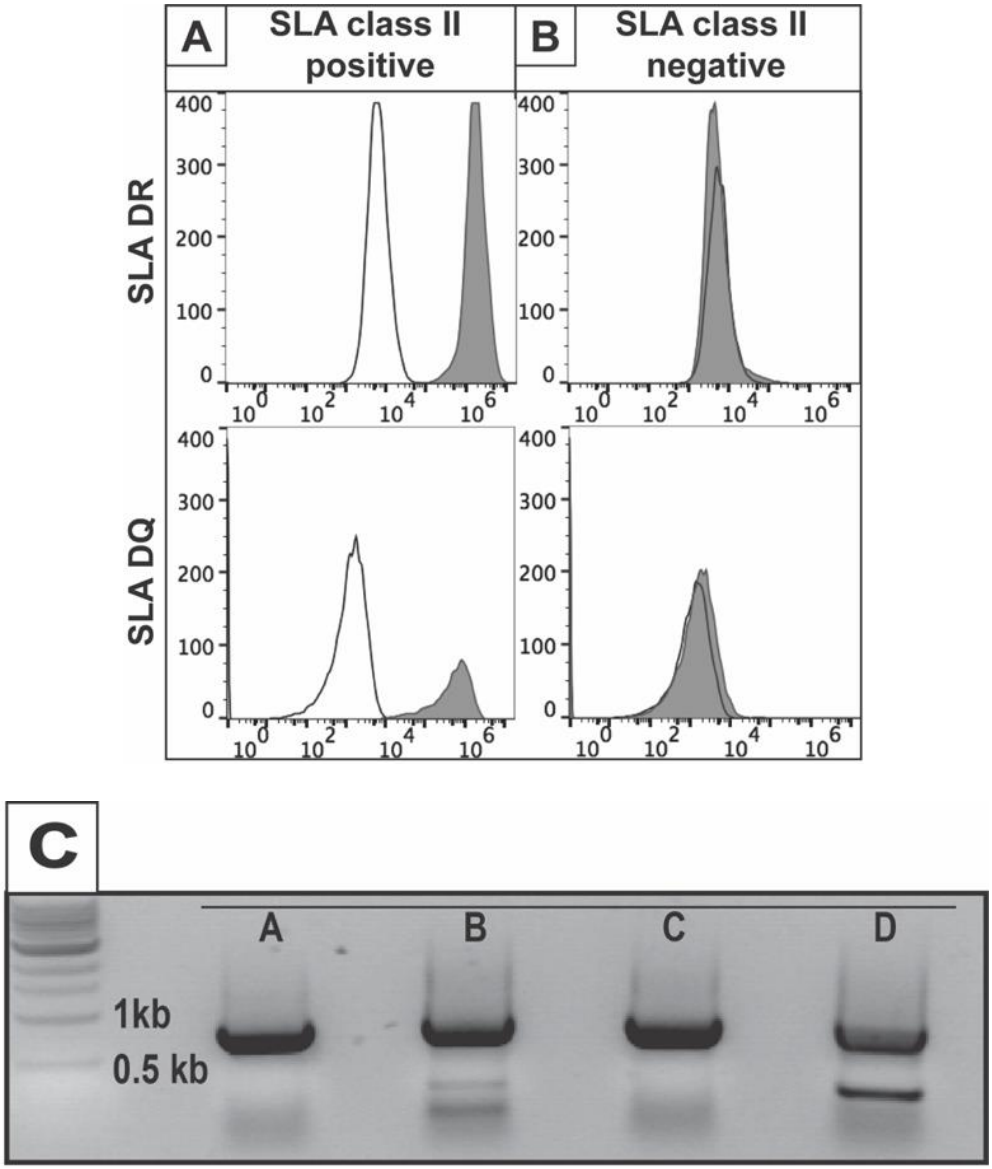


Figure 2. Demonstration of the Human Anti-Pig IgG Antibody Binding From 104 Individuals to SLA Class II Negative and SLA Class II Positive Cells. The mean and the standard deviation of the median fluorescence from human antibody binding to each cell type is shown. 19 sera samples lacked antibodies towards class II HLA and 85 samples contained antibodies specific for class II HLA proteins. The significance of antibody binding was compared using the Kruskal-Wallis test using Dunn's corrections for multiple comparisons.

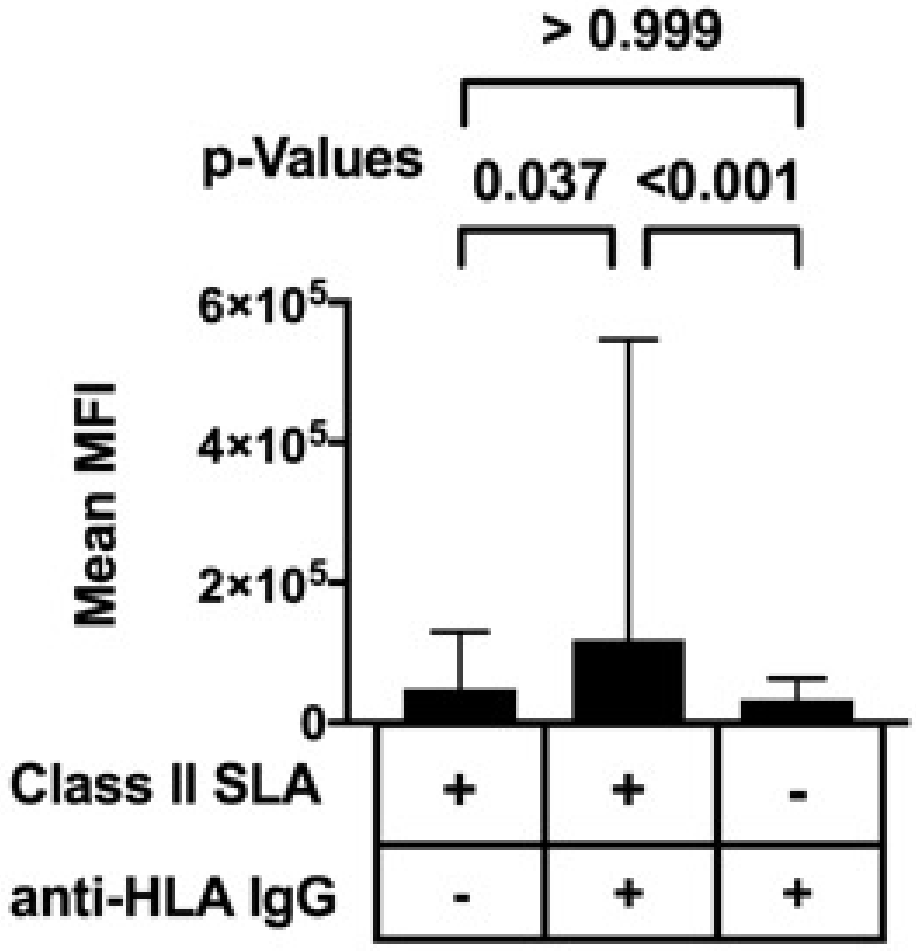
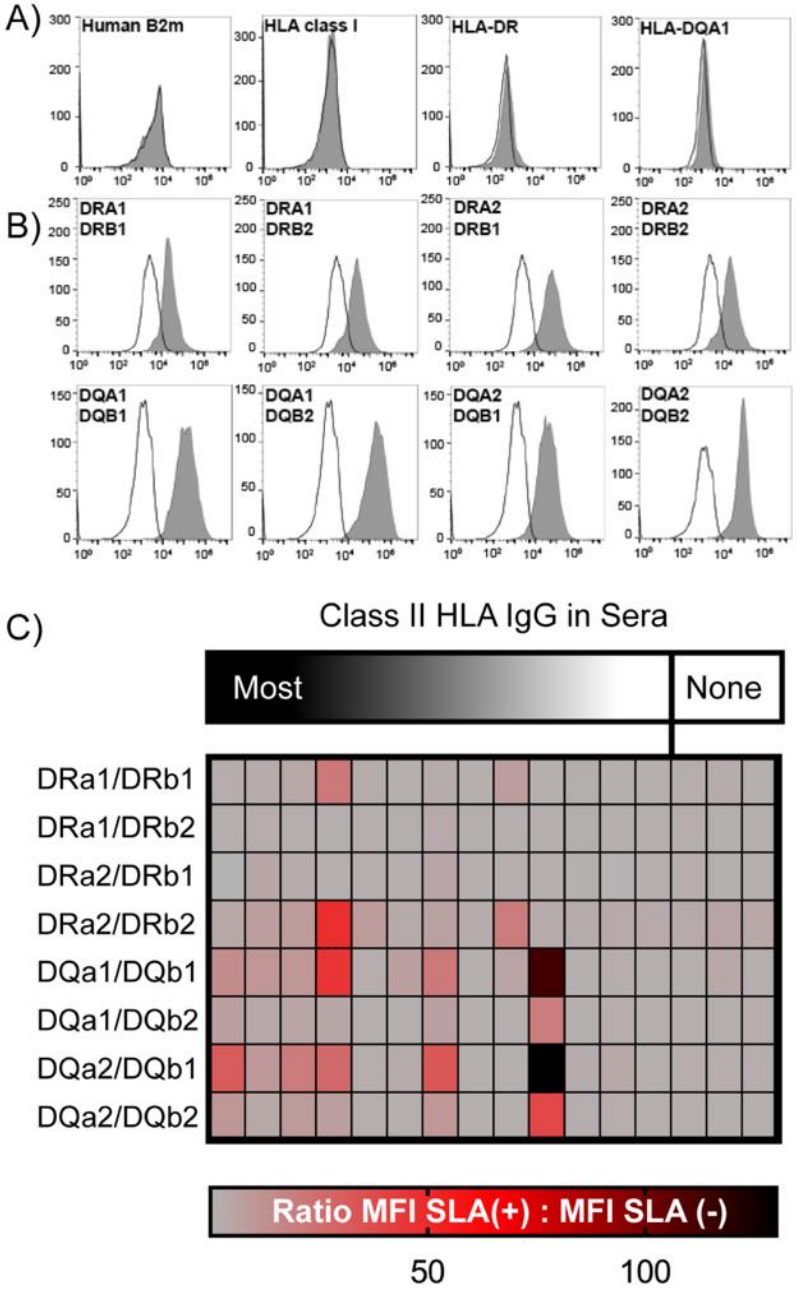


Figure 3. Human Antibody Binding to Either SLA-DR or SLA-DQ. Human embryonic kidney cells (HEK 293) cells were engineered to lack expression of β_2-microglobulin to eliminate cell surface class I HLA proteins. These cells were transfected with individual pairs of DR and DQ alpha and beta chains from the two SLA haplotypes present in the fibroblasts used in figure 2. All four combinations of two DRα and two DRβ transgenes were studied. Four pairs of DQα and DQβ transgenes were also evaluated. A) Phenotypic analysis of parent HEK cells. White histograms represent background staining of cells with an isotype control antibody. Grey histograms represent staining with antibodies for human β_2-microglobulin, class I HLA, and class II HLA (-DR and -DQ). B) Cells were stained with antibodies specific for SLA-DR or -DQ to validate cell surface expression of the indicated α/β pairs (grey histograms). White histograms represent negative control staining of the parent HEK293 cell devoid of β_2-microglobulin and class II SLA. C) Each cell line described in panel B was stained with 16 different human sera, and the presence of cell associated human IgG detected with fluorescent goat anti-human IgG. Each column represents an individual serum sample. Each row represents a different cell line. Three sera lacked any class II HLA reactive antibodies (None), and 13 sera contained IgG specific for varying numbers of class II HLA proteins. Median fluorescent intensities (MFI) of IgG binding to the different SLA class II expressing cells, SLA(+) and to SLA-deficient cells, SLA(-), were calculated. The SLA(+):SLA(-) ratio is shown in the heat map. D) The allele names of the class II SLA and their respective notations in panels A and B are indicated.



D)

Allele Name	Notation
DRB1*1001	DRb2
DRB1*0403	DRb1
DQB1*0601	DQb2
DQB1*0303	DQb1
DQA*0204	DQa1
DQA*0101	DQa2
DRA*020102	DRa1
DRA*w04re01	DRa2

EXAMINING THE BIOSYNTHESIS AND XENOANTIGENICITY OF CLASS II SLA PROTEINS

by

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Abstract

Genetically-engineered pig organs could provide transplants to all patients with end-stage organ failure, but antibody-mediated rejection remains an issue. This study examines the class II swine leukocyte antigen (SLA) as a target of epitope-restricted antibody binding. Transfection of individual alpha or beta heavy chains into HEK 293T cells resulted in both traditional and hybrid class II SLA molecules. Sera from individuals on the solid organ transplant waiting list were tested for antibody binding and cytotoxicity to this panel of class II SLA single antigen cells. A series of elution studies from a SLA-DQ cell line were performed. Human sera contain antibodies specific and cytotoxic against class II SLA. Our elution studies revealed six sera that bind the SLA-DQ molecule in an epitope restricted. Site-specific mutation of one of these epitopes resulted in statistically decreased antibody binding. Humans possess preformed, specific, and cytotoxic antibodies to class II SLA that bind in an epitope-restricted fashion. Site-specific epitope mutagenesis may decrease the antibody binding of highly-sensitized individuals to pig cells.

Introduction

Xenotransplantation, the use of genetically-engineered pigs as organ donors, could allow all patients with end-stage organ failure to receive a life-saving transplant. However, strong human antibody responses against pig tissue prevent this goal from being realized (1,2). Recent advances in genetic engineering have made it possible to inactivate three carbohydrate-modifying genes which produce xenoantigens on pig tissues. The low xenoantigenicity of these animals may have eliminated the humoral immune barrier for many people. For others, additional targets of humoral immunity remain (3,4). Swine Leukocyte Antigens (SLA) also contribute to xenoantigenicity(4,5). Swine major histocompatibility complex (MHC) genes encode these molecules, which are homologs of Human Leukocyte Antigens (HLA). Class I HLA and SLA proteins consist of a polymorphic membrane-bound MHC protein associated with the invariant β_2-microglobulin and a short peptide fragment. Class II proteins contain MHC-encoded polymorphic membrane-bound alpha and beta chains and a short peptide(6). Multiple class I and class II MHC genes, each locus containing many alleles, contribute to significant variation among HLA and SLA proteins. The strong antigenicity of HLA in allotransplantation originates from this polymorphism. Despite the variability of SLA and HLA, they contain significant structural and amino acid sequence identity. Consequently, we hypothesize that homologous proteins share epitopes, which enable cross-reactivity of HLA-specific human antibodies with pig cells.

To examine whether or not human antibody binds to class II SLA proteins, we previously created cell lines expressing unique combinations of class II alpha and beta chains (5). These transgenes encoded open reading frames of the class II proteins, SLA-

DR and SLA-DQ, homologs of HLA-DR and HLA-DQ. Pigs do not contain genes homologous to the human HLA-DP loci (7). We used these reagents to show that SLA-DR and -DQ proteins can be xenoantigens (5). Here we extend these analyses by expressing class II SLA heterodimers with swine CD74, also known as the invariant chain. This chaperone facilitates biosynthesis of class II MHC proteins, raising their cell surface abundance (6).

This report demonstrates: 1) that co-expressing pig CD74 and class II SLA alpha/beta heterodimers improved detection of xeno-reactive human antibodies. 2) Human antibody binding to class II SLA proteins initiates complement mediated cell killing. 3) Though prior sensitization to class II HLA contributes to the generation of class II SLA antibodies it is not essential. 4) Human antibody binding to a specific class II epitope can be reduced by mutagenizing amino acids, which contribute to the antigenic determinant.

Materials and Methods

Research Oversight

All human sera were collected and used in accordance with IRB-approved protocols.

Culture of Parent HEK 293T Cell Line

HEK 293T cells (ATCC CRL-3216) were cultured in minimum essential media (MEM-*α)* (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan UT) in collagen-I-coated plates (Becton Dickinson, Bedford, MA) at

37°C and 5% CO_2. Cells were confirmed to be class II HLA negative by incubation with an anti-HLA-DR Ab (Clone L243) and anti-HLA-DQA1 (Clone DQA1) and analyzed using BD Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA).

Creation of β_2m Deficient HEK 293T Cells Expressing Class II SLA Molecules

Previously we sequenced the SLA-DRα/β and -DQα/β genes in a single pig(8). The bicistrionic expression vector, pBudCE4.1 (Thermo Fisher Scientific, Waltham, MA), was engineered to express cDNA encoding 16 potential class II SLA heterodimers arising from the haplotypes present in that animal (Tables 1 and 2). The alpha chains of the alleles were inserted behind the CMV promoter and the beta chains were inserted behind EF-1a promoter of pBudCE4.1 using restriction enzyme digestion and DNA ligation. A second plasmid, pCDNA3.1+/Hygro (Thermo Fisher Scientific, Waltham, MA) encoding the swine invariant chain was also created using restriction enzyme digestion and DNA ligation. These plasmids were introduced into HEK 293T cells made deficient in class I HLA expression previously described by Ladowski et al(5).

The class I HLA deficient cells were transfected using a calcium phosphate protocol: 1 x 10^6 cells were plated into a 6-well dish and transfected by adding a cocktail of 214 uL water, 31 uL 2 M CaCl2, 2.5 ug DNA, and 250 uL of 2x HBS. The cells were placed into selection with either Zeocin alone or Zeocin and Hygromycin for swine invariant chain transfectants, grown in MEM-a + 10% FBS and sorted at the UAB Comprehensive Flow Cytometry Core on a BD FACSAria II for class II SLA expression as described above. The sorted cells were analyzed by a monoclonal anti-CD74 (Cerclip.1) antibody (Novus Biologicals, Littleton, Colorado, USA).

Human Antibody Binding to Class II SLA Single Antigen Cells

64 human sera samples, 26 sensitized towards class II HLA and 38 with no sensitization, were chosen for study of haplotype specific antibody binding on the panel of class II SLA single antigen cells. Sera treatment and methods of flow cytometric analyses were described previously (5).

CDC Assay on Class II SLA Single Antigen Cells

14 of the 64 flow cytometry samples were screened by complement-dependent cytotoxicity. 7 had class II HLA sensitization and 7 lacked allosensitization. The CDC assay was performed as described by Diaz et al., with minor modifications(9). Briefly, Cells were added to each well with $1x10^5$ in 25ul HBSS of Nunc™ 96-Well Polypropylene MicroWell™ Plate (Thermo Fisher Scientific, Waltham, MA), and incubated at 4°C for 30min with 25ul/well of human sera treated with or without DTT (2.5mM in final) for 30min at 37°C. The cells were washed at 3 times with HBSS, then treated with 50ul/well of 11-fold dilution of Low-Tox®-H Rabbit Complement (Cedarlane, Burlington, NC) for 90min at 37°C. The cells were stained with FDA (0.5ug/ml) / PI (2.5mg/ml, Sigma-Aldrich) at 4C for 15min. Data were collected using a BD Accuri C6 Flow Cytometer and software (BD Accuri, Ann Arbor, MI, USA).

Antibody Elution from Cells Expressing SLA-DQ DQβα*0101 + DQβ*0601

Of the 64 flow cytometry samples, 6 were chosen for antibody binding and elution from a target cell. Briefly, 20×10^6 HEK 293T cells expressing SLA-DQα1*0101, SLA-DQβ2*0601, and swine invariant chain were incubated with 100 uL

of serum for 30 minutes at 4°C. Unbound antibodies were removed by washing twice with phosphate buffered saline. Citric acid/phosphate buffer (pH 3.3) was added for 2 minutes to elute bound antibodies. Cells were pelleted by centrifugation and the supernatant was neutralized with Tris-buffered saline, pH 8.0, and concentrated to the original 100 uL serum volume using Vivaspin 6 30,000 MWCO centrifugal concentrators (Sartorius, New York, NY). These samples were screened on a class II HLA Luminex bead panel (One Lamda, Canoga Park, CA). In each case, antibody binding and elution from the HEK β_2m KO parent cell, and re-binding to the class II HLA Luminex bead panel was performed as a negative control.

Antibody Binding of HLA-DQ4,5,6 Sensitized Sera on the SLA-DQ Expressing Cell Line

Sixty three class II HLA sensitized sera samples of discarded and de-identified material from the University of Alabama-Birmingham Histocompatibility Lab were provided based on the presence (42 samples) or absence (21 samples) of sensitization to HLA-DQ(4,5,6). Assays evaluating human antibody binding to cells expressing SLA-DQα1*0101, SLA-DQβ2*0601, and CD74 were performed as previously described (5).

*Creation of the Cell Line Expressing SLA-DQα1*0101 and Mutated SLA-DQβ2*0601*

The 55Arg residue of SLA-DQβ2*0601 was converted to 55Pro in the pBUDCE4.1 SLA-DQα1*0101/SLA-DQβ2*0601 plasmid using the QuickChange XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA) and the following primers: Forward: 5'-GTGAGTGACCCCGCTGGGGCCGCCGGACGCCGACTAC-3' and Reverse: 5'-GTAGTCGGCGTCCGGCGGCCCCAGCGGGGTCAC-3'. This plasmid

encoding the R55P mutant was transfected with pCDNA3.1+/Hygro encoding swine invariant chain the HEK 293T β_2m KO cells with the calcium phosphate protocol described above. Transfected cells were placed into selection with Zeocin and Hygromycin, grown in MEM-a + 10% FBS, and sorted at the UAB Comprehensive Flow Cytometry Core on a BD FACSAria II for SLA class II expression with the CerCLIP.1 monoclonal antibody labeled with PE (Clone: CerCLIP.1, Thermo Fisher Scientific, Waltham, MA). A second monoclonal SLA-DQ antibody was also used to evaluate the SLA-DQ 55Pro mutant expression (Clone TH81A5, Washington State Monoclonal Antibody Center, Pullman, WA)

*Human Antibody Binding to Cells Expressing SLA-DQα1*0101, 55P Mutant SLA-DQβ2*0601and CD74*

21 sera samples from the cytotoxicity, elution, and the HLA-DQ4,5,6 sensitization studies suspected to have anti-SLA-55Arg antibodies with sufficient volume for further analysis were tested for immunoglobulin binding to the parent β_2m KO HEK 293T cell expressing SLA-DQα1*0101, 55P Mutant SLA-DQβ2*0601and CD74. Flow cytometry was performed as previously described (5).

Statistical Analysis of Antibody Binding

Flow cytometry files were analyzed in FlowJo V10 (FlowJo LLC, Ashland, GA). Antibody binding results were reported as median fluorescence intensity (MFI) (Figures 1, 4, and 5) or ratio of MFI compared to MFI of sera on parent cell (Figures 2 and 6). Cytotoxicity killing and SLA class II expression were determined by placing a

fluorescence gate on the negative control. Graph and data analyses were completed using Prism 7 for Macintosh (GraphPad Software Inc., La Jolla, CA, USA). The resulting data did not approximate normal distributions even after logarithmic transformation. Therefore, the Wilcoxon and Mann-Whitney with correction by the Dunn's multiple comparison tests were used to compare group means. SLA class II molecule structure predictions were created in SWISS-MODEL (University of Basel, Basel, Switzerland) and visualized in UCSF Chimera (University of California, San Francisco, CA).

Results

Pig CD74 increases cell surface expression of class II SLA proteins

Hek293 cells were used to express pig transgenes. These cells do not transcribe class II HLA genes and were engineered to lack expression of class I HLA proteins. Consequently, these cells exhibit low background binding to human sera, including those which contain HLA-specific immunoglobulin (5). Transgenes encoding various combinations of class II SLA alpha and beta chains were inserted in the HEK cells. Table 1 contains the name, abbreviation, and accession number of each allele studied. Table 2 contains the specific alpha-beta alleles making up the various SLA molecules examined in every figure. Some cells were also transfected with a transgene encoding pig CD74 to examine class II SLA expression in its presence (Figure 1). The invariant chain increased cell-surface density of all pairs a class II SLA-α/β heterodimers (Figure 1A and Supplemental Figure 1).

In humans and mice, some cross-pairings of class II α proteins with class II β proteins from different gene loci can give rise to "hybrid" class II proteins (10,11). In

pigs, this could result in SLA-DRα paired with SLA-DQβ and SLA-DQα paired with SLA-DRβ. All combinations of SLA-DRα/DQβ and SLA-DQα/DRβ were expressed in the presence or absence of pig CD74. Two hybrid molecules (pairs 9 and 10, table 2), containing the same DQα chain, accumulated on the cell surface (Figure 1A, Supplemental Figure 1 and Supplemental Figure 2). Their DRβ chains had 99% amino acid identity. Other hybrids did not express at the cell surface even in the presence of CD74 (supplementary data 2).

As an independent measure of class II SLA expression, these cell lines were also stained with the monoclonal antibody CerCLIP.1. This antibody recognizes the "clip" fragment of human invariant chain that associates with the peptide binding groove of class II HLA molecules(12). In the absence of CD74, every class II positive cell line yielded a background median fluorescence intensity (MFI) of approximately 1,000 (Figure 1B). Co-expression of invariant chain and class II SLA increased CerCLIP.1 binding in all SLA molecules tested except for one hybrid (pair 9, Figure 1B). This data indicates that CerCLIP.1 cross-reacts with pig CD74 and can be used to probe for cell surface expression of class II SLA molecules.

Evaluation of human antibody reactivity towards class II SLA.

Our previous work demonstrated that human IgG specific for class II HLA also cross-reacts with SLA-DR and -DQ (5). The role of the invariant chain was not evaluated. Here we extended these analyses to determine if CD74 affected the xenoantigenicity of class II SLA. We also tested greater numbers of human sera than in our previous report and included evaluations of IgM binding. In the presence of invariant

chain, IgG from sera containing class II HLA antibodies frequently cross-reacted with class II SLA (Figure 2D, dot plots). In contrast to our prior results, sera lacking anti-HLA antibodies occasionally contained IgG capable of recognizing class II SLA (Figure 2C dot plots). Multiple samples also contained IgM capable of binding class II SLA molecules regardless of the presence of class II HLA antibodies (Figures 2A and 2B). Hybrid SLA-DRβ and DQα molecules did not yield obvious IgM binding, but they were recognized by IgG (Figures 2A and 2B). Heatmaps below the dot plots show individual sera reactivity towards each class II SLA molecule (Figures 2A, and 2B). Sera displayed in the heatmaps, were chosen based on robust interaction with at least one class II SLA protein (MFI ratio of class II SLA positive to class II SLA negative control cell ≥ 10). Some samples contained antibodies reactive with most class II SLA proteins, while others exhibited a more restricted pattern.

Class II SLA antibodies drive complement-mediated cytotoxicity

Human sera were incubated with the panel of cells expressing various class II SLA molecules and pig invariant chain (Figure 3). We examined the cytotoxic activity of human sera lacking (Figure 3A) or containing (Figure 3B) class II HLA-specific antibodies in the presence of rabbit complement. Residual lytic activity, despite DTT-mediated inactivation of IgM, showed that IgG specific for class II SLA drove cytotoxicity in multiple samples. (Figures 3C and 3D). IgM specific for class II SLA also killed cells as shown by the ability of DTT to reduce cell death (Figure 3E).

Examining antibody cross-reactivity with class II HLA and SLA proteins

IgG specific for class II HLA proteins were tested for cross-reactivity with class II SLA. Six human sera were incubated with class II SLA positive Hek cells. Cells expressing pig CD74, SLA-DQα1*0101, and DQβ2*0601 were used because they contained abundant cell surface class II SLA protein. After washing, bound antibodies were eluted and used to probe beads containing individual class II HLA molecules (Figure 4). Four of the six sera contained cross-reactive antibodies primarily recognizing HLA-DQ4,5,6. The other sera recognized either HLA-DQ2,4 or HLA-DQ2,4,6,7,8,9. No cross-reactivity occurred between SLA-DQα1*0101/DQβ2*0601 molecules and HLA-DR or HLA-DP (not shown).

Comparing amino acid sequences of the cross-reactive molecules (SLA-DQα1*0101/DQβ2*0601, and HLA-DQ4,5,6) identified a common arginine at position 55 (55R) in the β chains from these particular HLA and SLA molecules. In addition, 55R contributes to an epitope found in HLA-DQ4,5,6 (13), and is located at the surface of the β chain (Figure 5A). These observations form the hypothesis that 55R resides in a cross-reactive epitope found in HLA-DQ4,5,6 and class II SLA.

To test this idea, cells expressing pig CD74, SLA-DQα1*0101, and SLA-DQβ2*0601 were incubated with human sera containing antibodies specific for class II HLA. Samples with HLA-DQ4,5,6 reactivity exhibited greater binding to the SLA-DQ positive cells than did allosensitized sera lacking antibodies specific for HLA-DQ4,5,6 (Figure 5B). Next, we produced a mutant SLA-DQβ2*0601, having a proline at residue 55 (55P) instead of arginine. We reasoned this change would not impair biosynthetic competence of the SLA-DQ variant because several class II HLA β chain alleles contain

55P (13). Compared to the non-mutant SLA-DQ 55R variants, the 55P molecules showed similar cell surface expression (Figure 6B) and binding of the invariant chain clip peptide (Figure 6A).

Lastly, 21 human sera were tested against cells expressing either the 55P or the 55R SLA-DQ variants (Figure 6B). Again, these sera contained HLA-DQ4,5,6 reactive antibodies. In a single sample, IgG binding increased on the 55P mutant compared to 55R (Figure 6B, *). In three samples, the 55P mutation reduced antibody interaction (Figure 6B, #; black bar values < 0) to background levels (Figure 6B, #; no red bar values > 0). For the remaining 17 sera, the 55P mutant also diminished immunoglobulin binding relative to the 55R variant (Figure 6B, black bar values <0). Antibody reactivity towards 55P was not completely eliminated in these samples as residual binding exceeded levels observed with SLA-deficient cells (Figure 6B, red bar values > 0).

Discussion

Initial pig-to-human transplantation experiments failed because preformed antibodies recognized glycan antigens on the donor tissue. Consequently, pigs have been engineered to express human complement regulatory proteins and lack expression of a single gene (14). This gene, GGTA1, produces an enzyme which transfers galactose to cell surface carbohydrates in an α1-3 linkage (αGal). Though pigs deficient in Gal expressed additional xenoantigens, the hope was that human complement regulators would inhibit antibody-mediated tissue damage. Inactivating other pig genes, CMAH and β4GalNT2, have further reduced xenoantigen expression when evaluated against most humans. The carbohydrates produced by the pig GGTA1/CMAH behave as

antigens in humans because people lack these glycans. Their deficiency arises as a consequence of evolutionary inactivation of GGTA1/CMAH in people (15,16). The reason for β4GalNT2 creating pig xenoantigens remains less clear because this gene remains functional in most humans (17).

In vitro analyses suggest that elimination of multiple xenoantigens may bypass antibody-mediated rejection in the absence of human complement regulatory transgenes (18). Our preliminary work using donor pigs with deficient GGTA1 and β4GalNT2 genes, and no human transgenes, supports this concept. A rhesus macaque has survived over 400 days with a life-supporting kidney from this double knockout pig [manuscript in preparation]. These results suggest that avoiding pig antigens, targeted by pre-existing human antibodies, may prevent donor-specific antibodies (DSA) from rejecting a xenograft.

This report shows that class II SLA molecules are xenoantigens. Human antibodies recognizing these proteins initiate complement-mediated cell lysis (Figure 3) and cross-react with shared epitopes found on HLA and SLA (Figures 4-6). Mutating an arginine in the SLA-DQ beta chain (55R) to proline (55P) eliminated SLA reactivity of three human sera. Cell surface expression of this mutant was minimally affected (Figure 6). This proof-of-principle shows that it is possible to eliminate MHC-driven xenoantigenicity without creating SLA-deficient animals. The 55P mutation reduced, but did not eliminate, IgG binding to the SLA-DQα1*0101/DQβ2*0601 protein in 17 of 21 human sera. This may result from incomplete disruption of a single antigenic determinant or from sera having IgG which recognize multiple epitopes. Further mutagenesis of the class II molecule could resolve this issue.

Careful histocompatibility analyses that match donors and recipients provide an engineering-independent method of bypassing DSA. The practice of looking for HLA-specific antibodies which may attack donor tissue has been key to the success of human allotransplantation. Recent data in pig to NHP xenotransplant models indicate careful cross-matching may improve xenotransplant outcomes (19) . Rather than performing complex genome editing, it may be possible to avoid class II SLA xenoantigenicity by careful donor-recipient pairing. However, in the case of people who have IgG that recognize HLA-DQ4,5,6 this may not be possible. These alloantibodies frequently target 55R in the SLA-DQβ chain (Figure 6B), and most, if not all, SLA-DQβ contain 55R. We found 100 SLA-DQβ protein sequences in the NCBI protein database. Of these, 94 contained arginine at position 55 (20). A second analysis showed that all 43 SLA-DQβ protein sequences in the IMGT database contained 55R (21). Therefore, switching animals to find a non-reactive SLA-DQ genotype for patients with antibodies recognizing the 55R epitope may be difficult.

Reagents created in this study have improved detection of humoral responses against class II SLA. As with other species the invariant chain improves cell surface expression of pig class II SLA proteins. These tools permitted the identification of amino acids having partial or major contributions to a xenoreactive epitope. Despite identifying SLA-DR, -DQ, and hybrid (DRβ/DQα) molecules as targets of xenoreactive human immunoglobulin, further refinements will be valuable. Though powerful, expressing various combinations of alpha and beta chains generates different molecules with variable levels of cell surface expression. This may skew which SLA appear to be the target of human antibodies towards highly expressed molecules. As an example, allele

pair 8 (Table 2) yields abundant SLA-DQ at the cell surface (Figure 1). Though we detected antibodies against every allele combination, pair 8 generated the most frequent and robust positive responses (Figures 2 and 3). Elevating the expression of the other class II SLA molecules may be needed to define their true role as xenoantigens.

As our understanding of pig antigens matures, issues of humoral xenoantigenicity appear increasingly similar to those facing alloantigenicity. Carbohydrates can be targets of DSA with α-Gal, Neu5Gc, and the β4GalNT2-derived glycans forming pig antigens (3,4). Carbohydrates of the ABO blood group comprise antigens on human tissue (22). DSA against carbohydrate-based xeno- and allo-antigens exist before exposure to the donor tissue (23). In addition, pig and human MHC synthesize protein antigens which can be targets of DSA (4,5,24). Generating HLA-specific antibodies typically requires exposure to foreign human tissue through transfusions, pregnancy, or organ transplantation. This report suggests that while HLA/SLA cross-reactivity may increase by allosensitization, human IgG and IgM can also arise in the absence of HLA-specific antibodies (Figures 2 and 3). Perhaps SLA antibodies generated in the absence of HLA reactivity reflect exposure to MHC-derived proteins from other species either through the food supply or vaccination.

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Table 1: Class II SLA Alleles.

SLA Allele	Accession #
DRα*020102	AIH07184.1
DRα*w04	AIH07183.1
DRβ1*0403	AIH07189.1
DRβ1*1001	AIH07190.1
DQα1*0204	AIH07186.1
DQα1*0101	AIH07185.1
DQβ1*0303	AIH07187.1
DQβ1*0601	AIH07188.1

These alleles represent two haplotypes from a single pig(8).

Table 2: Allele Combination Nomenclature.

SLA Allele Combination	Pairing
DRα*020102 + DRβ1*0403	1
DRα*020102 + DRβ1*1001	2
DRα*w04 + DRβ1*0403	3
DRα*w04 + DRβ1*1001	4
DQα1*0204 + DQβ1*0303	5
DQα1*0204 + DQβ1*0601	6
DQα1*0101 + DQβ1*0303	7
DQα1*0101 + DQβ1*0601	8
DRα1*020102 + DQβ1*0303	9
DRα1*w04 + DQβ1*0303	10

The various pairings of the alpha and beta chains of DR and DQ are indicated. Pairing numbers will be used to simplify the labeling of figures.

Figure 1: Pig CD74 Increases Cell Surface Expression of Class II SLA Proteins. Hek293 cells expressing 10 different class II SLA α and β heterodimers in the presence (black bars) or absence (red bars) of pig CD74 were tested for: (A) Binding to a monoclonal antibody, specific for class II SLA and **(**B) Binding of CerCLIP.1, a monoclonal antibody specific for invariant chain peptides associated with class II SLA. CD74 expression significantly increased antibody binding (p=0.002 for both assays, Wilcoxon matched-pairs sign rank test). Specific alleles making up each alpha-beta combination are indicated in table 2. DR indicates SLA-DRα/DRβ pairs, DQ represents SLA-DQα/DQβ pairs, and hybrid represent SLA-DRβ/DQα pairs.

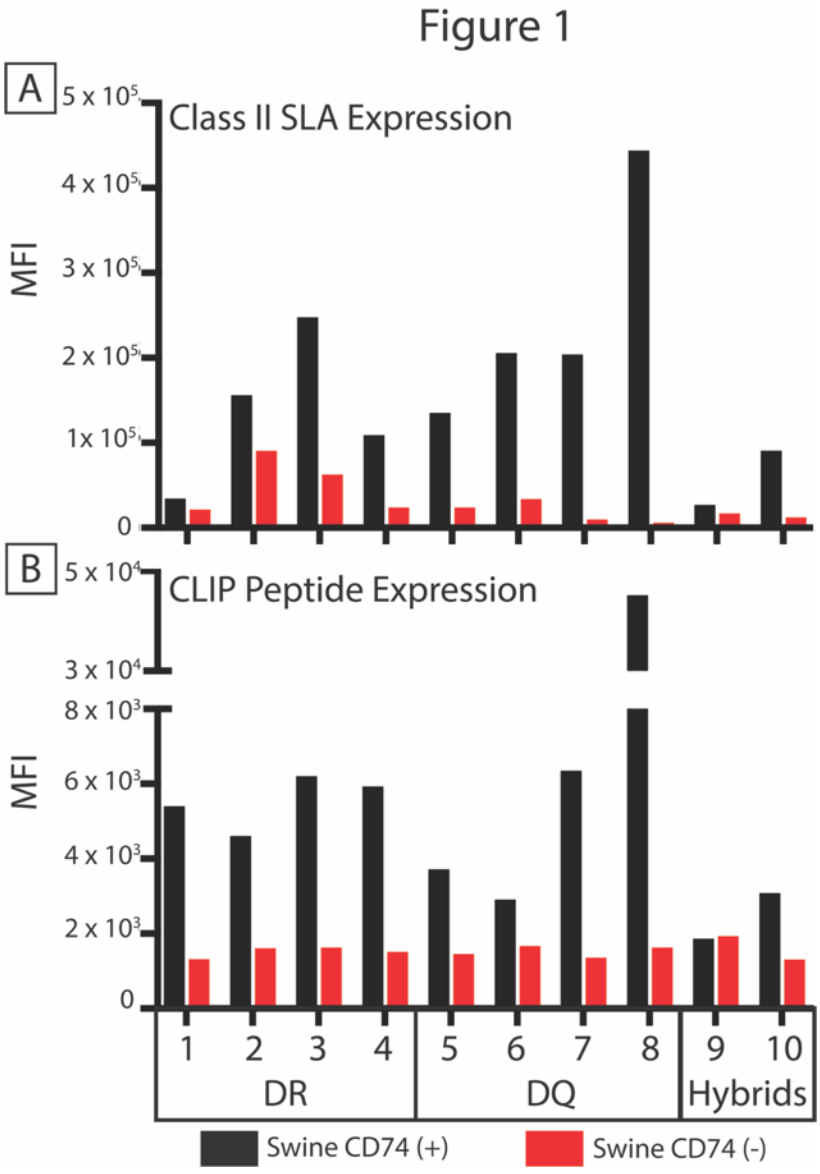


Figure 2: Evaluation of Human Antibody Binding to Class II SLA Co-expressed with Swine CD74. Human sera were incubated with cells expressing pig CD74 and various class II SLA proteins or with parent cells lacking both class II SLA and CD74. 26 sensitized sera contained antibodies specific for class II HLA. 38 un-sensitized sera lacked anti-HLA antibodies. Fluorescent antibodies detected human immunoglobulin binding to cells (panel A, IgM binding-unsensitized sera; panel B, IgM binding-sensitized sera; panel C, IgG binding unsensitized sera; panel D, IgG binding-sensitized sera). Dot plots show the Log_{10} of MFI ratios comparing immunoglobulin binding to SLA positive cells versus SLA-deficient parent cells. Values greater than 0 indicate more binding to class II positive cells. Log_{10} values less than 0 represent greater immunoglobulin binding to the parent cell line. When antibodies bind equally to both cell types the $Log_{10} = 0$. Heatmaps below the dot plots show binding results of sera having at least a single $Log_{10} \geq 1$. Each row represents an individual serum tested against every class II SLA heterodimer. Table 2 describes the class II allele-pair combinations. Gray squares represent class II (+) vs class II(-) MFI ratios ≤ 1. All other squares indicate increased binding to class II expressing cells. DR indicates SLA-DRα/DRβ pairs, DQ represents SLA-DQα/DQβ pairs, and H represent SLA-DRβ/DQα pairs.

Figure 2

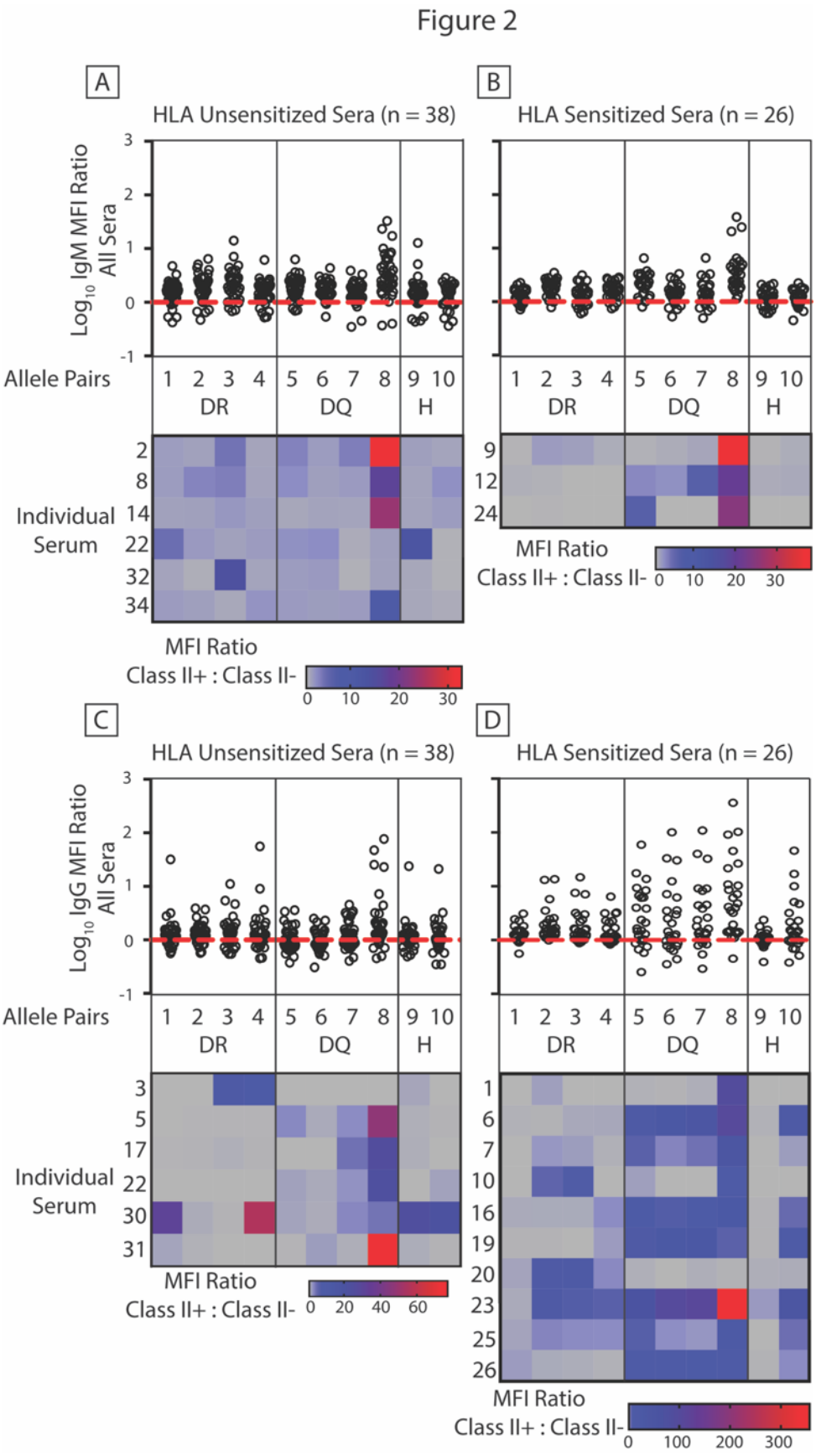

A
HLA Unsensitized Sera (n = 38)
B
HLA Sensitized Sera (n = 26)
Log10 IgM MFI Ratio All Sera
Allele Pairs
1 2 3 4 5 6 7 8 9 10
DR DQ H
Individual Serum
2 8 14 22 32 34
9 12 24
MFI Ratio Class II+ : Class II-
0 10 20 30
C
HLA Unsensitized Sera (n = 38)
D
HLA Sensitized Sera (n = 26)
Log10 IgG MFI Ratio All Sera
3 5 17 22 30 31
1 6 7 10 16 19 20 23 25 26
0 20 40 60
0 100 200 300

Figure 3: Human Antibodies Specific for Class II SLA can Initiate Complement Mediated Cytotoxicity. 7 sera lacking class II HLA antibodies and 7 sera containing class II HLA antibodies were incubated with cells expressing pig CD74 and individual combinations of class II SLA alleles. Table 2 describes the class II allele combinations. Parent cells were Hek cells devoid of CD74 and class II SLA. Rabbit complement was added and dead cells counted as an indicator of antibody initiated complement cytotoxicity (percent killing). (A) Cytotoxicity of sera lacking class II HLA antibodies. (B) Cytotoxicity of sera containing class II HLA antibodies. DTT was added to inactivate IgM in panels C (sera lacking class II HLA antibodies) and D (sera containing class II HLA antibodies). DR indicates SLA-DRα/DRβ pairs, DQ represents SLA-DQα/DQβ pairs, and H represent SLA-DRβ/DQα pairs. Parent represents killing against cells lacking both class II SLA proteins and CD74. Panel E shows the reduction of cell killing for a single serum against cells expressing all 10 class II SLA proteins before and after DTT treatment (±DTT comparison p = 0.001, Wilcoxon test).

Figure 3

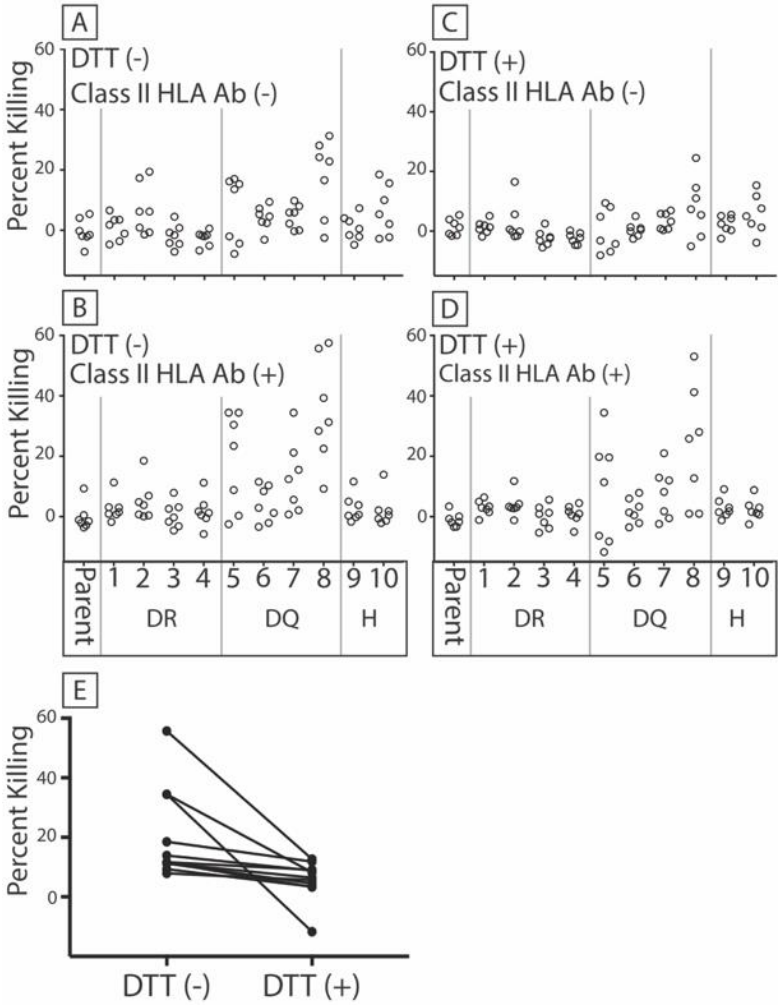


Figure 4: Examining Cross-reactivity of Human Antibodies with Class II SLA and Class II HLA Proteins. 6 sera containing class II HLA specific antibodies were incubated with a cell line expressing pig CD74, SLA-DQα1*0101, and -DQβ2*0601. IgG eluted from this cell line cross-reacted with beads containing HLA-DQ antigens but not HLA-DR or HLA-DP. The median fluorescence of antibody binding to beads containing the different HLA-DQ proteins are shown.

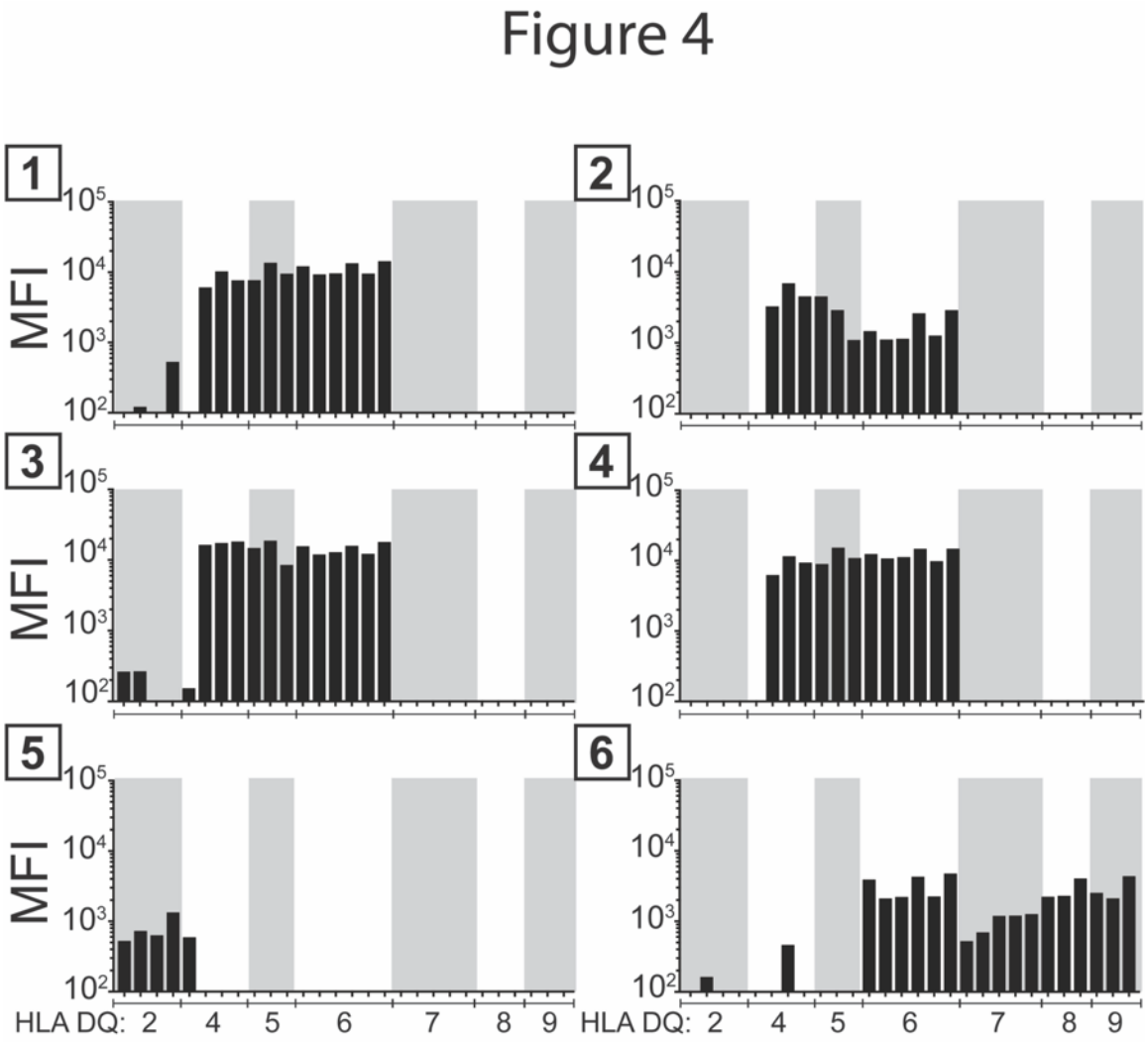


Figure 5: Amino Acid 55 Contributes to a Class II SLA Epitope which Cross-reacts with Human IgG. Following the results in Figure 4, sequence alignments of HLA-DQ4,5,6, SLA- DQα1*0101/DQβ2*0601 were performed. These suggested that arginine at position 55 contributed to the cross-reactive epitope in DQβ and in the HLA beta chains in HLA-DQ4,5,6. (A) Structural prediction highlighting the putative location of the 55R residue in the SLA- DQα1*0101/DQβ2*0601 molecule. This residue was mutated to a proline (R55P) which is found in class II HLA molecules other than -DQ4,5,6 HLA molecules. (B) Human IgG binding to a cell line expressing CD74, SLA-DQα1*0101, and SLA-DQβ2*0601. All tested sera contained some class II HLA-binding IgG. The samples were split into two groups based on the presence (n = 42) or absence (n = 21) of HLA-DQ4,5,6 reactivity. The presence of antibodies against HLA-DQ4,5,6 increased binding to SLA-DQα1*0101, and DQβ2*0601 (Mann-Whitney test, p= 0.030).

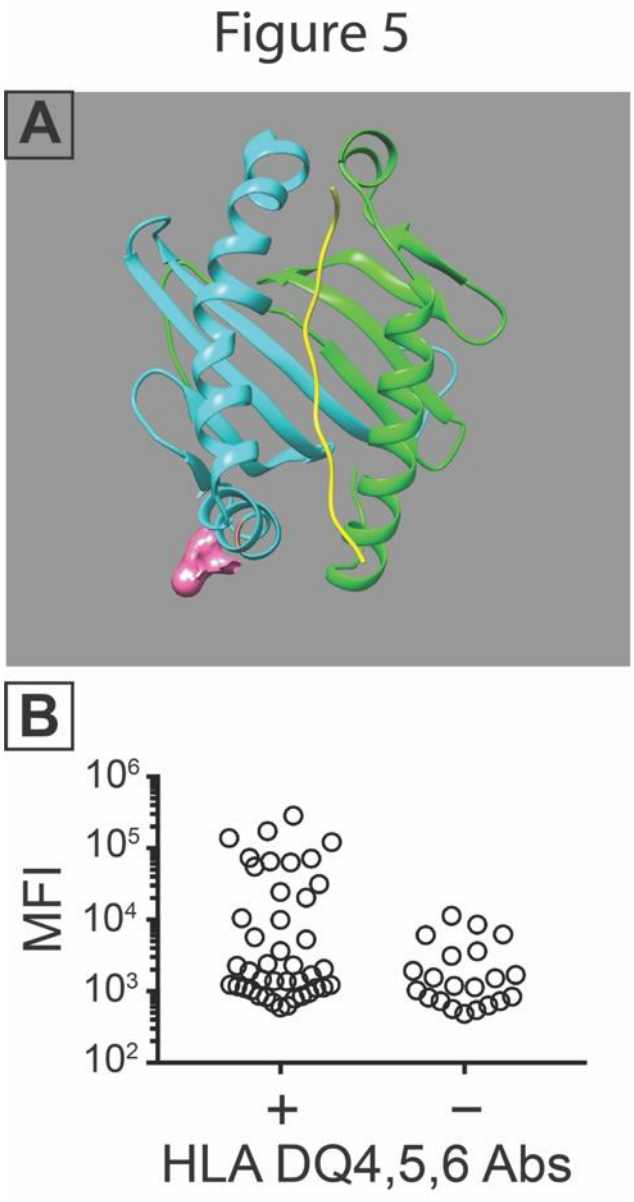
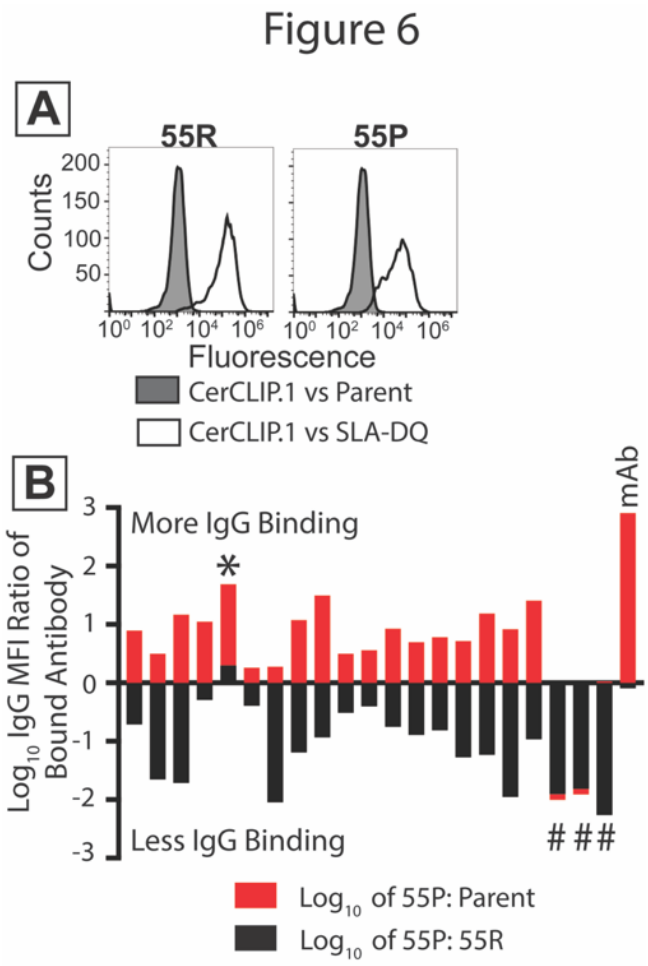
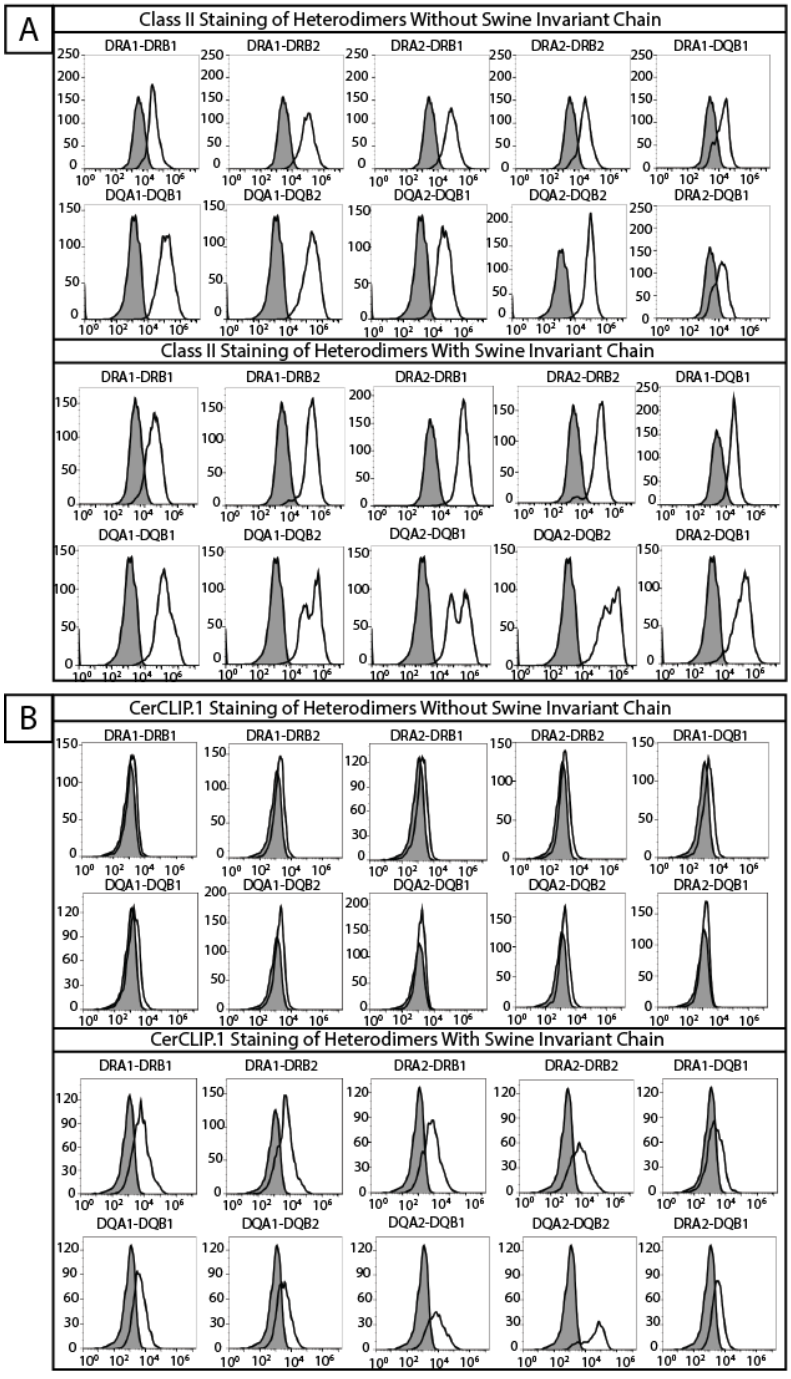


Figure 6: Mutation of Arginine 55 in SLA-DQ Beta Reduces Class II SLA-Based Xenoantigenicity. Cell lines expressed CD74, SLA-DQα1*0101, and mutant or unmodified SLA-DQβ2*0601. Mutant molecules contained a β chain having proline at position 55 (55P). Wild type class II contained arginine at position (55R). (A) White histograms show binding of the CerCLIP.1 antibody to cells expressing either 55R or 55P. Gray histograms show CerCLIP.1 binding to CD74/SLA-deficient HEK cells. (B) 21 human sera were incubated with cells expressing either the 55R or 55P variants of SLA-DQ. These sera were selected from prior experiments suggesting they reacted with SLA-DQα1*0101/ SLA-DQβ2*0601. Data from each sample is represented by one red and one black bar (stacked on top of each other). MFI ratios of fluorescent anti-human IgG were calculated to compare binding to 55P versus 55R molecules. The $\log_{10}$ values of these ratios were plotted for each serum (black bars). The same analysis compared IgG binding to the 55P cells versus SLA-negative parent cells (red bars). The 55P mutation partially reduced IgG binding to the SLA-DQ protein in 17 samples. Three samples had no detectable antibody staining of 55P mutants (#). One sample showed greater binding to the 55P mutant than to 55R (*). The mAb sample indicates the binding of a monoclonal antibody specific for SLA-DQ. This antibody recognized SLA-DQ 55P variants (red bar) almost identically to 55R variants (black bar, Log_{10} of 55P/55R = -0.09).

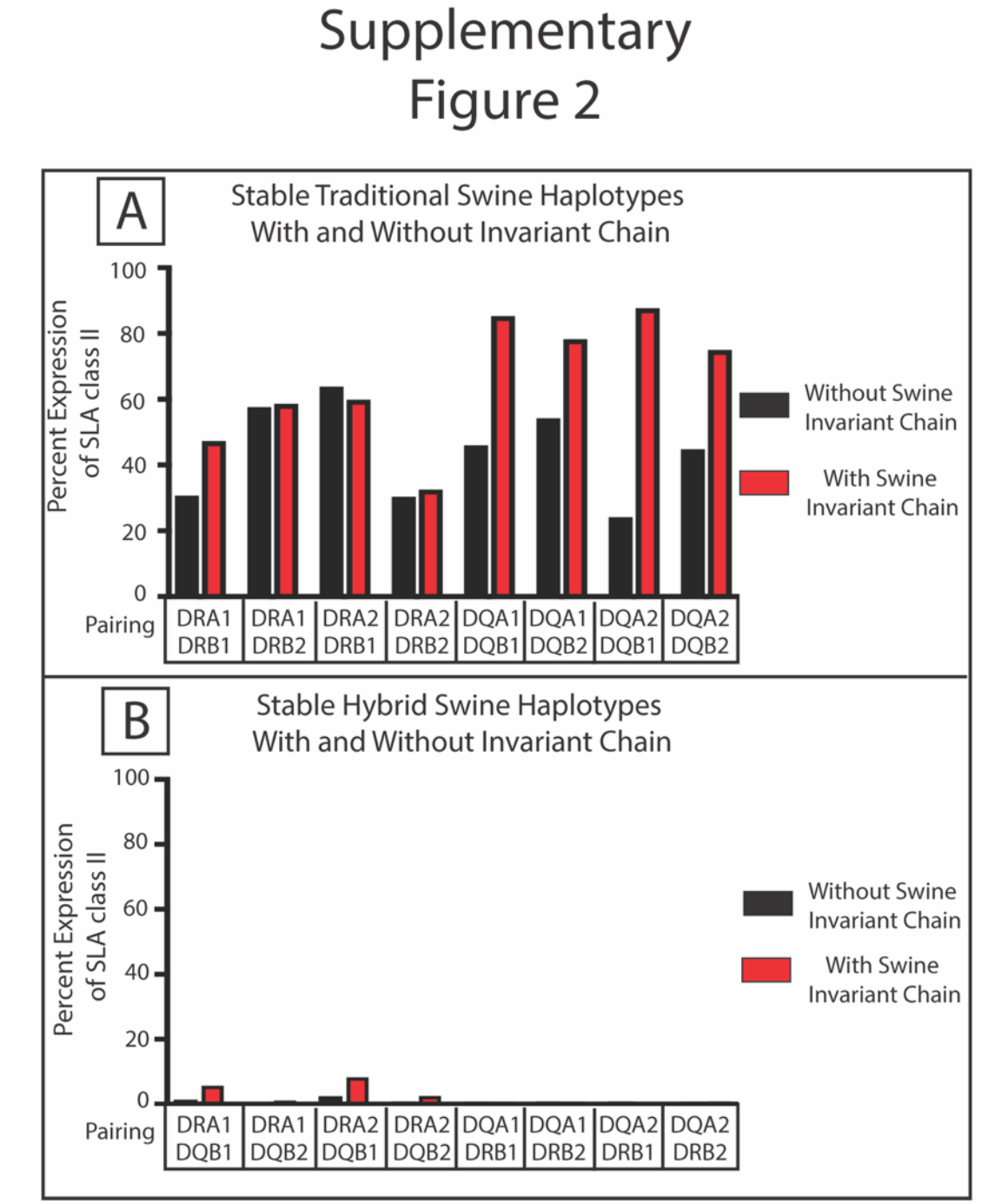


Supplementary Figure 1: Post-flow cytometry sorting phenotype of class II SLA single antigen cells. Cells were stained with antibodies specific for **A)** SLA-DR or -DQ or **B)** CerClip.1 to validate cell surface expression of the indicated α-β pairs (white histograms). Grey histograms represent negative control staining of the parent HEK293 cell devoid of β2-microglobulin and class II SLA.

Supplementary Figure 1



Supplementary Figure 2: Antibiotic selection stable expression of SLA class II in HEK 293T cells transfected with sixteen different SLA class II alpha-beta plasmid combinations, with (red) or without (black) dual transfection of the swine invariant chain. The traditional cis- and trans-haplotype pairing are shown in A and the hybrid haplotype pairings are shown in B.



CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation sought to clarify the role of SLA class II as a potential xenoantigen and generate the appropriate reagents to perform histocompatibility testing for future xenotransplant recipients.

Principal Findings

CIITA transfected swine cells and SLA class II heavy-chain transfected human embryonic kidney (HEK) cells were developed to confirm SLA class II as a xenoantigen, with both systems demonstrating human sera antibody binding. The latter model was used to demonstrate an increase in expression of MHC class II in the presence of invariant chain and the existence of hybrid class II molecules, an occurrence known for HLA but not SLA. SLA-specific antibodies were shown to be cytotoxic to the single antigen SLA class II HEK cells, and the antibodies eluted from these cells bound SLA class II in an epitope-restricted pattern, most commonly to an arginine residue at position 55 of the SLA-DQB chain. That –DQ is the more immunogenic protein mimics findings in allotransplantation (175,176). Mutation of this amino acid epitope resulted in significantly decreased antibody binding for the sera tested but was amino acid and sera specific with a glutamic acid appearing to decrease binding the most. Avoiding individuals with a HLA crossmatch indicative of antibodies to the position 55 arginine is a strategy to avoid positive crossmatches in the highly HLA sensitized patient population.

Creation of a pig with 55R mutated to another amino acid, such as proline or glutamic acid is an alternative strategy to generate class II mutated pigs.

Context of the Field of Xenotransplantation

The Past

To achieve clinical xenograft success, AMR needed to be overcome. In the 1990s and early 2000s, the limitations identifying and purifying pig embryonic stem cells, coupled with a paucity of genetic engineering, made the development of antigen knockout pigs difficult. The pigs generated during this time relied on random integration, the incorporation of a protein driven by an exogenous promoter into the embryo through embryo microinjection. Although relatively easy and inexpensive, this strategy results in imprecise gene insertion, no control over number of gene copies inserted, and variable or mosaic patterns of gene expression.

Two approaches were attempted to decrease the antibody barrier: preventing the consequences of the complement cascade through complement regulatory proteins (CRP) and competitive inhibition of the known xenoantigen, Gal. The CRP resulted in the birth of CD59, CD55, and CD46 pigs (69,99). These proteins act to inhibit MAC complex formation, prevent the development of the C3 convertase complex, and aid the degradation of C4b and C3b, respectively (69,177). The goal of glycosylation competitive inhibition was to deplete the pig cells of reagents to generate the Gal antigen by expression of alternative glycan enzymes, and resulted in the development of three single-gene knock-in pig models: endo-β-galactosidase C (EndoGalC), galactosidase-α (GLA), and β-1,4-N-acetylglucosaminyltransferase III (Gnt-III) (132). Cells from the

CRP and glycosylation competitive inhibition pigs successfully outlasted WT in cytotoxicity assays, but the survival of the organs in NHP models was short-lived with the Gal antigen intact at the cell surface.

The Present

Homologous recombination (HR), the targeted disruption of a gene by insertion of a plasmid with complementary 5' and 3' ends, is a precise method to mutate or knockout genes but very inefficient and expensive. Given the lack of pig embryonic stem cells for embryo transfers, the development of the technology to clone pigs through somatic cell nuclear transfer was necessary before HR could be pursued (177). In 2003, Phelps et al announced the birth of a Gal knockout healthy pig (77). As mentioned in the INTRODUCTION, the survival of GTKO organs lasted weeks to months compared to the 6-hour survival of WT organs (177). Nuclease-based editing technology decreased the cost associated with gene knockouts while increasing the efficiency and speed at which pigs could be generated. Nuclease-based editing resulted in the birth of the CMAH/GTKO pig, the GTKO/SLA class I, and the GT/CMAH/β4GalNT2 KO (132). As discussed previously, human antibody binding to PBMCS from the GT/CMAH/β4GalNT2 KO were the lowest of any genetically-modified pig.

Previous reports proposed SLA class II to be a xenoantigen, but prior to this dissertation that hypothesis was unconfirmed (178). Although the glycan antibodies may play a more important role in causing acute AMR, the history of allotransplantation suggests that anti-MHC class II antibodies will be critical in both the early and chronic stages of AMR.

The Future

As the field of xenotransplantation progresses, it is likely that the genetic engineering strategies available will provide the opportunity for complex, sophisticated, and precise manipulations of the pig genome. Examples are available in the rapidity with which KO pigs can be generated. To confirm the role of SLA class II in long-term organ survival, a live healthy class II KO pig is essential. Studies with MHC class II deficient mice requiring sterile living conditions suggest the generation of a healthy SLA class II knockout pig will be difficult and expensive (177). The development of the reagents described allowed for the discovery of specific SLA class II immunogenic epitopes – rather than generating a class II null pig, it is possible to KO the offending epitope or protein (e.g. leave SLA-DR intact but KO SLA–DQ or mutate 55Arg for individuals who are HLA-DQ4,5,6 sensitized). This strategy removes the antigen and affords the pig the immunological proteins necessary to prevent infections. Precise genome-editing strategies such as this, coupled with further modification of the pig genome, will result in personalized organs for recipients that may eventually be superior to allografts.

Future SLA class II Studies

Despite providing a solid foundation for future studies in SLA class II, there are limitations to the detailed experiments that could be addressed in further studies. In the sera antibody binding experiments it is difficult to standardize the level of expression of SLA class II, the studies relied on monoclonal antibody levels to approximate the level of protein expressed but the binding of antibodies is influenced by a variety of factors including the structure of the individual haplotypes, presentation of CLIP/peptides, and

conformation of antibody (177). A potential solution to this system is an independent marker of expression to standardize sera antibody binding – coupling the class II alpha or beta chain to a fluorescent protein to provide an unbiased indication of expression. An alternative strategy is the purification of SLA proteins and coupling standardized concentrations to microbeads, a reagent similar to the clinically-approved Luminex beads.

Given that the predominant antibody epitope appears to be SLA-DQ, studying the role and influence of SLA-DQα in sera antibody recognition and binding could also provide guidance as to how best genetically engineer the donor pig (179). Additionally, the confirmation of the other antibody epitopes, such as 71Thr on SLA-DQβ, needs to be confirmed. Finally, a few sera samples that were unsensitized to HLA proteins appeared to have SLA class II antibodies capable of binding and killing the cell. The etiology and epitope of these antibodies is unknown. It is suspected that diet could be a cause of these SLA class II restricted antibodies, but potential sensitization may have occurred through social interaction or surgical procedures utilizing pig-derived tissue (177).

Further studies on the biochemical construction of SLA class II are required. It is unknown what isoforms of pig Ii exist. Humans possess multiple isoforms of Ii, with varying distinct class II molecule affinities and pathways (177). The efficiency and role of SLA-DM and –DO in peptide presentation represent additional areas of investigation, disruption of these proteins may positively or negatively impact the class II presented peptides and result in suppressed or hyperactive T cell proliferation.

The focus of this dissertation was antibody binding to SLA class II, and it is well known that human T cells are capable of proliferating in response to swine cells (177).

The anti-pig T cell response is thought to be both CD4 and CD8 mediated, but the CD4 response to SLA class II appears to be the stronger and more critical to graft rejection of the two T cell subsets (137,180). The class II reagents described will be crucial for *in vitro* models of T cell-SLA class II interactions and may provide evidence that certain haplotypes or proteins are more stimulator to T cells, similar to SLA-DQ binding more antibodies than SLA-DR. The cell lines will also be beneficial in class II peptide presentation assays –class II presented peptides can be eluted from the cells and the amino acids sequence can be confirmed via mass spectrometry. Differences in what peptides the SLA class II molecules present may help guide selection to generate an ideal donor organ. Further strategies to influence class II peptide presentation or T cell proliferation can be analyzed *in vitro:* mutate the class II binding groove, -DM or –DO, the CD4 binding site, and sites of class II-TCR interaction. Potential amino acids for mutagenesis studies are described in **The Biosynthesis of MHC Class II** in the **INTRODUCTION.**

As suggested, live SLA class II knockout swine, although expensive and logistically complex, would provide organs to confirm the role of class II in antibody formation and potential graft failure in NHP transplants. To repeat a previous point, one strategy to avoid producing a class II null pig is disruption of either the SLA-DQ or –DR alleles, or mutation of the antibody epitopes. If these strategies result in defective class II molecules and unviable animals, an alternative strategy is to replace immunogenic with non-immunogenic alleles. The swapping of proteins could avoid antibody binding, although the 55Arg epitope is not a candidate for this strategy as it is expressed on all SLA-DQβ proteins. A final strategy to avoid antibodies would be the replacement of the

immunogenic SLA-DQ protein with the recipient matched HLA-DQ. It is unknown how human T cells would respond to HLA-DQ and –DQ presented peptides on a pig cell, requiring *in vitro* studies before the generation of a live animal.

Remaining Hurdles to Clinical Xenotransplantation

As the field prepares to submit applications for FDA approval, the remaining hurdles to clinical application will be briefly addressed.

The Zoonotic Concern

The primary zoonotic concern in xenotransplantation is transmission of porcine endogenous retroviruses (PERVs) from pig to human but recent advances in our understanding of PERV have rendered this a minor issue. Endogenous retroviruses are copies of retroviral DNA that have integrated into the genome of the host and have been found in all mammalian cells (181). Although up to 62 copies of PERV have been detected in primary pig cell lines (177), many of these viruses are defective due to deleterious mutations, and the ease at which PERV infects human cells *in vitro* is controversial (182). All known infectious PERV species capable of growth in human cell lines have been classified into the γl retroviral family and consist of subfamilies A, B, and C.

The PERV subfamilies possess high homology in the *gag* and *pol* retroviral genes, but significant dissimilarity in the *env* gene results in variable host cell growth. The experiments performed by Oldmixon et al and Wood et al demonstrated that while PERV-A and -B are capable of infecting both human and pig cells *in vitro*, PERV-C

possesses a narrow host range and only infects pig cells (182). However, in transmission studies performed by Wood et al of co-cultured pig cells with human targets, the most detected version of PERV was a recombinant PERV-A and –C (PERV-A/C). The authors suggest that the –C locus is necessary to produce a human-tropic replication competent PERV (183). A Martin et al study isolated PERV-A/C recombinants and demonstrated that 145 amino acids of the transmembrane component of the PERV-C *env* are conserved in PERV-A/C recombinants, suggesting a requirement of this region to drive the recombination process (72,184). Careful selection of a PERV-C negative swine herd may significantly reduce the associated zoonotic risk with pig organ donors without the need for genetic engineering (185).

PERV-A, -B, and –A/C may be infectious *in vitro*, but the ability to infect human or NHPs *in vivo* has not been demonstrated. Switzer et al tested 21 Old World and 2 New World NHP exposed to pig xenografts, including six hearts, six skin grafts, and six pig islet cell grafts (186). The NHPs were screened by polymerase chain reaction (PCR), Western blot, and reverse-transcriptase PCR (RT-PCR) for presence of PERV and all plasma, peripheral blood mononuclear cell (PBMC), and tissue samples were negative for PERV sequences or antibodies. Heneine et al tested 10 human diabetic patients who received pig fetal islets for evidence of PERV by PCR serology, PCR, and RT-PCR (187). The one-year survival of xenograft tissue was confirmed in five patients by pig C-peptide excretion and mitochondrial DNA (mtDNA) in patient sera. All tests were negative for markers of PERV infection, both at the early (day 3 to day 180) and the late (4 to 7 years) time points. Studies have also examined the sera and PBMCs of immunosuppressed NHPs inoculated with high titer PERV, patients exposed to an

extracorporeal bioartificial liver containing a membrane deviced composed of pig hepatocytes, and diabetic patients enrolled in the first clinical pig encapsulated xeno-islet transplant program (188). All studies returned negative results for the presence of PERV in the human and NHP recipients.

In the theoretical event of PERV *in vivo* infection, clinically-approved anti-retrovirals have been shown to efficiently inhibit PERV replication. Of note, the life-cycle of PERV can be inhibited at reverse transcription of RNA to DNA and the integration of the retroviral DNA into the host genome (189). If necessary to completely eliminate PERV from the pig genome, Niu et al have shown the ability to mutate the PERV loci with CRISPR/Cas9 nuclease technology and generate a live pig (69,99).

Organ Selection

Following FDA approval, the kidney is likely to be the first whole organ used in the clinical xenotransplantation trials. There is an immediate and pressing need for kidney donors, and an alternative available in dialysis if the graft should fail. Additionally, the availability of dialysis provides time to meticulously evaluate and screen potential recipients – maximizing both the patient's safety and odds of successful graft outcomes.

Screening Candidates for Xenotransplantation

Appropriate MHC matching between donor-recipient pairs is a proven method to improve transplant graft outcomes, and developing reagents to screen and avoid high titer antibodies in recipients is crucial for histocompatibility screening. Given the low human

sera antibody binding to cells from the GT/CMAH/β4GalNT2 KO pig, this is likely the background on which the first clinical trials will take place. Studies have shown that commercially-available, FDA-approved immunosuppressants are sufficient to control the human T cell response to pig tissues (190). Lessons from allotransplantation demonstrate the necessity to select patients with no-to-low antibodies to the donor organ, requiring careful screening of potential recipients. Using the reagents described above, candidates can be evaluated for class II antibodies and serially monitored for signs of *de novo* DSA formation or graft rejection.

Conclusion

Successful xenotransplantation will provide a second chance at normal life for hundreds of thousands experiencing end-stage organ failure. In order to prevent acute and chronic rejection, a meticulous understanding of potential xenoantigens is required. This dissertation addresses and confirms a previously hypothesized xenoantigen, the SLA class II. Following confirmation of the class II antigenicity, a strategy to prevent antibody binding was tested. The reagents detailed in this study will help screen and select candidates for clinical xenografts.

The future of xenotransplantation is bright, and few hurdles remain before clinical trials. Dr. Thomas Starzl, the father of transplantation, wrote "procedures that were inconceivable yesterday, and are barely achievable today, often become the routine of tomorrow." The transplantation of genetically-modified pig organs into human recipients appears to be ready for routine application.

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APPENDIX A

IACUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 27-Apr-2017

TO: Tector, Alfred Joseph

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 27-Apr-2017.

Protocol PI:	Tector, Alfred Joseph
Title:	Xenokidney Development and Regulatory Approval (XDARA-K)
Sponsor:	UNITED THERAPEUTICS CORPORATION
Animal Project Number (APN):	IACUC-20862

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 26-Apr-2020.

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
CH19 Suite 403 | CH19 Suite 403
933 19th Street South | 1530 3rd Ave S
(205) 934-7692 | Birmingham, AL 35294-0019
Fax (205) 934-1188 |

APPENDIX B

IRB APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The Assurance number is FWA00005960 and it expires on November 8, 2021. The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56.

Principal Investigator:	Tector, Alfred Joseph
Co-Investigator(s):	Easlick, Juliet L. Hauptfeld, Vera Ladowski, Joseph M Martens, Gregory Tector, Matthew F Wang, Zheng-Yu
Protocol Number:	**X170410006**
Protocol Title:	*Histocompatibility Testing (Xenokidney Development and Regulatory Approval)*

The IRB reviewed and approved the above named project on 5/11/17. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.

IRB Approval Date: 5/11/2017

Date IRB Approval Issued: 5/11/17

IRB Approval No Longer Valid On: 5/11/18

HIPAA Waiver Approved?: Yes

Expedited Reviewer
Member - Institutional Review Board
for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

470 Administration Building
701 20th Street South
205.934.3789
Fax 205.934.1301
irb@uab.edu

The University of
Alabama at Birmingham
Mailing Address:
AB 470
1720 2ND AVE S
BIRMINGHAM AL 35294-0104

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

DATE: August 22, 2016

MEMORANDUM

TO: A. Joseph Tector
Principal Investigator

FROM: Cari Oliver, CIP
Assistant Director
Institutional Review Board for Human Use (IRB)

RE: Request for Determination – Not Human Subjects Research
IRB Protocol N160805010 – Kidney Xenotransplant Development and Regulatory Approval

A member of the Office of the IRB has reviewed your Application for Not Human Subjects Research Designation for above referenced proposal.

The reviewer has determined that this proposal is **not** subject to FDA regulations and is **not** Human Subjects Research. Note that any changes to the project should be resubmitted to the Office of the IRB for determination.

470 Administration Building
701 20th Street South
205.934.3789
Fax 205.934.1301
irb@uab.edu

The University of
Alabama at Birmingham
Mailing Address
AB 470
1720 2ND AVE S
BIRMINGHAM AL 35294-0104

Application for Designation of Not Human Subjects Research (NHSR)

- This form is to be used to request a determination by the IRB (or designated reviewer) of whether an activity is research involving human subjects.
- Complete every numbered item, using a font different from the items for your **responses**.
- Retain the order, numbering, and general layout of this form.
- Please direct questions or comments to the Office of the IRB at 205-934-3789 or irb@uab.edu.

GENERAL INFORMATION

1. Title of Project **Kidney Xenotransplant Development and Regulatory Approval**

2. Principal Investigator	Name	**A. Joseph Tector**		
	Department/Division	**Surgery / Transplant**		
	Mailing Address	**LHRB 752, 1720 2nd Ave. S, Birmingham Al 35294-0000**		
	Telephone	**205-934-5747**	BlazerID	**jtector**
3. Contact Person	Name	**Matt Tector**		
	Telephone	**317-471-9577**	Fax **NA**	BlazerID **mtector**

4. Is this activity funded in any way? ☒ Yes ☐ No

If yes, *attach* 1 copy of completed funding application and complete (a)-(d):

a. Grant or Contract Title		**Kidney Xenotransplant Development and Regulatory Approval**
b. PI of Grant or Contract		**Devin Eckhoff MD**
c. OSP Proposal Number		**000511202**
d. Funding Source	Gov't Agency or Agencies	**[name]**
	UAB Departmental Funds	**Surgery**
	Other	**United Therapeutics**

OTHER INVESTIGATORS, SUPERVISORS

5. Is anyone listed as a Co- or Other investigator on this project? ☐ Yes ☐ No

If yes, indicate Co- or Other and complete (a)-(c) for each investigator. Copy/paste the three rows below as necessary for additional investigators.

a. Name	**Co-** ☒ **Other** ☐	**Joseph Tector**
b. Job Title		**Professor**
c. Primary UAB Dept., or non-UAB Employer		**Transplant Surgery**
a. Name	**Co-** ☐ **Other** ☒	**Matt Tector**
b. Job Title		**Assistant Professor**
c. Primary UAB Dept., or non-UAB Employer		**Surgery**
a. Name	**Co-** ☐ **Other** ☒	**Zheng Yu Wang**
b. Job Title		**Assistant Professor**
c. Primary UAB Dept., or non-UAB Employer		**Surgery**
a. Name	**Co-** ☐ **Other** ☒	**Greg Martens**
b. Job Title		**research associate**
c. Primary UAB Dept., or non-UAB Employer		**surgery**

a. Name	Co- ☐ Other ☒	**Joseph Ladowski**
b. Job Title		**research associate**
c. Primary UAB Dept., or non-UAB Employer		**surgery**
a. Name	Co- ☐ Other ☒	**Sue Downey**
b. Job Title		**Research Technician**
c. Primary UAB Dept., or non-UAB Employer		**Surgery**

6. Is the principal investigator (named in Item 2) a UAB student? Yes ☐ No ☒

If yes, provide name, phone, and BlazerID of student's supervisor, and ***obtain signature*** of supervisor.

Name **[name]** Telephone **[telephone]** BlazerID **[blazer ID]**

Signature of Student's Supervisor:

CRITERIA FOR DETERMINATION

7. Is the activity a systematic investigation, including research development, testing and evaluation, designed to develop or contribute to generalizable knowledge?	☒Yes ☐No
8. Does the activity involve obtaining information about living individuals?	☒Yes ☐No
9. Does the activity involve intervention or interaction with any living individuals?	☐Yes ☒No
10. Does the activity involve information that is *individually identifiable*, that is, "the identity of the subject is or may readily be ascertained by the investigator or associated with the information"?	☐Yes ☒No
If yes, is the information private, that is, "about behavior that occurs in a context in which an individual can reasonably expect that no observation or recording is taking place, and information which has been provided for specific purposes by an individual and which the individual can reasonably expect will not be made public"?	☐Yes ☐No
11. Does the activity involve one or more human subjects and any use of a drug other than the use of a marketed drug in the course of medical practice; that is, is it subject to FDA IND regulations?	☐Yes ☒No
12. Does the activity involve one or more human subjects and any evaluation of the safety or efficacy of a medical device; that is, is it subject to FDA IDE regulations?	☐Yes ☒No
13. Are the results of the project to be submitted later to, or held for inspection by, the FDA as part of an application for a research or marketing permit?	☒Yes ☐No

ACTIVITIES INVOLVING HUMAN MATERIALS

To help the IRB determine that your project does not need further review, complete the following items.

14. Does the activity involve *only* cadaveric materials?	☐Yes ☒No
If yes, *attach* documentation from source.	
15. Does the activity involve *only* blood products from the Red Cross or other blood banks?	☐Yes ☒No
If yes, *attach* documentation from source.	
16. Does the activity involve potentially identifiable human materials, such as those from an autopsy?	☐Yes ☒No
If yes, describe the materials, their origin, the coding system, and plans for use: **[description]**	

17. Briefly describe the proposed research, including what materials you are obtaining and the source of those materials:

RED CROSS and UAB Pheresis Labs: We hope to perform research analyzing the compatibility of human blood or components of human blood with various pig cells, tissues, and molecules. This data may also become part of an FDA application for a clinical trial in Xenotransplantation. We would like to collect blood from blood banks (including the Red Cross) and also clinical labs that are part of UAB. The products we would access are collected by the blood banks and clinical labs as part of their normal services, but are to be discarded as by products or excess material from their standard processing.

UAB HLA LAB: **We are attempting to modify the genes in pig cells to make pig cells compatible with the human immune system. We plan to mix human sera/plasma with pig cells to evaluate if human antibodies attack modified pig cells. We hope to collect excess diagnostic samples from the HLA typing lab to serve as a source of the plasma and sera that we will analyze. We would like to collect as many samples as possible from as many patients as possible with no special criteria in terms of sex, race/ethnicity. We also hope to collect data with each sample that indicates if the given sample contains antibodies that react with human HLA molecules. We would like the PRA score and if available the specific human alleles that each sample recognizes. We will ask the HLA lab to create a coded key to ensure that we are able to distinguish samples/data from one another. The HLA will not allow us access to that key.**

Mail or deliver all IRB materials and correspondence to
Room 470, Administration Building (AB)
701 20th Street South, Birmingham, AL 35294-0104
Phone 205-934-3789 — Fax 205-934-1301

ORIGINAL, DATED SIGNATURE OF PRINCIPAL INVESTIGATOR			
Signature:		**Date:**	08/03/2016

For IRB Use Only

Notes

APPENDIX C

COPYRIGHT TRANSFER FORMS

February 28, 2018

Joseph Ladowski
University of Alabama-Birmingham
1900 University Blvd
Birmingham

Email: jmladows@uab.edu

Dear Dr. Ladowski:

The American Association of Immunologists, Inc., grants permission to reproduce Figures 1-6 from the article, "Examining the Biosynthesis and Xenoantigenicity of Class II SLA Proteins," published in *The Journal of Immunology,* vol. 200, in press, 2018, in your PhD thesis, contingent on the following conditions:

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