CHARACTERIZATION OF ANTIBODY BINDING TO SWINE LEUKOCYTE ANTIGEN CLASS II

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Master of Science
in the Translational Science Program,
Indiana University

June 2016

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree of Master of Science.

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ACKNOWLEDGEMENTS

It is difficult to express in words the gratitude one can have for those who impact your life in such a positive way. Over the past two years I have learned a tremendous amount about research, academia, and life. I would like to thank Dr. Mark Payne and Ms. Carrie Hansel for the opportunity to pursue this Master's in Translational Research.

Of course, it would be impossible to pursue this work without my advisors: Dr. Janice Blum, Dr. Matt Tector, and Dr. A. Joseph Tector. I have made more mistakes than most graduate students so specifically I would like to thank these three for their tireless patience. I am consistently astounded by their impressive intelligence, direction, and drive. If I can incorporate even a fraction of their knowledge and passion into my own life, I will have a long and fruitful career.

Finally, I would like to thank my family, especially my wife, Alicia, and son, Jackson. My research required long-hours and weekends and unfortunately these two felt the full burden of that. Despite this, my wife's tireless support and encouragement are truly appreciated, even if not voiced nearly enough.

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Though the elimination of carbohydrate xenoantigens has reduced the antibody barrier to clinical xenotransplantation, identification of additional targets of rejection could further increase the immunologic compatibility of pig tissues with humans. Many patients in need of organ transplantation have antibodies to proteins encoded by the human major histocompatibility complex (MHC) which have high similarity to their swine homologs. The goal of this thesis was to determine if the class II genes of the swine MHC can bind human antibodies.

To characterize antibody binding effect to class II swine leukocyte antigens (SLA), a constitutively positive SLA class II cell was created through transfection with the human class II transactivator (CIITA). Cells expressing only SLA-DR or SLA-DQ were also created using the CRISPR/Cas9 gene knockout tools. These various lines were incubated with human sera and tested for binding to IgM and IgG in a flow cytometry crossmatch (FCXM).

The results demonstrate reliable antibody binding to each of the SLA class II –DR and –DQ derivatives. A two-way paired t-test revealed statistical difference in total sera binding between to the DR(+)DQ(+) and DR(-)DQ(-) clones for IgG ($p = 0.0059$) but not IgM ($p = 0.2460$). Looking at the subset of individuals with and without anti-HLA class II sensitization, statistical difference was noted for IgG ($p = 0.0229$) but not IgM ($p = 0.3045$). Examining further the role of DR(+) vs DQ(+), statistical analysis revealed difference in the DR(+)DQ(-)

vs. the DR(-)DQ(+) FCXM ($p = 0.0099$), the DR(+)DQ(-) vs. the DR(+)DQ(+) FCXM ($p = 0.0192$), and the DR(-)DQ(-) parent vs. DR(+)DQ(+) FCXM ($p = 0.0329$). No difference was found in the DR(-)DQ(+) vs. DR(+)DQ(+) FCXM ($p = 0.1601$).

The results of this project suggest that SLA class II, specifically SLA-DQ, could be a target of antibody binding and cross-reactive anti-HLA class II antibodies may be capable of binding SLA class II.

A. Joseph Tector MD, PhD, Chair

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ABBREVIATIONS

αGal Galα1-3Galβ1-4GlcNAc-R
AMR ..... Antibody-Mediated Rejection
APC ..... Antigen Presenting Cell
β2M β2-MIcroglobulin
β4GalNT2 ..... β1,4N-Acetylgalactosaminyl Transferase
Cas9 ..... CRISPR-Associated
CIITA ..... Class II Transactivator
CLIP ..... Class II-Associated Invariant-Chain Peptide
CREB ..... cAMP Response Element-Binding Protein
CREG ..... Cross-Reactive Group
CMAH ..... Cytidine-Monophosphate-Acetylneuraminic Acid Hydroxylase
CRISPR ..... Clustered Regularly Interspersed Short Palindromic Repeat
crRNA ..... CRISRP RNA
DSAbs ..... Donor Specific-Antibodies
ER ..... Endoplasmic Reticulum
FBS ..... Fetal Bovine Serum
FCXM ..... Flow Cytometry Crossmatch
HLA ..... Human Leukocyte Antigen
IFN-γ ..... Interferon-γ
IU ..... Indiana University
Jak ..... Janus Kinase
MFI ..... Median Fluorescence Intensity
MHC ..... Major Histocompatibility Complex
NHP ..... Non-Human Primate
PAM ..... Protospacer Adjacent Motif
PBMC ..... Peripheral Blood Mononuclear Cell
PRA ..... Panel Reactive Antibody
pre-crRNA ..... Pre-CRISPR RNA
RBC ..... Red Blood Cell
RT-PCR ..... Reverse-Transcriptase Polymerase Chain Reaction
SLA ..... Swine Leukocyte Antigen
sgRNA ..... Single-Guide RNA
tracrRNA ..... Transactivating crRNA
TCR ..... T-Cell Receptor
WT ..... Wild-Type

INTRODUCTION

Transplant Organ Shortage

As of March, 2016 there are over 121,000 patients in need of a lifesaving organ transplant. Of these patients, over 100,000 await a kidney, and 12 of those potential kidney recipients die each day (Organ Donation and Transplantation Statistics, 2015). This list does not include the necessity for pancreatic islets, eye tissue, red blood cells, skin tissue, and neuronal cells as well. To address the discrepancy between organ donors and potential recipients, xenotransplantation, the use of animals as organ donors, could be a possible supply for organs. Originally it was thought that Non-Human Primates (NHPs), such as baboons and chimpanzees, would be a suitable donor but ethical concerns and the potential risk that a NHP-confined virus could potentially infect human cells make pigs a more suitable source. Additionally, pigs have a shorter gestational period and time to maturity, similar physiologic size for kidneys transplants, larger litters, and cost less to raise and maintain (Cooper, 2012).

Barriers to Xenotransplantation

The original Wild-Type (WT) xenografts failed rapidly due to hyperacute rejection mediated by recipient preformed antibodies to some of the donor xenograft carbohydrates. The first and most well-known of these carbohydrates is Galα1-3Gal β 1-4GlcNAc-R commonly referred to as (αGal) (Galili, 1993). Disruption of α-1,3-galactosyltransferase, the enzyme responsible for synthesizing αGal epitopes within the Golgi apparatus, resulted in significantly decreased xenoantibody binding, but acute rejection remained a problem (Chen,

et al., 2005). The disruption of two other enzymes is theorized to further decrease the xenoantigen barrier, Cytidine-Monophosphate-Acetylneuraminic Acid Hydroxylase (CMAH) and β1,4N-acetylgalactosaminyl Transferase (β4GalNT2). Mutation of CMAH and βGalNT2 on an αGal-deficient background diminished antibody binding on Peripheral Blood Mononuclear Cells (PBMCs) to levels that may be considered clinically acceptable. Unfortunately a group of individuals remain with persistently elevated antibody binding (Estrada, et al., 2015). Given that the MHC genes of humans and swine are highly similar and the fact that many humans have antibodies to non-self human MHC, it is theorized this residual binding could be to proteins encoded by the swine MHC.

The Major Histocompatibility Complex (MHC)

The classical MHC genes, responsible for presenting peptide antigens to T-cells, are commonly divided into two classes of relevance to transplantation: class I and II. The human versions are referred to as Human Leukocyte Antigens (HLA) and the porcine is referenced as Swine Leukocyte Antigens (SLA). Generally MHC class I presents endogenous peptides, commonly self-proteins or invasive viral particles, and MHC class II presents exogenous peptides (Parham, 2005).

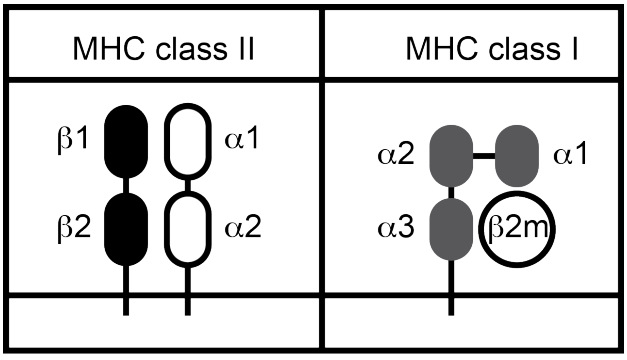

Figure 1: Representation of the structure of the MHC class II (left image) and MHC class I (right image)

MHC class I is present on the majority of nucleated cells as a heterodimer composed of the class I heavy chain bound to the protein B2-microglobulin (β2m) (**Figure 1**) (van Endert, 1999). While class I is found on nearly every cell, constitutive expression of MHC class II is typically considered to be restricted to professional antigen-presenting cells (APCs). Work by Daar et al has shown that this model might not be accurate and that class II expression can be found on multiple organ systems (Daar, Fuggle, Fabre, Ting, & Morris, 1984). In addition, stimuli such as Interferon-γ (IFN-γ) can drive the expression of class II MHC genes by non APC (Steimle, Otten, Zuffarey, & Mach, 1993).

MHC Class II

Class II molecules, like class I, are heterodimers constructed in the Endoplasmic Reticulum (ER) with a binding pocket capable of binding longer peptides than the MHC class I binding pocket (13-25 compared to class I's 8-10 amino acids). In the ER, the newly synthesized alpha and beta chains of MHC class II bind to a third chain, the invariant chain (Guagliardi, et al., 1990). The invariant chain assists class II protein folding in the ER (Cresswell, 1994) and blocks the class II binding pocket to inhibit peptide loading of class II. The invariant chain also directs trafficking of class II molecules to the endocytic pathway (Riberdy, Newcomb, Surman, Barbosat, & Cresswell, 1992) where it is cleaved by proteases such as cathepsin S, leaving a small fragment named the class II-associated Invariant-Chain Peptide (CLIP) still bound to the class II binding pocket. CLIP can then be removed by a specific class II glycoprotein,

HLA-DM, and the class II binding pocket is then capable of sampling endocytic vesicle antigens (Denzin & Cresswell, 1995) (Kropshofer, et al., 1996).

Role of CIITA in MHC Class II Creation

Transcription of the class II proteins is controlled by presence of the class II transactivator (CIITA), an “on/off switch” for class II creation. Multiple promoters, leading to multiple CIITA transcripts, regulate CIITA. These promoters are selectively activated in various cell types and lead to either inducible or constitutive expression. IFN-γ has been shown to induce class II expression through CIITA via the Janus kinase (Jak)-Stat pathway (Lee & Benveniste, 1996). Binding of IFN-y to its cell-surface receptor activates Janus kinase 1 (Jak1) and Jak2, allowing the Jak molecules to phosphorylate Stat1, a transcription factor located in the cytoplasm which when activated translocates to the nucleus and activates the IFN-γ-responsive promoters of CIITA.

Once CIITA is created, it serves as a coactivator with a few other transcription factors necessary for appropriate MHC-II regulation: RFX5, RFX-AP, RFX-ANK, the NF-Y complex, and cAMP Response Element-Binding protein (CREB). RFX5, RFX-AP, and RFX-ANK are components of the greater RFX complex which binds the X1-box region of the MHC II promoter, while NF-Y factors binds the Y region and CREB binds the X2 region (Zhu, et al., 2000) **(Figure 2).** The assembly of these pieces creates an “enhanceosome” to which the CIITA binds and activates gene transcription of the MHC class II. Previous work by Steimle et al has also shown that transfection with CIITA cDNA can

render a cell constitutively class II positive (Steimle, Otten, Zuffarey, & Mach, 1993).

The Immune Response to the MHC

The MHC is a very polymorphic region of the genome and different MHC molecules and classes (A, B, C) can differ by only a few amino acid substitutions. In allotransplantation, MHC-mismatched recipients can produce a humoral antibody-mediated response that can lead to rapid, hyperacute rejection. This occurs in “sensitized” patients that contain antibodies specific for foreign MHC as a consequence of prior exposure during pregnancy, blood transfusions, or allografting. A foreign MHC is any MHC structure that differs from the self-MHC found on autologous cells. Non-MHC antibodies can also occur when a recipient is exposed to antigens such as those on a bacteria or a foreign sugar, as is the case when an A- or O-blood type is transfused with B-blood type Red Blood Cells (RBCs) covered in the B-antigen. These bound antibodies activate the complement cascade resulting in cell damage or death. The occurrence of this complement cascade within the vessels of an allo- or xenograft, covered in MHC class I, results in hyperacute rejection (Dalmasso, et al., 1992). A similar process found in xenotransplantation when a baboon recipient recognizes αGal sugars on

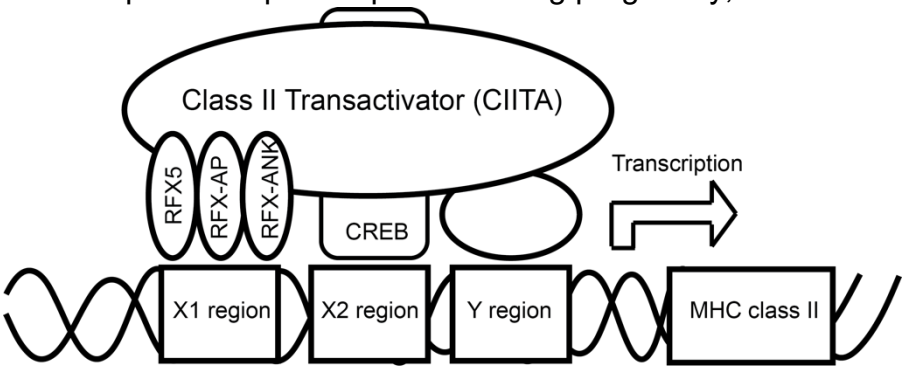

Figure 2: CIITA and the MHC class II promoter

the surface of the pig xenografts. De novo antibody production, following exposure to the transplanted organ, can also cause problems for grafts. In allotransplantation the de novo production of antibodies to MHC class II results in late-stage AMR and transplant glomerulopathy (Willicombe, et al., 2012).

Similarities Between SLA and HLA

Using the National Center for Biotechnology Information Blast Alignment Search Tool to compare the protein sequences of HLA (DRB1*03, -DQB1*02, -DRA*01:01:01:01, and –DQA1*01:01:01) to SLA (DRB1*0403, -DQB1*0303, -DRA*w04e01, and –DQA*0204), revealed over 75% identity between the two species**.** The HLA genes were chosen because they are common in the human population and the SLA types are those found in Indiana University (IU) Xenotransplant Lab swine: (Reyes, et al., 2014). These similarities suggest that anti-HLA antibodies could cross-react with SLA and II (Varela, Mozo, Cortes, Blanco, & Canedo, 2003). Additionally, future recipients may have previously created SLA-specific antibodies and TCRs through previous exposure to pig particles, either through diet (Hartig, Haller, Sachs, Kuhlenschmidt, & Heeger, 2000) or vaccination (Kumar, et al., 2013). Although previous studies have shown human antibody binding to porcine tissue or cells, no reliable reagents exist for xenotransplant patient screening compared to allotransplantation.

Tools to Measure the Immune Response

To prevent rejection of donor grafts, recipients are tested for the presence of donor-specific antibodies (DSAbs). One of these tests, the cellular crossmatch, involves incubating recipient sera with donor cells and adding rabbit complement.

If DSAbs are present in the recipient sera, they activate complement, causing lysis of donor cells. Additionally, recipient sera can be tested against a representative panel of individual HLA alleles. Antibody binding to specific alleles in this panel allows us to determine what HLA-specific antibodies the potential recipient possesses. We can then calculate the number of positive results and express this as a percentage referred to a Panel Reactive Antibody (PRA). The PRA provides an estimate of how sensitized the recipient is to HLA molecules. Finally, sera can be incubated with beads coupled to various HLA alleles and analyzed by flow cytometry to observe the level of antibody binding to beads (Mulley & Kanellis, 2011). An adaptation of this used in xenotransplantation, the Flow Cytometry CrossMatch (FCXM), involves incubating sera on cells with a target antigen, staining the cells for antibody binding of IgM and IgG, and running the cells on flow cytometry. These tools can help determine what, if any, antibodies are present in potential recipients so grafts can be matched with the recipient least likely of rejecting the organ. Possessing a close match decreases the likelihood of graft rejection, as the recipient will not reject HLA types found in the body (Terasaki & Ozawa, 2004).

Determining Antibody Binding to Class I or Class II

An assay exists to determine the prevalence of anti-SLA class I antibodies in patient sera by performing a FCXM comparing SLA class I deficient PBMCs to PBMCs from a class I intact pig. If the antibody binding is higher on class I intact versus deficient cell, it is suspected those are anti-class I antibodies. Unfortunately, a reliable assay to determine the antibody and cellular response to

SLA class II does not exist: a SLA class II deficient pig does not exist, the assays in existence for class II rely on swine PBMC whose phenotypic makeup is not fully characterized (for example, both T and B-cells in swine contain class II MHC). Adherent cell models utilize either cells expressing reduced levels of class II MHC as a consequence of expressing a dominant negative variant of CIITA. IFN-y is used to stimulate class II production in some assays, but expression can vary widely.

The CRISPR/Cas9 System

In recent years, scientists have used the type II clustered regularly interspersed short palindromic repeat (CRISPR) system and *Streptococcus pyogenes* CRISPR-Associated (Cas) protein to manipulate the eukaryotic genome. In the endogenous state, the bacterium first incorporates DNA from invading plasmids and viruses into the CRISPR locus amongst a period of short 20 bp long palindromic repeats. The incorporated DNA and palindromic repeat will eventually serve as the Pre-CRISPR RNA (pre-crRNA). The pre-crRNA will be bound by a Transactivating crRNA (tracrRNA), and processed into CRISPR RNA (crRNA) by RNAase III. The tracrRNA:crRNA complex recruits the Cas9 nuclease and the tracrRNA:crRNA:Cas9 complex then binds a DNA sequence that is both complementary to crRNA and capable of binding the Protospacer Adjacent Motif (PAM) found after the crRNA sequence. Following successful binding, the Cas9 generates a double-strand break in the DNA and the trimer complex unbinds. Repair mechanisms fixing these breaks are error prone, introducing mutations at a low frequency (Hsu, Lander, & Zhang, 2014).

The CRISPR-Cas9 system's development as a genome editing tool took off in 2012 when the Doudna and Charpentier labs combined the tracrRNA and crRNA into a Single-Guide RNA (sgRNA). This advance allowed for a simple and rapid generation of plasmids that could efficiently mutate target DNA (Jinek, et al., 2012).

Project Goals

In order to develop an assay that determines whether SLA class II serves as a potential target for antibody binding three reagents needed to be created: 1) an II immortalized fibroblast cell line expressing class II SLA but lacking class I SLA; 2) SLA DR+ DQ-; and 3) SLA DR- DQ+ cell lines to further characterize the antibody preference to SLA class II. I hypothesized that there are antibodies capable of binding SLA class II, these antibodies are likely cross-reactive with HLA class II antibodies, and there is no preference to either subset of class II. If successful, this experiment will suggest SLA class II could be a potential antibody target in clinical xenotransplantation.

MATERIALS AND METHODS

Culture of Parent Cell Line

An SV40 T antigen immortalized fibroblast cell line derived from a SLA class I and galactose-*α*1,3-galactose (Gal) deficient pig was chosen as the parent cell line (Reyes, et al., 2014). The cells were confirmed to be Gal, SLA class I, class II negative by incubation with an isolectin *Griffonia simplicifolia* IB4 (IB4 Lectin) Alexa Flour 647 (Invitrogen, Grand Island, NY, USA), an anti-SLA class I-FITC (AbD Serotec, Raleigh, NC), an anti-SLA class II DR-FITC (AbD Serotec, Raleigh, NC), and an anti-SLA class II DQ Ab (AbD Serotec, Raleigh, NC) and analysis using BD Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA) (**Figure 3**). These cells 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, 5% CO_2 and 10% O_2.

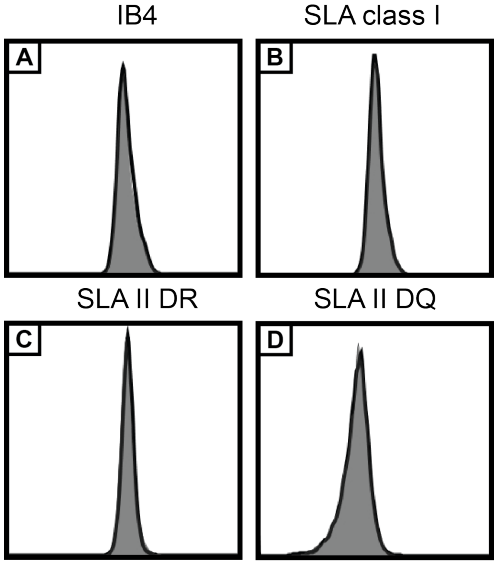

Figure 3: Phenotype of parent fibroblast deficient in Gal (A), SLA class I (B) and SLA class II DR (C) and DQ (D) as measured by lectin and monoclonal antibody staining. Gray histograms are the cells of interest and the white unfilled histograms represent isotype controls

Transfection and Selection

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. The donor plasmid, pCDNA3 myc CIITA (Plasmid #808)

was a gift from Matija Peterlin (Addgene plasmid #14650) (Kanazawa, Okamoto, & Peterlin, 2000) consisting of the human version of CIITA in a PCDNA3 plasmid backbone. Another CIITA plasmid, pCol2 flu CIITA, was tried in the cell line but failed to induce class II expression. Differential promoter strength (CMV vs Col II) could explain the difference in the plasmids' ability to induce class II. Three days post transfection cells were screened on a BD Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA) using a mouse 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 at the Indiana University (IU) Flow Cytometry Resource Facility. The cells were placed into selection using the antibiotic Geneticin, G418 (Invitrogen, Carlsbad, CA). Cells were cultured from the 96-well plate to individual 10-cm plates and 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 with mouse anti-pig SLA class II DQ **(**AbD Serotec, Raleigh, NC). Two clones were selected, one that demonstrated a class II positive DR(+)DQ(+) phenotype and another with a class II negative DR(-)DQ(-) phenotype, both resistant to G418 selection.

Sequencing

DNA sequencing analysis of the MHC class II+ cell line was performed with genomic DNA isolated with the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA). Reverse-transcriptase Polymerase Chain Reaction (RT-PCR) amplification of the class II alleles was

performed using the primers and conditions described by Reyes et al (Reyes, et al., 2014) and the RT-PCR products were sent for Sanger sequencing to Genewiz, Inc (Genewiz Inc., South Plainfield, NJ).

Human Antibody Binding for Class II

Sera samples were obtained from 183 patients on the IU transplant waitlist patients with IRB-approval. These samples were heat inactivated at 57°C for 30 min and the sera was absorbed for 30 minutes on 50% packed WT pig RBCs to reduce background binding by removing any pig-specific non-MHC antibodies. 12.5 uL of absorbed sera was incubated for 30 min 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 and donkey anti-human IgM Alexa Flour 647 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 30 min 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.

Generation of sgRNA CRISPR/Cas9 Vectors

Oligonucleotide pairs for the targeted sites in SLA class II DR and DQ were annealed to generate short double-strand DNA fragments with BbsI compatible overhangs. These fragments were then ligated into a BbsI digest pX330 plasmid. The chosen oligonucleotides used to construct the sgRNA expression vectors targeting class II are as follows: targeting exon 1 of the DQB1 chain forward 5'-TGTCTGGGATGGTGGCTCTG-3' and reverse 5'-

CAGAGCCACCATCCCAGACA-3'; targeting exon two of DRB1 forward 5'-GGAGCAGAAGCGGGCGGAGG-3' and reverse 5'-CCTCCGCCCGCTTCTGCTCC-3'; targeting exon two of DQA forward 5'-GGCTGTCAATCAGGTTCCTG-3' and reverse 5'-CAGGAACCTGATTGACAGCC-3'; targeting exon two of DRA forward 5'-TGCACTGGCCAACATAGCTG-3' and reverse 5'-CAGCTATGTTGGCCAGTGCA-3' (**Figure 4**). pX330, a bicistronic expression vector containing both the Cas9 gene and the BbsI cut site was purchased from Addgene (Plasmid #42230, http://www.addgene.org/42230/).

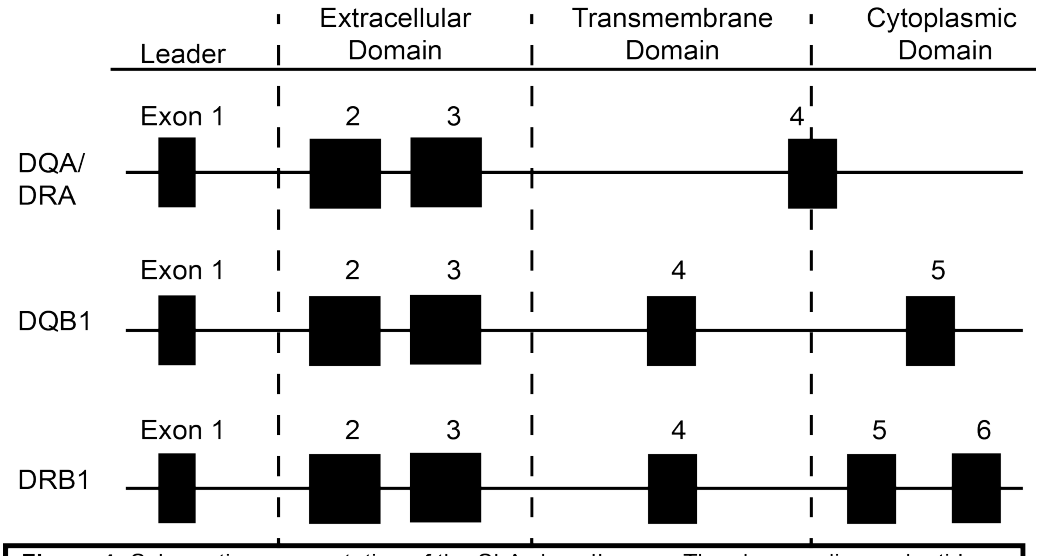

Figure 4: Schematic representation of the SLA class II genes.The chosen oligonucleotides used to construct the sgRNA CRISPR expression vectors targeting class II are as follows: targeting exon 1 of the DQB1 chain, targeting exon two of DRB1, targeting exon two of DQA, targeting exon two of DRA

Generation of DR(+)DQ(-) and DR(-)DQ(+) Cells

The DR(+)DQ(+) was cotransfected with either the two plasmids described above targeting the beta chain or the two plasmids targeting the alpha chains. For this transfection, the Neon transfection system (Life Technologies, Grand Island, NY, USA) was used according to manufacturer's instructions and 2 µg each plasmid was used. The cells transfected with the plasmids targeting the

class II alpha chain were sorted by the IU Flow Cytometry Resource Facility based on the absence of DR and presence of DQ, as determined by the monoclonal antibodies described above. The cells transfected with the plasmids targeting the class II beta chain were sorted for DR expression and absence of DQ, as determined by the monoclonal antibodies described above.

Human Antibody Binding for DR or DQ

Sera samples were obtained from 44 patients on the IU transplant waitlist patients with IRB-approval. 12.5 uL of absorbed sera was incubated for 30 min 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 DR(+)DQ(+), the DR(+) DQ(-), the DR(-)DQ(+), or the DR(-)DQ(+) parent 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 Laboratoies Inc., West Grove, PA, USA) for 30 min 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.

Statistical Analysis of Binding

Antibody binding results were reported as Median Fluorescence Intensity (MFI) of the FL4 channel. Graph and data analyses were completed using Prism 6 for Macintosh (GraphPad Software Inc., La Jolla, CA, USA). Of the 183 samples for the class II positive and negative FCXM, 122 samples were easily categorized into two groups based on the presence or absence of anti-HLA class II antibodies and used for statistical analysis. The 44 samples used for the

DR(+)DQ(-) and DR(-)DQ(+) FCXM were also analyzed for the anti-HLA class II sensitization but only three samples were found to have anti-HLA class II antibodies, not enough for a reliable statistical comparison. Human serum antibody binding assays were analyzed using a two-tailed paired or unpaired t-test comparing single MFI results for each individual with significance set at $p < 0.05$.

RESULTS

Development of a Class II Positive and Negative Clone

Integration of the human class II transactivator (CIITA) gene into a porcine fibroblast cell line successfully drove expression of both SLA DR and DQ, swine do not contain SLA-DP genes. Phenotypic staining of the parent cell line and resulting clones is shown in **Figure 5**.

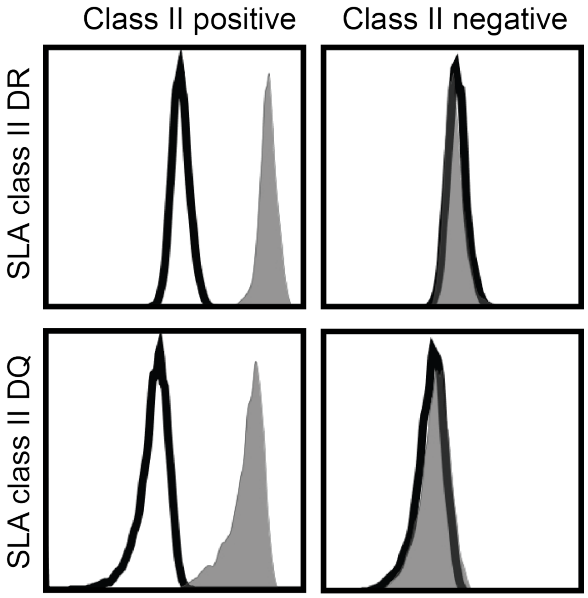

Figure 5: Phenotype of SLA class II positive and the SLA class II negative cell lines as determined by monoclonal antibodies.The class II positive cell is depicted on the left and the class II negative cell on the right. Gray histograms are the cells of interest and the white unfilled histograms represent isotype controls

Sequencing Results

The MHC background of the parent pig was previously described by Reyes et al (Reyes, et al., 2014). As expected, the class II+ cell line expressed transcripts from all present alleles: DQA*0101, DQA*0204, DQB*0303, DQB1*0601, DRA1*020102, DRA*w04re01, DRB*0403, and DRB1*1001 (**Figure 6**).

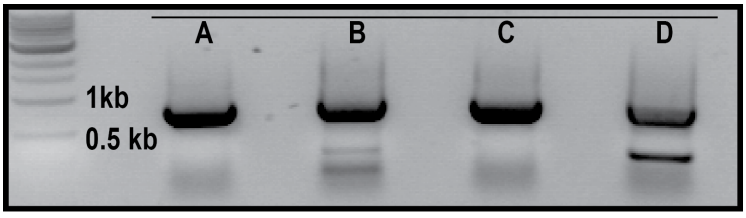

Figure 6: Results from the RT-PCR using gDNA isolated from the SLA class II positive cell. Conditions and primers used were previously described by Reyes et al. Shown are the bands that resulted from the DRA primers (A), DRB1 primers (B), DQB1 primers (C), and DQA primers (D). In each case the upper band was sent off for Sanger sequencing to confirm the integrated human CIITA gene drove expression of all class II haplotypes.

Antibody Binding to SLA Class II Positive and Negative Cells

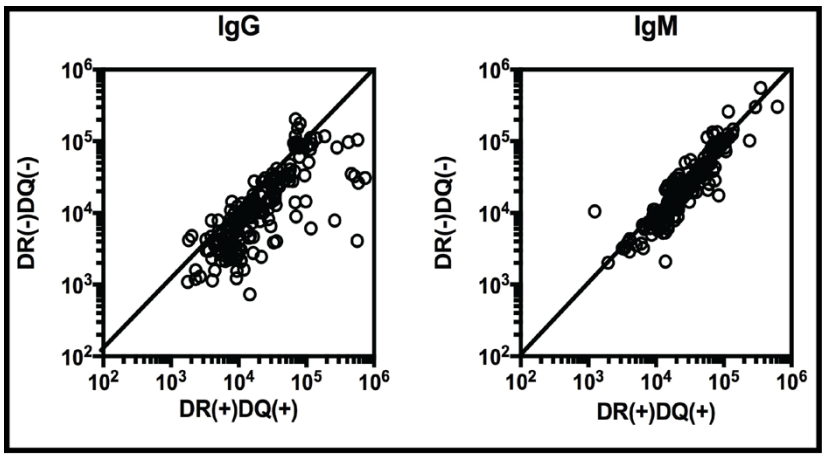

Figure 7: Demonstration of the human anti-pig IgG and IgM antibody binding from the 183 individuals on the Indiana University transplant waiting list to SLA class II negative (y-axis) and SLA class II positive (x-axis) cells. A two-way paired t-test shows significance for IgG (p = 0.0059) but not IgM (p = 0.2460). The diagonal line bisecting the graph represents equal binding to both a SLA class II positive and class II negative cell.

Cells lacking expression of SLA class II demonstrated decreased IgG binding compared to cells with 100% SLA class II DR and DQ expression (**Figure 7**) with no difference in IgM. A two-way paired t-test revealed statistical difference in total sera binding between a positive and negative cell for IgG (p = 0.0059) but not IgM (p = 0.2460). The antibody binding to a class II+ cell versus a class II- cell varies among sera: some individuals possess large quantities of antibodies capable of binding SLA class II and others possess almost none.

Anti-HLA Class II Sera Antibody Binding

122 of the 183 total patients screened in this study had accessible anti-HLA class II antibody data. Of these samples with known anti- HLA antibody type, 60 (49%) were found to have anti-HLA class II antibodies and 62 (51%) were found to have no anti-HLA class II antibodies. Increased binding is found

when comparing those individuals with anti-HLA class II antibodies to those without (**Figure 8**). The difference in binding to the SLA class II positive cell was compared in a two-way unpaired t-test amongst individuals with and without anti-HLA class II antibodies for IgG ($p = 0.0229$) and IgM ($p = 0.3045$).

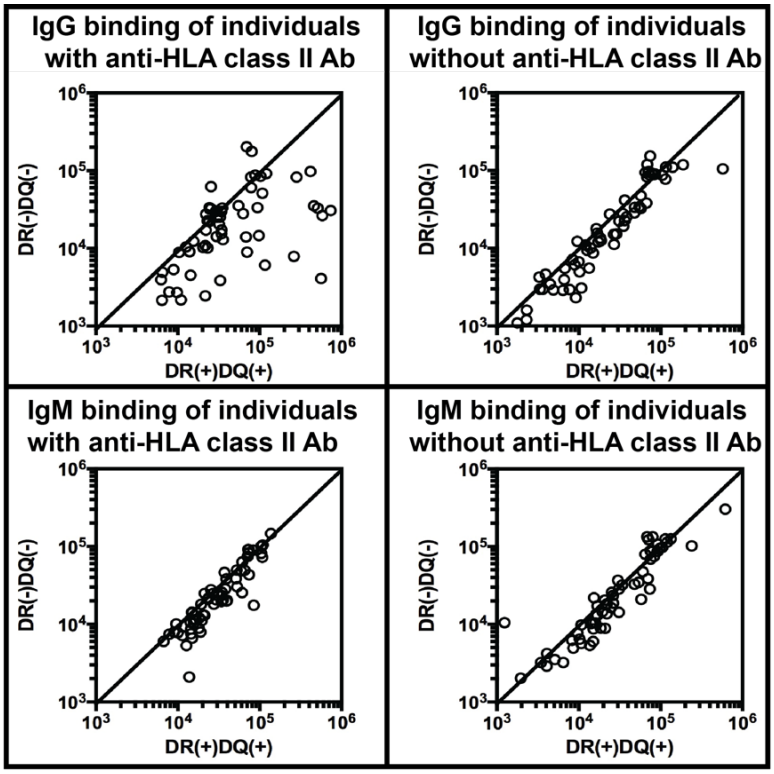

Figure 8: Human-anti-pig IgM and IgG binding of the 122 individuals on the Indiana University transplant waiting list with (n = 60, left column) and without (n = 62, right column) anti-HLA class II antibodies.The difference in binding to the SLA class II positive cell was compared in a two-way unpaired t-test and revealed statistical difference for IgG ($p = 0.0229$) but not IgM ($p = 0.3045$).The diagonal line bisecting the graph represents equal binding to both the SLA class II positive and class II negative cell.

Development of DR(+)DQ(-) and DR(-)DQ(+) Cell Lines

Transfection with CRISPR-Cas9 plasmids targeting either the alpha or beta chain of the class II molecule successfully generated DR(+)DQ(-) and DR(-)DQ(+) cell lines as depicted by the phenotypic staining in **Figure 9.**

Antibody Binding to DR(+)DQ(-), DR(-)DQ(+), DR(+)DQ(+), and DR(-)DQ(-) Parent Cell Lines

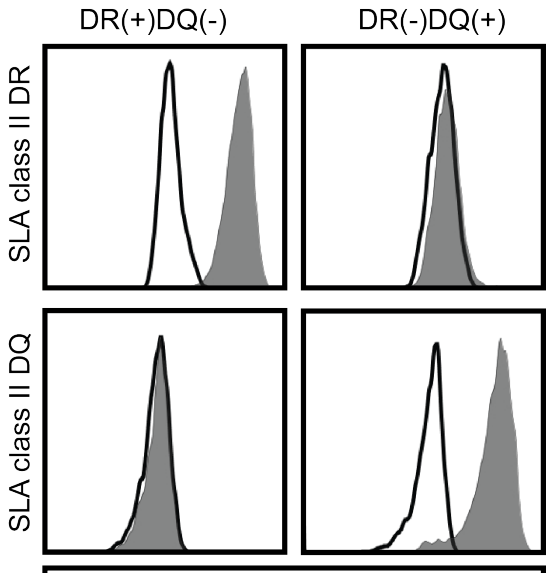

Figure 9: Phenotype of SLA class II DR(+)DQ(-) and DR(-)DQ(+) cell lines as determined by monoclonal antibodies.The DR(+)DQ(-) cell is depicted on the left and the DR(-)DQ(+) cell on the right. Gray histograms are the cells of interest and the white unfilled histograms represent isotype controls

The results of the previous experiments revealed the importance of IgG analysis and subsequently only examined IgG for the class II subset FCXM. Statistical difference, as determined by a two-way paired t-test, was detected in the DR(+)DQ(-) vs. the DR(-)DQ(+) FCXM ($p = 0.0099$), the DR(+)DQ(-) vs. the DR(+)DQ(+) FCXM ($p = 0.0192$), and the DR(-)DQ(-) parent vs. DR(+)DQ(+) FCXM ($p = 0.0329$). No difference was found in the DR(-)DQ(+) vs. DR(+)DQ(+) FCXM ($p = 0.1601$). Binding patterns are depicted in **Figure 10.**

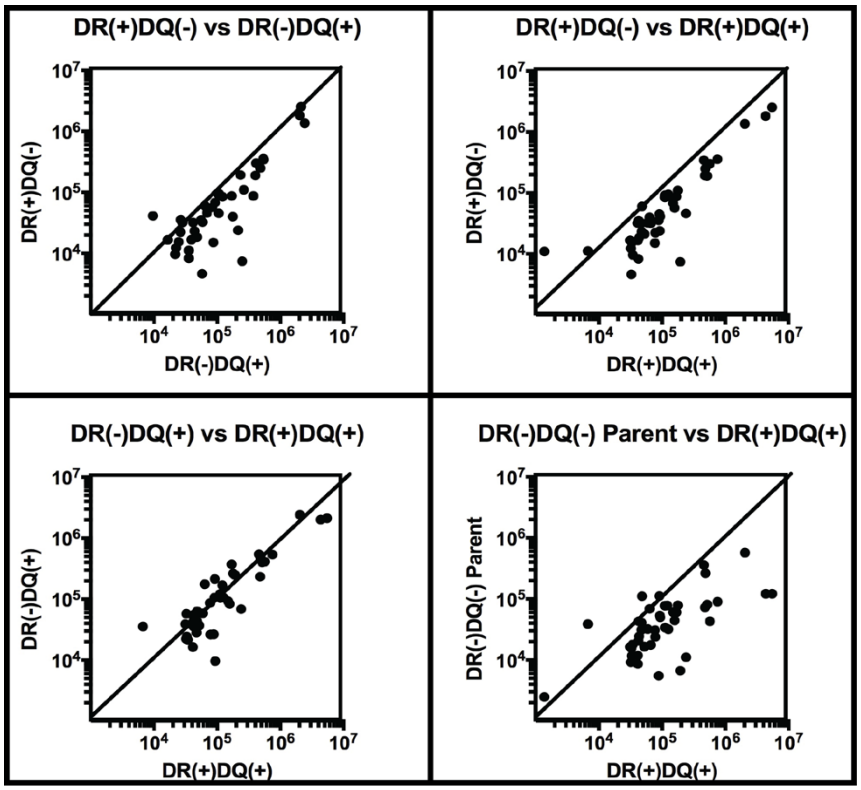

Figure 10: Human-anti-pig IgG binding to DR(+)DQ(+), DR(-)DQ(+), DR(+)DQ(-), DR(-)DQ(-) cell lines of 44 individuals on the Indiana University transplant waiting list. Statistical difference, as determined by a two-way paired t-test, was detected in the DR(+)DQ(-) vs. the DR(-)DQ(+) FCXM (top left, p = 0.0099), the DR(+)DQ(-) vs. the DR(+)DQ(+) FCXM (top right, p = 0.0192), and the DR(-)DQ(-) parent vs. DR(+)DQ(+) FCXM (bottom right, p = 0.0329). No difference was found in the DR(-)DQ(+) vs. DR(+)DQ(+) FCXM (bottom left, p = 0.1601).The diagonal line bisecting the graph represents equal binding to the cell types tested.

DISCUSSION

Following successful elimination of the swine surface glycans, and in an effort to develop even less immunogenic xenografts, focus will turn to potential protein xenoantigens: the MHC. The MHC is one of the chief sources of rejection in clinical allotransplantation and the relationship between donor-specific antibodies (DSAbs) to MHC and graft rejection is well-documented (Ponticelli, 2012). Numerous groups have also reported on the negative effect of HLA-mismatches and graft survival (Duquesnoy, et al., 2003). The results of the 183 sera FCXM study suggest that the swine MHC has the potential to contribute to the human anti-pig humoral xenogenic response. Additionally, the genetically conserved nature of the MHC across species, specifically the MHC class II, and the statistically significant difference in binding to SLA class II for individuals with anti-HLA class II antibodies, suggest that an antibody cross-reactivity exists between human leukocyte antigen HLA and SLA class II. If correct, future patients will need to be screened for both antibodies specific for SLA as well as anti-HLA antibodies capable of binding SLA.

Furthermore, the development of DR(+)DQ(-) and DR(-)DQ(+) cell lines allowed for the evaluation of what, if either, subset of class II results in the stronger immune response. Surprisingly, the lack of statistical difference between DR(-)DQ(+) and DR(+)DQ(+) imply that SLA class II DQ will be the more immunogenic group. This perhaps makes sense, given the history of cross-reactive groups (CREGs) and HLA's role in allotransplantation. To briefly summarize, a CREG is an antibody that is capable of recognizing an amino acid

at a particular position in the HLA molecule. If other polymorphic HLA molecules possess that exact positional amino acid group, the CREG antibody is capable of binding, and therefore an antibody can “cross-react” across numbers of MHC molecules. Of the 21 known cross-reactive groups (CREGs) to HLA DQB, 10 are also found in SLA DQB (47.6%). Of the 60 known CREGs to HLA DRB, 11 are found in the SLA DRB (18.3%). The higher percentage of cross-species DQB CREGs means that of individuals with antibodies to HLA DQ, these antibodies are likely to also bind SLA DQ.

In conclusion, this project resulted in the successful development an immortalized cell line that can be used for analysis of the role that SLA class II plays a role as a xenoantigen. This cell was used to demonstrate the existence of human-anti-pig antibodies, a possible anti-HLA/anti-SLA cross-reactivity, and the relative strength of SLA DQ as a target of antibody binding. Future studies will involve evaluating SLA class II as a target of T-cell response and to what extent this lymphocyte proliferation is due to cross-reactivity between HLA-specific TCRs and SLA class II. Finally, further inquiry is required to characterize the allelic patterns of xenoimmunogenic reactivity.

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CURRICULUM VITAE

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EDUCATION

2014 – 2016
Indiana University-Purdue University Indianapolis, Indianapolis, IN
M.S. of Translational Science, June 2016

2012 – current
Indiana University School of Medicine, Indianapolis, IN
M.D., anticipated May 2020

2008 – 2012
The University of Chicago, Chicago, IL
B.S. in Biological Sciences, Specialization in Endocrinology

RESEARCH AND TRAINING EXPERIENCE

June 2015 – current
Medical Scientist Training Program, Indiana University SOM, Indianapolis, IN
Research Fellow, Tector Xenoimmunology Lab
- Translational research on MHC swine leukocyte antigen class II

June 2014 – June 2016
CTSI MD/MS Fellowship in Translational Science, Indiana University SOM, Indianapolis, IN
Research Fellow, Tector Xenoimmunology Lab
- Translational research on MHC class II knockout porcine fibroblasts

June 2013
Hartford Hospital, Hartford, CT
Research Fellow, Division of Cardiovascular Surgery
- Clinical research on mitral valve repairs and replacements
- Developed a protocol for open heart surgery in clopidogrel-exposed patients

May – Sept. 2013
Riley Children's Hospital, Indianapolis, IN
Research Fellow, Division of Cardiovascular Surgery
- Clinical research comparison of Apical Aortic Conduit vs. Transcatheter Aortic Valve Replacement

June – Aug. 2012
Lutheran Hospital, Fort Wayne, IN
Summer Research Fellow, Division of Cardiovascular Research

- Clinical research on the risk factors for tracheostomy survival

Feb – Aug. 2010
Yin Research Lab, The University of Chicago, Chicago, IL
Research Assistant
- Research on the efficacy of phage display for novel protein discovery

June – Aug. 2009
Midwestern Alliance for Health Education, Indiana University Purdue University Fort Wayne, Fort Wayne, IN
Research Fellow, Division of Cardiovascular Research
- Clinical research program of long-term outcomes in carotid endarterectomies

PUBLICATIONS

Butler JR, Skill NJ, Priestman DL, Platt FM, Li P, Estrada JL, Martens GR, **Ladowski JM**, Tector M, Tector AJ "Silencing the Porcine iGb3s Gene Does Not Affect Gala3Gal Levels or Measures of Anticipated Pig-to-Human and Pig-to-Primate Acute Rejection" *Xenotransplantation*, 2016 DOI: 10.1111/xen.12217 (in press)

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PRESENTATIONS & POSTERS

Ladowski JM, Butler JR, Martens GR, Li P, Blankenship R, Reyes L, Wang ZY, Blum JS, Tector M, Tector AJ "Generation of a Swine Leukocyte Antigen Class II Cell Line Using Human Class II Transactivator" Poster Presentation, *IUSM Alumni Association Research Poster Symposium* May 2015

Butler J, Martens G, Li P, Estrada J, Wang Z, Lutz A, **Ladowski JM**, Burcin E, Tector M, Tector A. "The Fate of Human Platelets Exposed to Porcine Renal Endothelium: A Single-Pass Model of Platelet Uptake in Domestic and Genetically Modified Pig Kidneys." Oral Presentation, *Indiana Chapter American College of Surgeons* April 2015

Ladowski, Joseph M and Ladowski, Joseph S. "Retrospective Analysis of Bovine Pericardium (Vascuguard®) for Patch Closure in Carotid Endarterectomies." Poster Presentation, *Society of Clinical Vascular Surgery* April 2010

Ladowski, Joseph M and Ladowski, Joseph S “Retrospective Analysis of Bovine Pericardium (Vascuguard®) for Patch Closure in Carotid Endarterectomies." Oral Presentation, *Midwest Alliance for Health Education* Aug 2009

PROFESSIONAL EXPERIENCE

Medical Student Service Group, Indiana University SOM
Feb 2015 – June 2016
Junior Chair
- Chair of the student-run service learning group
- Responsible for 25 different service projects/initiatives

In-Training, A Medical Student Journal
June 2015 – current
Editor-in-Chief
- Responsible for editing and publishing all articles
- Manage a board of over 40 medical students across the globe
- Served as a *Medical Student Editor* from Feb 2013 – Feb 2015

Literature and Medicine, Indiana University SOM
April. 2013 – June 2015
President
- A medically oriented book club for IUSM student
- Focused on presenting and reviewing works of literature and film

American Medical Association, Indiana University SOM
Jan. 2013 – June 2014
Class Representative
- Class representative for IUSM at the AMA National conferences
- Organized a variety of activities from speaker series to information sessions

Medical Ethics, Indiana University SOM
April 2013 – Jan 2015
Co-chair
- A medical-oriented Ethics program
- Arranged speaker series, debates, and policy discussions

Transplant Student Interest Group, Indiana University SOM
April 2013 – Jan 2015
President
- An organ and tissue donation interest group
- Organized an “On-Call Pager Program” for students to observe procurement and the transplantation procedures

Medical Spanish-Society of Latinos, Indiana University SOM
Jan. 2013 – May 2014
President

- Medical Spanish club to teach clinical medical Spanish to students
- Extracurricular and volunteer activities to introduce the Hispanic culture

Indiana State Medical Association, Indiana University SOM
April 2013 – May 2014
Committee on Physician Assistance Representative

- Worked with a Physician-oriented addiction recovery program
- Met once a month to provide consults to participants of the program

HONORS, AWARDS, FELLOWSHIPS

Medical Scientist Training Program, Indiana University SOM
NIH sponsored MD/PhD, June 2015 – current

- Awarded through the Indiana University MSTP program
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CTSI MD/MS Fellowship in Translational Science, Indiana University SOM
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- Awarded through the Indiana Clinical and Translational Science Institute
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- Awarded through the IUSM Scholarship Committee
- Presented to one IUSM student based on academic merit