ABSTRACT

Improving Transplant Efficacy

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Director: Bob Kane, Chemistry

The development of tissue and organ transplantation has been an important achievement in medicine. Extensive research into medications and preservation techniques has improved the success of transplants. However, ongoing research continues to find new ways to improve transplant efficacy and durability. Our area of transplant research involves the bioconjugation of immunosuppressant medications onto the surface of pancreas islet cells prior to transplantation. This thesis will introduce current advances into organ transplantation as well as significant challenges that remain. Chapter 2 will describe our research aimed at demonstrating the use of a novel anti-inflammatory medication in islet cell transplantation. Chapter 3 will present our investigations of chemical linkers as model studies to be extrapolated onto other tissue and organ types. Our research ultimately aims to create a more protective and safe biological environment for transplanted tissues.

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IMPROVING TRANSPLANT EFFICACY

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By

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TABLE OF CONTENTS

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CHAPTER ONE

Introduction and Background

The field of organ transplantation has seen great advances in the recent decades. However, research is still ongoing to decrease the rate of rejection and improve transplant efficacy. This literature review will describe the current organ preservation and transplant techniques and allude to current research initiatives to increase transplant success.

The first organ transplant was performed on a kidney in 1954. The success of this kidney transplant propelled the field of transplant biology. Because of the importance of the kidney in organ transplantation, we will frequently look at kidney preservation and transplant techniques seen in protocols today[1].

Types of Organ Rejection

Organ transplantation and immunosuppression are important research foci. Before addressing ways to improve transplant efficacy, a thorough understanding of tissue rejection mechanisms must be understood. Three stages of transplanted tissue rejection are described within the transplant literature: hyperacute rejection, acute rejection, and chronic rejection. Hyperacute rejection occurs within minutes to days post-transplant. Pre-existing donor specific antibodies against recipient Human Leukocyte Antigens (HLA) or endothelial cells cause this reaction. In kidneys, hyperacute rejection presents with neutrophil infiltration and platelet margination along the endothelium of kidney capillary beds[2].

However, hyperacute rejection is rare in modern transplantation due to careful donor-recipient cross matching. In cases of poor cross matching, immune system activation occurs through antibody deposition onto HLA antigens, subsequent complement activation, and recruitment of inflammatory cells leading to blood clot formation. This leads to capillary blockage and tissue necrosis[2,3,4].

Acute rejection occurs within days to months after transplantation and is caused by alloantibodies and alloreactive T cells that respond to HLA antigens. Here, cytotoxic CD8 T cells destroy tissues that present alloantigens. Antibody-mediated rejection also persists at this stage and features alloantibodies that induce complement activation, neutrophil recruitment, and inflammation and coagulation that lead to tissue necrosis. Today, acute rejection is mediated through immunosuppressive therapies administered post-transplant[3,4].

The final phase of rejection, chronic rejection, represents the main cause of tissue graft failure today. Graft failure in this stage occurs due to chronic inflammation. Smooth muscle within the tunica media of organ vasculature proliferates and causes occlusion and ischemic damage. Activated T cells secrete pro-inflammatory cytokines and cause the production of alloantibodies that activate the classical complement pathway. This results in chronic damage and eventual organ rejection[3,4].

Though chronic antibody mediated rejection has been recognized in kidney transplantation, it needs to be defined for other organ types. It is often missed due to limitations of current diagnostic criteria[2].

Vascular Anastomosis

To succeed in organ transplantation, vascular anastomosis must be successfully performed. The vascular anastomosis time (AT) should be reduced as much as possible to preserve tissue health. Marzouk, K. and colleagues reported that an AT greater than 29 minutes in kidney transplantation leads to particular worse outcomes and an increase in delayed graft function (DGF). DGF is typically characterized as a need for dialysis within a week post renal transplantation. Also significant, longer ATs correlated with higher serum creatinine levels. Higher serum creatinine levels are consistent with slower recovery of kidney function[5]. This could likely be due to increased ischemic time which results in higher levels of immune activation.

The quality and method of anastomosis is foundational in securing a productive graft. After successful anastomosis, graft survival becomes dependent on immune interventions. Though this thesis focuses mainly on immunosuppressant measures for improving transplant efficacy, it is important to know the foundations of organ transplantation in order to understand the limiters and aggravators of inflammation so they can be effectively mediated.

Ischemia Reperfusion and Tissue Injury

Another important consideration in transplantation is cold ischemic time. Ischemic reperfusion injury (IRI) results from a cessation of blood flow to a tissue followed by a reintroduction of warm blood flow. It is characterized by tissue injury and non-specific immune system activation. Immune system activation results from both ischemia and reperfusion. Ischemia, and the subsequent reduction in blood flow, leads to depletion of ATP, impaired oxidative metabolism, a generation of reactive oxygen

species, and inhibition of sodium potassium ATPase pumps[6]. This is the initial non-immune tissue injury that activates the innate immune system (figure 1). Anti-inflammatory drugs and antioxidants have been used with attempts to reduce tissue injury[7].

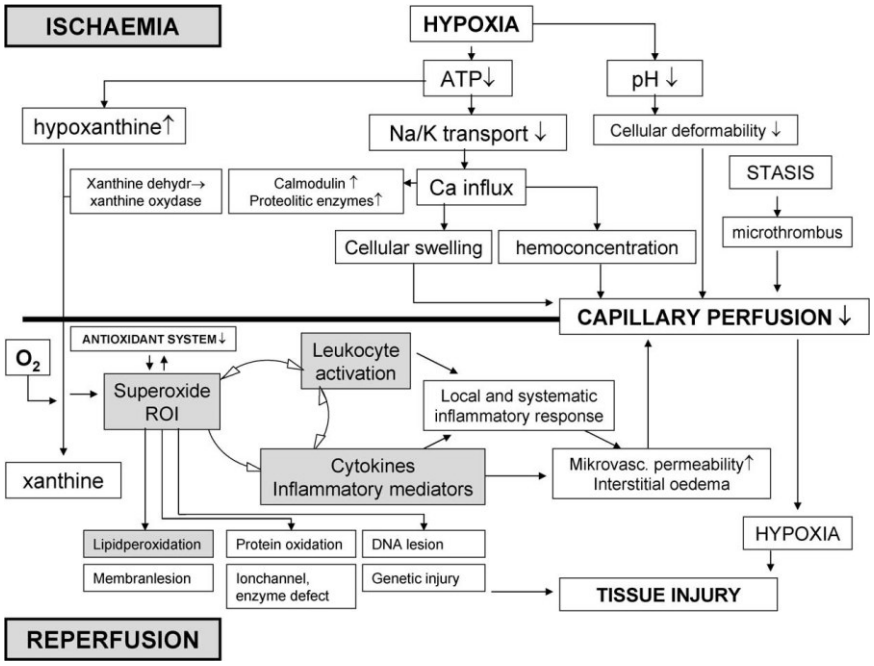

Figure 1: Effects of Ischemia/Reperfusion on Tissue[8]

After these metabolic disturbances, IRI may activate innate immunity by engaging toll-like receptors (TLRs) using endogenous ligands. Activation of TLRs impacts both short and long-term graft survival. TLRs recognize motifs that are present on many microorganisms. TLRs are present on macrophages, dendritic cells, neutrophils, B cells, and natural killers cells[9].

DAMPS, or damage associated molecular patterns, also active TLRs. The most widely studied DAMPs are HMGB1 and heat shock proteins. Degraded protein

fragments can also activate TLRs and further exacerbate innate immune activation[10]. The specific pathway of a commonly studied TLR, TLR4, will be highlighted in chapter 2.

The figure above notes the damage caused by ischemia and reperfusion. After cold ischemic time, the reintroduction of warm blood flow, reperfusion, also takes a toll on tissue health. Reperfusion of the transplanted organs activates leukocytes. Activated leukocytes lead to the release of reactive oxygen intermediates, which then lead to the release of cytokine inflammatory mediators. This further activates leukocytes and a positive feedback circle begins (figure 1)[8].

For reasons stated above, an important aspect of successful organ transplantation involves reducing the effects of IRI. Though IRI is not completely diminished in current transplant procedures, a variety of interventions can be used to significantly decrease its after effects. This could include reducing reactive oxygen intermediates, cytokine concentrations, and activation of innate immune cells. A discussion of these IRI interventions follows.

Solutions used for Organ Preservation

Before organ transplantation and prior to removal of the donated organ, a preservation solution is flushed into the organ to preserve the tissue during transfer. Several solutions have been used to preserve organs during transplantation. The University of Wisconsin (UW) solution is a standard solution in the United States. It has been shown to reduce the rates of delayed graft function (DGF). The University of Wisconsin solution resulted in a DGF rate of 33.9%. Other solutions have also emerged that yield similar results. The Celsior solution resulted in 31.3% reduction in DGF.

These solutions are key in reducing ischemia reperfusion injury during transplant procedures[11].

The UW solution, and similar solutions, were developed with three purposes in mind: to maintain an osmotic concentration using metabolically inert substances, provide additional administration of colloid carrier hydroxyethylstarch (HES), and reduce oxygen radicals with the use of scavengers[12].

Polyethylene Glycol (PEG) solutions can also prove beneficial when added to organ preservation solutions. PEG solutions are also the most applicable to this study. Addition of a PEG solution has been shown to attenuate the inflammatory injury due to IRI and enhance the quality of pig kidney transplantation. PEG may be helpful due to its formation of a hydrogel membrane coat on tissue surfaces. PEG has also been shown to reduce lipid peroxidation. This may impair hydrogen peroxide-induced apoptosis in the kidney epithelial cells. Lastly, these researchers concluded that the addition of PEG to preservation solutions reduces MHC class II expression in epithelial tubule cells, blocking T cell activation in the pig model[13].

Researchers in the Lee group used PEGylation for successful islet transplantation in rats. It was discovered that PEGylated islets survived an average of 32 days, compared with unmodified islets, which experienced rejection in 2 weeks. PEGylated islets also showed a reduction in immune cell infiltration in the surrounding tissue[14]. This could be due to PEGs ability to "camouflage" the transplanted tissue from the immune system.

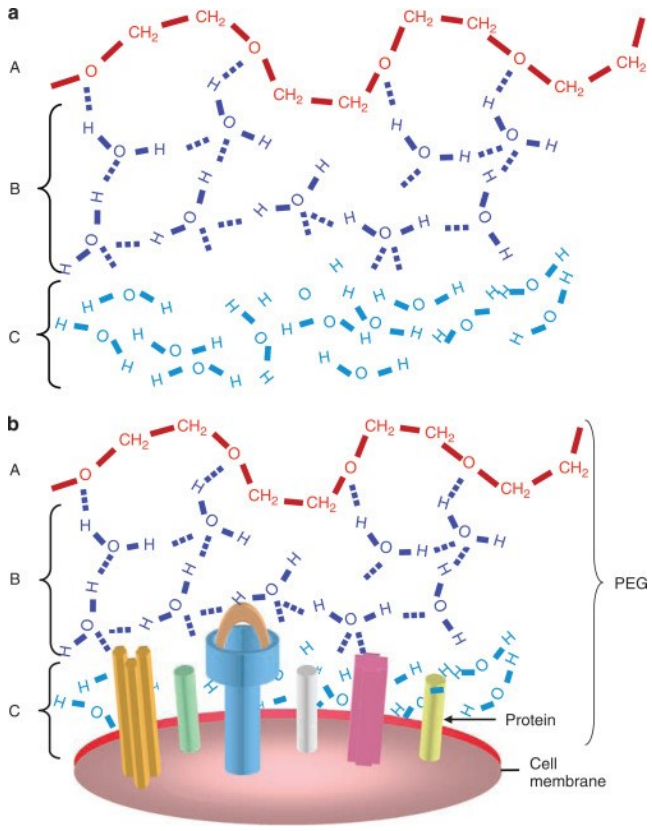

Figure 2: a. A) PEG molecular backbone B) Water molecules bonded to PEG with long-lasting hydrogen bonds C) Water molecules with short-lasting hydrogen bonds
Figure: b. PEG structure on cell surface [15]

As noted above, there are a variety of standardized organ preservation solutions. Some are specifically indicated depending on the organ being transplanted. Pricing and transplant program preference also dictate which solution is used. Regardless, each solution is used with the purpose of reducing immune system activation and increasing tissue viability throughout the transplant procedure.

Cold Storage and Machine Perfusion

Cold organ storage is currently used in transplant procedures. It involves subjecting the transplanted tissue to a constant 4 degree Celsius environment. The organ is then placed in a preservation solution and remains static. However, exacerbation of

metabolic injury and probability of graft dysfunction increases with cold temperatures, so its use remains controversial[16]. Cold storage is meant to reduce the metabolic rate, reduce oxygen requirements, and preserve tissue functionality. However, storage in a cold environment generates reactive oxygen species and activates the complement cascade. Researchers show that prolonged cold storage time increases complement deposition on a kidney tubule graft (figure 3)[17].

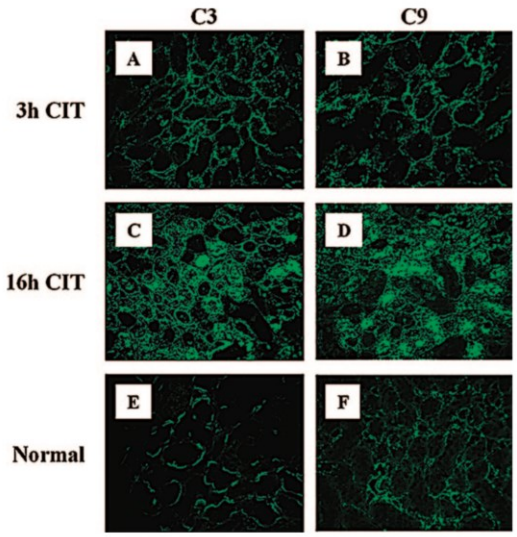

Figure 3: Complement activation (green) on graft of kidney tubule[17]

Machine perfusion is an alternative method to preserve organs during transplant procedures and involves constant perfusion of the organ tissue. Researchers in the Peng group used a porcine model to demonstrate that machine perfusion yields better protection of function and metabolic activity in transplanted tissue when compared with the cold storage method[18].

Researchers compared cold storage to machine-perfusion preservation in cadaveric renal transplantation. Results indicated that there was no significant difference

in post-transplant dialysis requirements, serum creatinine levels, or distribution of graft losses. Because machine-perfusion preservation is more expensive, they recommend cold storage[19].

Ongoing research continues to investigate the benefits of both cold static storage and machine perfusion. One technique may be more beneficial in the transplant of certain organs depending on local immune cell presence. However, even with improvement to both techniques, an innate immune response is still expected. Therefore, a pharmaceutical treatment plan is also necessary.

Medications used in Transplantation

Current organ transplant protocols place patients on immunosuppressant medications after transplant to prevent organ rejection. There are a variety of medications that can be effective in immune system modulation. The timing, dosage, and approach to immunosuppression all require consideration when creating an effective medication plan for the patient. A few medications that can provide important post-transplant protection will be discussed: rapamycin, tacrolimus, and repertaxin.

Rapamycin is an immunosuppressant used to prevent organ rejection. It targets mTOR, a ubiquitous kinase-containing complex that promotes cell growth. Researchers demonstrated that the administration of T_{reg} cells with rapamycin inhibits the proliferation of CD4+ and CD8+ T cells. This combination treatment almost completely inhibited the expression of IFN-γ. This is significant since IFN-γ plays a critical role in up-regulating other immune cells[20].

More specifically, rapamycin's potent immunosuppressive function derives from its ability to disrupt cytokine signaling resulting from mTOR stimulation. Normally, this cytokine signaling leads to lymphocyte growth and differentiation[21]. Administration of rapamycin was shown to induce a prolongation of the G1 phase in T lymphocytes. It also prevented the synthesis and accumulation of ribosomal proteins. This is the basis by which rapamycin delays immune cell growth and preserves transplanted tissue[22].

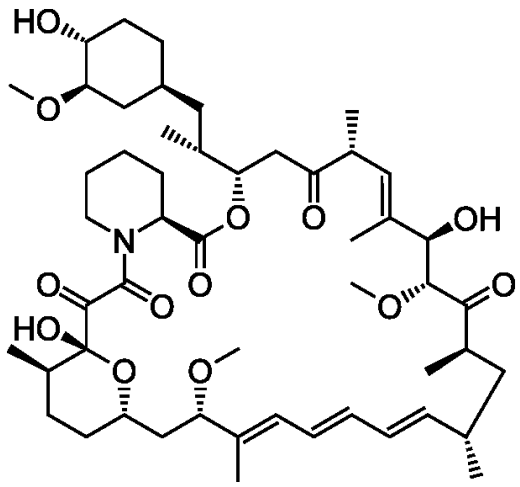

Figure 4: Chemical structure of rapamycin

Tacrolimus (FK-506) is another immunosuppressant medication used in transplant protocols. Its prolonged-release form was first approved in 2007. Prolonged-release tacrolimus is well tolerated in patients having undergone solid organ transplant[23].

Research highlights the potential for personalized management of post-transplant regimens to improve patient prognosis. Parabolic personalized dosing was used to effectively manage patients by keeping tacrolimus blood trough levels within the target ranges. This treatment modality could prove extremely beneficial where patient response

to immunosuppressive therapy is variable, as seen in individual immune responses to transplanted tissue[24].

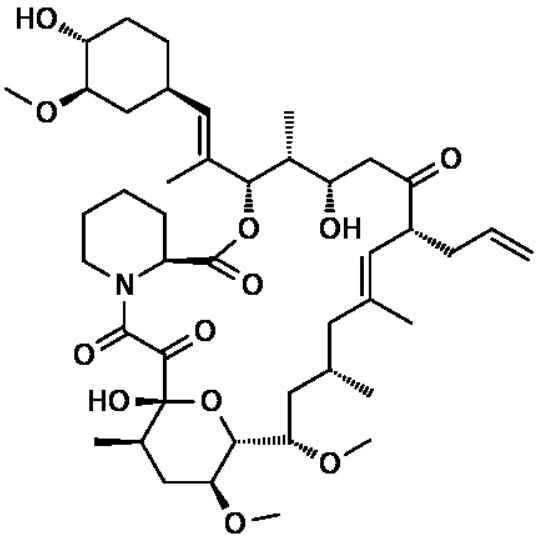

Figure 5: Chemical structure of tacrolimus (FK-506)

Repertaxin is an inhibitor of CXCR2 signaling. A study by Cugini and colleagues investigated the effects of blocking CXCR2 signaling in a rat model of renal post transplant IRI injury. Administration of repertaxin lowered the number of graft infiltrating granulocytes. This is significant since granulocyte infiltration into renal grafts contributes to dysfunction[25]. CXCR signaling pathways are responsible for the production of neutrophil-attracting chemokines. Using repertaxin to block these chemokines could potentially inhibit tissue damage caused by neutrophil infiltration.

Most likely due to its inhibition of CXCR signaling, repertaxin was also seen to prevent increases in serum creatinine levels. Serum creatinine stabilization suggests that repertaxin prevents renal dysfunction due to IRI in transplantation[25]. Due to these promising findings, repertaxin is now undergoing clinical trials for prevention of primary

graft dysfunction after lung transplantation[26]. Hopefully, this medication will emerge as a beneficial post-transplant immunosuppressant for many tissue types.

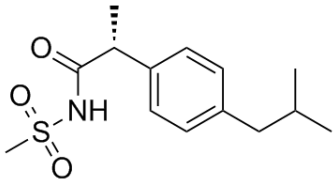

Figure 6: Chemical structure of repertaxin

Challenges that Remain

Despite the many interventions mentioned above, research remains ongoing with attempts to improve transplant results. New immunosuppressant drugs are being tested and current drugs are being administered in new, more effective ways. Ultimately, a balance between over- and under-stimulation of the immune system must be achieved[27]. Achieving this balance presents exciting opportunities for not only improved transplant results, but also increased access to transplants.

Ultimately, organ transplant recipients are in need of improved post-transplant therapy to prevent, as mentioned above, chronic organ rejection. No causative therapy is available to prevent chronic allograft rejection. Dysfunction in transplanted tissue due to chronic rejection occurs in as much as 50% of patients within three years of transplantation[28]. Researchers in the Lattmann group found that chronic rejection in the absence of immunosuppressive therapies is associated with devastating cytokine activation in the lung and liver of the host[29].

Treatment methods must be found to more effectively reduce chronic inflammation and prevent the chronic tissue damage that ultimately leads to organ rejection. For this reason, we will further examine potential therapies and initiatives that are being undertaken to suppress the immune system at a healthy and sustainable level post-transplant.

TLR4 Antagonists

Inhibition of Toll-like receptors could play a critical role in improving the efficacy of organ transplantation. Toll-like receptors are part of a family of receptors that play a role in both adaptive and innate immunity. TLR signaling activates dendritic cells (DCs) that mature and increase their antigen presentation. This signaling also increases DC migration to the lymph nodes where antigens are presented for T cell activation, thus recruiting the adaptive immune response. Researchers in the Howell group show that the action of these dendritic cells plays a critical role in liver allograft rejection. Offering further support, human studies have shown that TLR agonists released at the time of transplant can prevent induction of immune tolerance and stimulate rejection[30].

One specific Toll-like receptor, TLR4, plays a central role in memory T cell homeostasis. After transplantation, TLR4 plays a role in the formation of memory T cells for DAMPS. It has been shown that low monocyte expression of TLR4 before liver transplant correlates with reduced risk of rejection of transplanted tissue[30]. Since TLR4 is a molecule important in signaling a variety of immune responses, TLR4 antagonism may significantly reduce the immune response that leads to organ rejection. We will first examine the TLR4 pathway to gain a better understanding of its route of action.

Lipopolysaccharide (LPS) is an important ligand for TLR4 activation. Upon TLR4 activation, MyD88 associates with IL-1 receptor-associated kinase 4 (IRAK4). This activates TGF-β–activated kinase 1 (TAK1) and I-κB kinase (IKK) phosphorylates IκB. IκB subsequently degrades and releases NF-κB for translocation into the nucleus (Figure 7) [30]. NF-κB is ultimately responsible for the transcription of pro-inflammatory cytokines that induce further immune system activation.

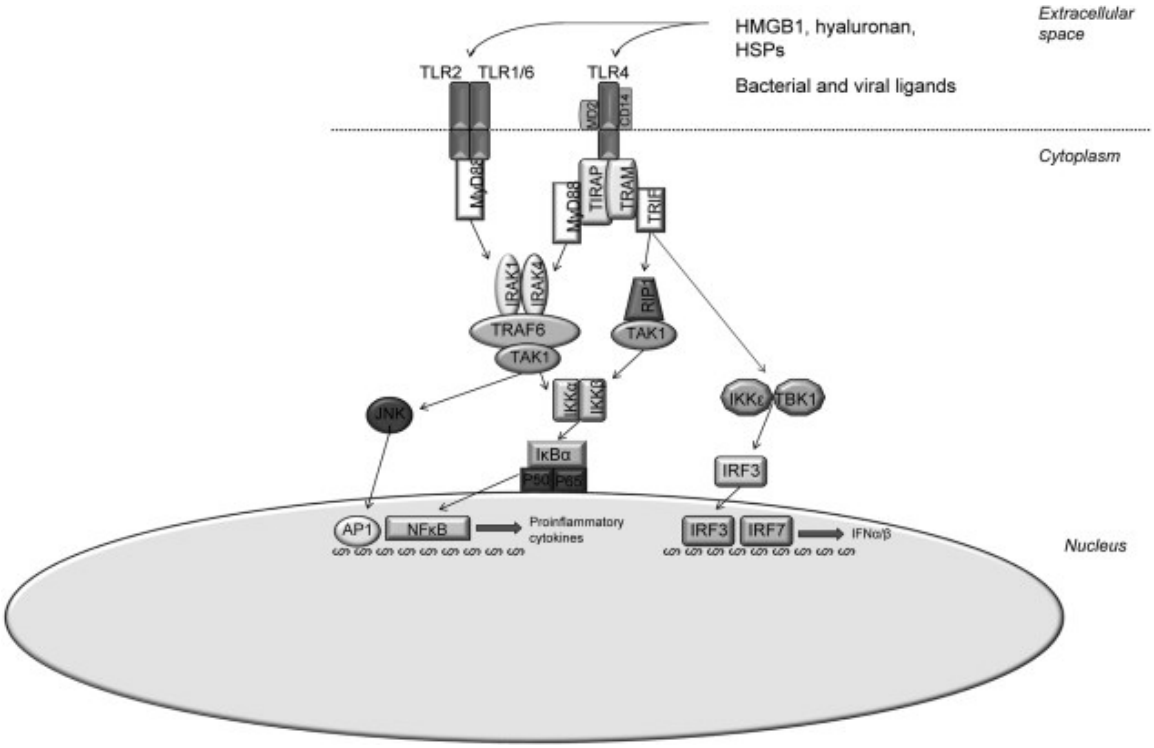

Figure 7: TLR4 activation pathway[30]

Inhibiting the TLR4 pathway could prove beneficial for improving transplant efficacy. There exist a variety of TLR4 antagonists. A study by Seunghyun, J. and colleagues found that Clozapine blocks NF- κB activation induced by LPS in microglial cells, the resident macrophages of the central nervous system. NF-κB was found to play an important role in regulating the inflammatory gene expression in microglia. Clozapine

(figure 8) specifically works to block the LPS-induced phosphorylation of IκBα and p65/RelA, two important TLR4 signaling molecules upstream of NK-κB, at two specific serine residues. Administration of Clozapine prevented stimulated expression of IL-1β, IL-6, *i*NOS, and COX2, all of which represent important inflammatory drivers. This suggests that inhibiting NF-κB nuclear translocation plays a critical role in inhibiting inflammation[31]. Because of its inhibition of these crucial mediators of the TRL4 pathway, and its promising chemical structure, clozapine presents therapeutic potential for immunosuppression post-transplantation.

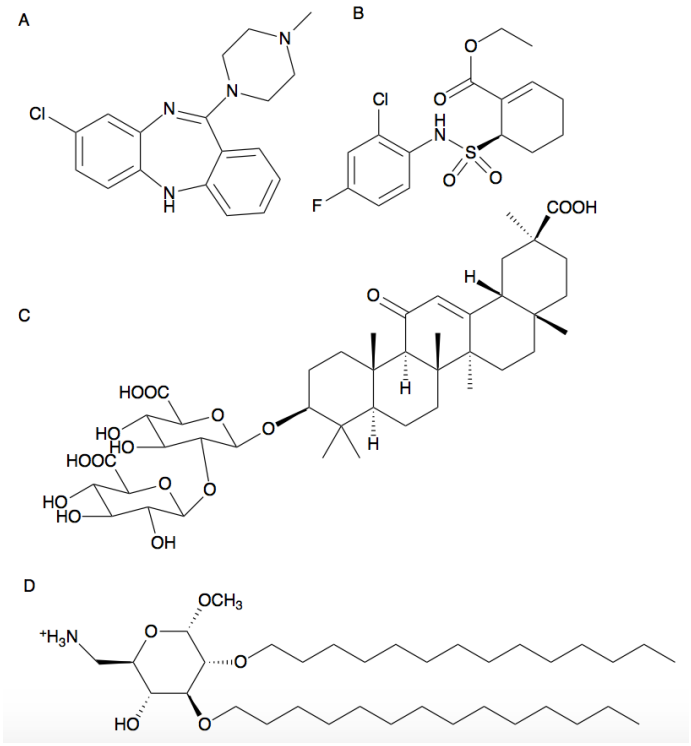

Figure 8: Chemical structures of clozapine (A), TAK-242 (B), glycyrrhizin (C), and IAXO-102 (D)

Another TLR4 inhibitor, TAK-242, plays a critical role in immunosuppression. This small molecule binds the intracellular domain of TLR4. TAK was found to reduce

the severity of chronic pancreatitis through TLR4 inhibition. Researchers induced chronic pancreatitis in a rat population and administered TAK to experimental rats. Results indicated that administration of TAK reduced the expression of TGF-β. Additionally, the expression of immune cell surface markers such as CD3, CD4, and CD8 decreased after administration of TAK. This suggests that TAK inhibits TLR4 sufficiently enough to reduce the prevalence of CD+ cells within the cell-mediated adaptive immune response[32].

Inhibition of TLR4 with TAK (figure 8) provides an encouraging mechanism for immunosuppression. Research by Akitaka, S. and colleagues suggests that TAK has the ability to reduce auto-inflammatory symptoms in systemic auto-inflammatory diseases. Administration of TAK to auto-inflammatory mice resulted in reduced expression of IL-6, a cytokine important for neutrophil recruitment. TAK also reduced expression of CXCL13, a chemokine important for attracting B cells to the lymph node for activation[33].

Researchers in the Fumihiro group studied a variety of TLR4 antagonists. Their research focused on TLR4 antagonists and their effects on central nervous system disease. Despite the focus on CNS diseases, these antagonists hold potential for other therapeutic effects after organ transplantation. Glycyrrhizin (figure 8) is a traditional herbal remedy that was seen to prevent TLR4 up-regulation. After administration of glycyrrhizin, suppression of cytokines such as IL-1β, IL-6, IL-8, and MCP-1 were seen[34].

Two other TLR4 antagonists were investigated: LPS-RS ultrapure and IAXO-102 (figure 8). LPS-RS ultrapure inhibits the interaction of MD-2 with TLR4. MD-2 is a scaffolding protein necessary for successful TLR4 signal initiation. IAXO-102 also interferes with MD-2; however, it interrupts the cell surface marker CD14 and its

interaction with MD-2. Since the interaction between MD-2 and CD14 initiates the TLR4 signaling pathway, interfering with these molecules provides a strong TLR4 inhibitory effect[34].

IAXO-102 was also found to play a potential role in reducing Abdominal Aortic Aneurysms (AAA) through immune system modulation. Researchers found that IAXO-102 has the potential to decrease experimental AAAs by preventing vascular inflammation through inactivation of NF-κB pathways. Vascular inflammation plays a crucial role in transplant rejection. Therefore, IAXO-102 could be a very strong candidate for use in immunosuppressive therapies after transplantation[35]. The potential for IAXO-102 to have positive therapeutic effects in both central nervous system disease and AAA supports its merit in other therapeutic instances where NF-κB pathway inhibition could be beneficial.

CHAPTER TWO

Biological Application – TAK in Islet Transplant

Many promising TLR4 antagonists exist. However, our lab chose TAK for further investigation. This is due to its clinical potential for improving islet transplant and its favorable chemical structure for the bioconjugation procedures.

Research at the Annette C. and Harold C. Simmons Transplant Institute is ongoing to improve the efficacy of clinical islet transplantation. Collaboration between this institute and the Baylor University Chemistry and Biochemistry Department is focusing on the ability of TAK to inhibit TLR4. TLR4 mediated inflammation was observed in a MIN6 cell line treated with LPS, TAK, and TAK plus LPS (figure 9 and 10). The MIN6 cell line is used as a model for simulating normal islets. It exhibits similar characteristics of glucose-stimulated insulin secretion and glucose metabolism. This cell line was treated and left to incubate for a period of four hours. The cells were then collected, RNA was isolated, and RT-PCR analysis was performed.

Significant inhibition of IP10, a primary TLR4 mediated cytokine, was shown. Another TLR4 mediated cytokine, TNFα, showed significant inhibition when treated with TAK and LPS. This data supports TAK's ability to significantly inhibit TLR4 activation in the presence of LPS. This data establishes a foundation for the use of TAK in further transplantation models (figure 9 and 10).

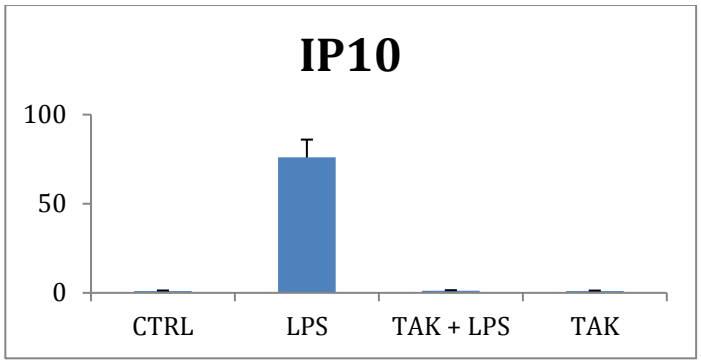

Figure 9: MIN6 cell line treated with LPS, TAK+LPS, and TAK. TAK+LPS presented p=0.0059.

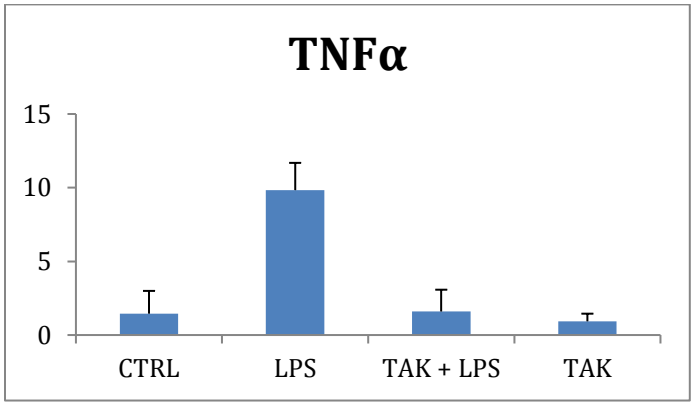

Figure 10: MIN6 cell line treated with LPS, TAK+LPS, and TAK. TAK+LPS presented p=0.0044.

An important step in islet transplantation is islet isolation. Islet isolation involves digesting the pancreas and isolating the islets to be infused back into the patient's hepatic portal vein. Islet isolation is rough on the islets and itself causes an inflammatory response. Therefore, this protocol must be done gently and with great care to limit tissue injury and preserve as many islets as possible for transplant into the patient. Research is ongoing in mouse models to find ways to protect the islets during the isolation procedure.

For this reason, we dissolved 3 μM TAK into the enzyme solution, collagenase, used to digest the pancreas. During islet isolation, collagenase is injected into the common bile duct (CBD). A clamp is placed after the pancreatic duct drains into the

CBD. This allows the collagenase solution to be transported only to the pancreas and the collagen within the pancreas to be fully digested. When the collagenase was mixed with TAK, both were injected into the mouse through the CBD and transported to the pancreas.

Common protocol for islet isolation was followed. After islet isolation, the islets were left to incubate. This allowed time for the newly added TAK to vary the RNA expression in the islets. After an incubation period of 4 hours, RNA was isolated from the islets and RT-PCR analysis was performed to quantify the activation of several important genes downstream from TLR4. Tissue factor, IP-10, ICAM, and MCP1 all showed significant reduction when compared to the control islets (figure 11). This suggests that TAK is effective at inhibiting TLR4 activation and supports its use in islet transplant protocols.

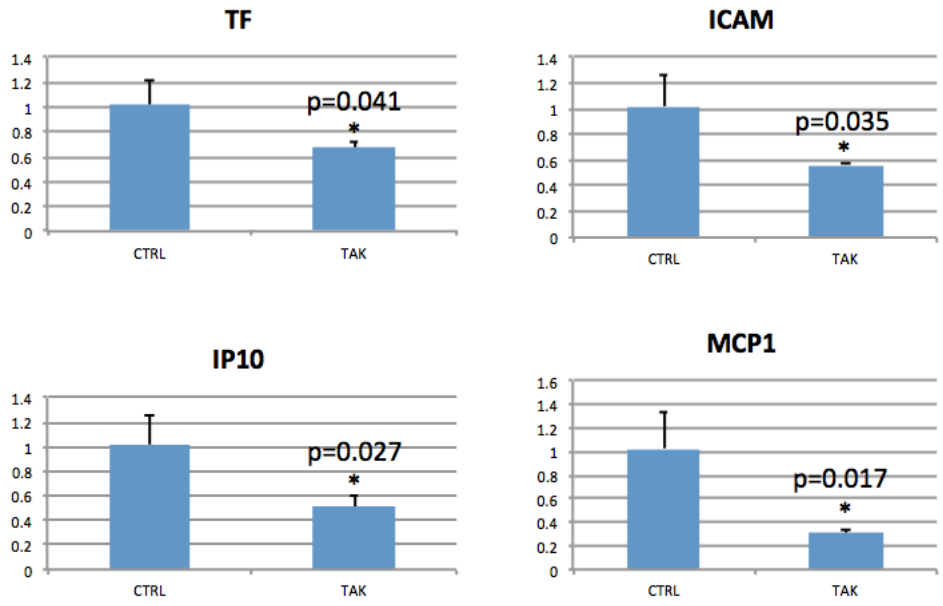

Figure 11: Islet isolation with Collagenase (ctrl) and TAK + collagenase (TAK) and levels of TF, ICAM, IP10, and MCP1 using RT-PCR analysis

As mentioned previously, NFκB is an important transcription factor in the TLR4 pathway. HEK-Blue hTLR4 cell lines were used to observe NFκB expression. HEK hTLR4 cells are normally obtained by co-transfection of human TLR4, MD-2, and CD14 co-receptor genes, along with an inducible secreted embryonic alkaline phosphatase (SEAP) into HEK293 cells. SEAP is a reporter gene placed under the control of an IL-12 p40 minimal promoter fused to five NFκB and AP-1-binding sites. Therefore, stimulation with a TLR4 ligand actives NFκB and AP-1, inducing the production of SEAP. Levels of SEAP are then observed with HEK-Blue detection media[36].

NFκB expression in HEK hTLR4 cells decreased when the media was treated with 3 μM TAK. NFκB expression was quantified using a colorimetric analysis of the HEK media in the well plate (figure 13). In both hypoxic and normoxic conditions, TAK was shown to decrease the coloration of the media, thus inhibiting expression of NFκB (figure 12). It was expected, however, that the cells in normoxic conditions would present lower levels of NFκB expression. Therefore, the experiment should be redone to produce more credible results.

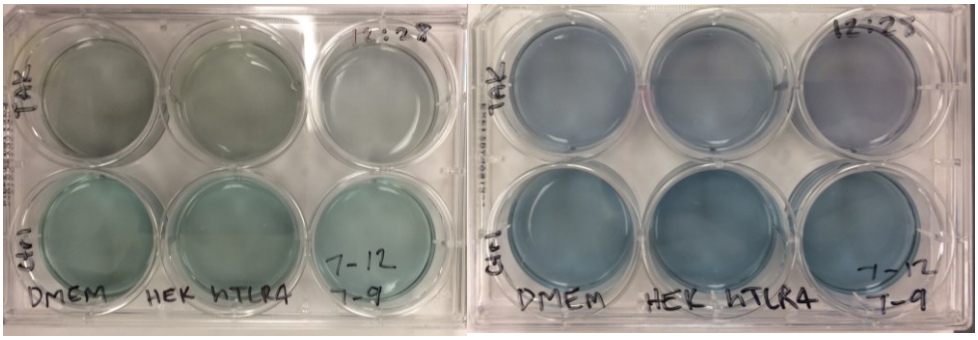

Figure 12: Left-HEK TLR4 cell line samples treated (TAK) and untreated (CTRL) placed in hypoxic conditions; Right-HEK TLR4 cell line samples treated (TAK) and untreated (CTRL) placed in normoxic conditions

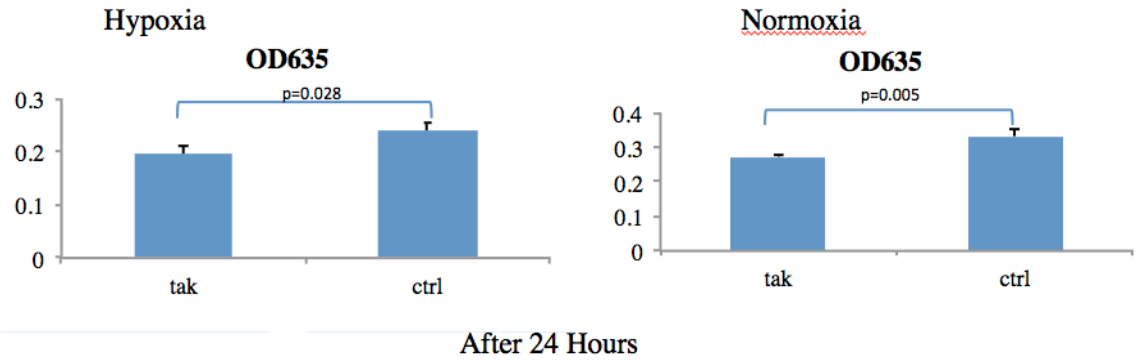

Figure 13: HEK TLR4 cell lines treated with 3 μM TAK in both conditions of hypoxia and normoxia

To determine if TAK can eventually be used clinically in transplant procedures, viability tests must be run to determine its effects on cell sustainability and function. A viability assay was run with MIN6 cells, a cell line that exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to normal islets. A sample was considered viable if over 80% of the cells survived after treatment. MIN6 cells were found to be viable when treated with TAK concentrations as high as 6 μM after 48 hours (figure 14). Since the working solution of TAK used in the isolation experiments is 3 μM, these results suggest that the working TAK solution itself is not harmful to MIN6 cell lines. Though this experiment provides promising results, it should also be performed on islets.

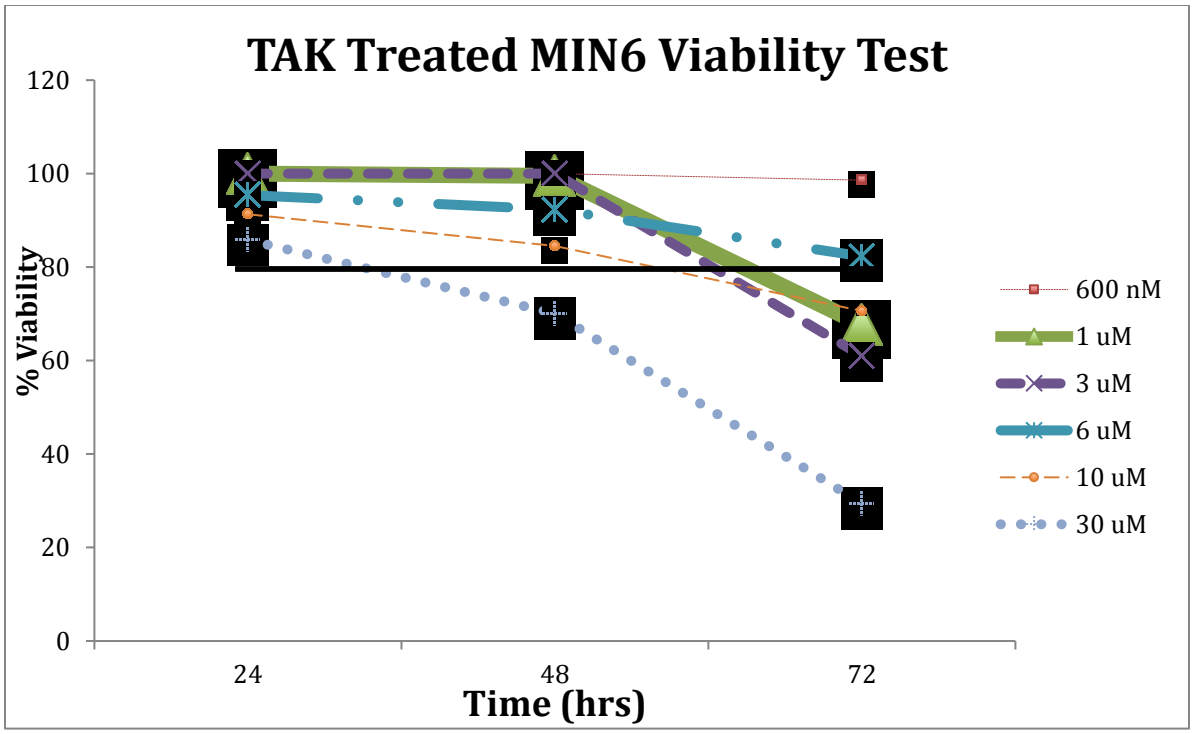

Figure 14: MIN6 cell line treated with different concentrations of TAK and monitored over 72 hours. Viability of 80% is marked with a black line.

CHAPTER THREE

Bioorganic Approach – Tissue Surface Modification

Going forward, our lab sought to use this information, combined with chemical conjugation techniques, to conjugate TAK to the surface of murine pancreas islet cells. We hoped that the bioconjugation of an immunosuppressant onto the surface of transplanted tissue would better improve transplant outcomes. First, organic synthesis was performed to create a linker molecule able to utilize click chemistry to attach to the surface of pancreas islets. The linker was synthesized to utilize β-elimination to cleave after transplantation (figure 15)[37].

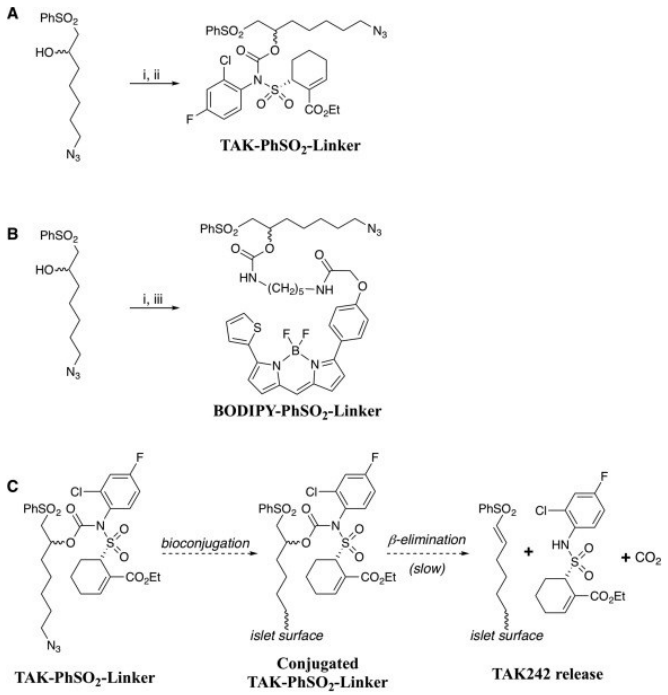

Figure 15: Cleavable prodrug chemistry (A) Synthesis of TAK-$PhSO_2$-Linker prodrug: (i) Triphosgene, pyridine, THF (ii) TAK-242, Et_3N, THF. (B) Synthesis of BODIPY-$PhSO_2$-Linker: (i) Triphosgene, pyridine, THF (iii) BODIPY-TR-cadaverine, Et_3N, THF. (C) TAK-$PhSO_2$-Linker conjugation to tissue surface and release of free TAK-242 by β-elimination[37]

After understanding the immunosuppressive effects of TAK, creating a cleavable linker, and establishing its ability to conjugate to the surface of islets, allo-islet isolation and transplantation were performed using mouse models. Islets in quantities of 100 were isolated and transplanted into Streptozotocin-induced diabetic mice. In the mouse, islets are commonly transplanted into the kidney capsule instead of the hepatic portal vein, where they vascularize. A control group, TAK treated islets, and TAK modified islets were introduced. After monitoring the blood glucose levels of the mice, the TAK modified islets resulted in the greatest return to a normoglycemic range, followed by the TAK treated islets. The TAK modified islets showed the greatest percentage of euglycemic mice after 28 days post-transplant (figure 16)[37].

An Intraperitoneal Glucose Tolerance Test (IPGTT) was performed after transplantation. This involves an intraperitoneal injection of glucose into the mouse and monitoring mice blood glucose levels every 30 minutes for 150 minutes. Initially, all mice will show hyperglycemic blood glucose levels. However, the experimental mice are expected to more quickly return to a normoglycemic range. As expected, the mice with modified islets demonstrated the lowest blood glucose levels 60-150 minutes after an intraperitoneal injection of glucose (figure 16)[37].

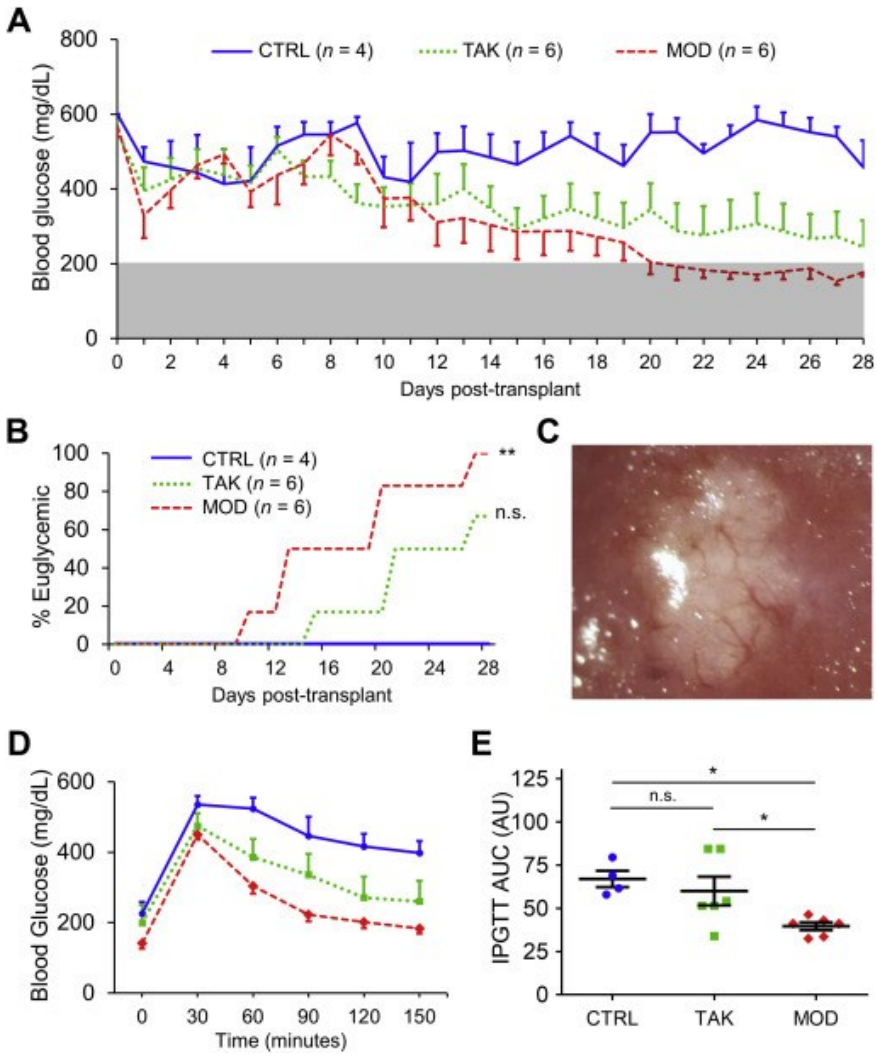

Figure 16: A) CTRL, TAK treated islets, and TAK modified islets B) Percentage of Euglycemic mice C) Transplanted islets inserted into mouse kidney capsule D) Mouse Blood Glucose levels measured during Intraperitoneal Glucose Tolerance Test (IPGTT) E) IPGTT test[37]

Because all mice receiving TAK modified islets achieved euglycemia ($p = 0.004$) and only 67% of the TAK-treated islets achieved euglycemia ($p = 0.052$), the potential for tissue surface modification creates exciting opportunities[37]. This data was published and will hopefully lead to further studies with clinical applications.

These promising results featuring TAK modified islets support further investigations into tissue surface modification in transplantation. Little research is

available investigating tissue modification in the way we will propose. For this reason, we believe it is important to create a foundational model that showcases a variety of chemical linkers on several commonly transplanted tissue types. We hope this data, along with the promising TAK modified islet results, will support further investigation into tissue surface modification and local drug release.

In past experiments, we utilized two different dyes, a stable Alexa Fluor and a cleavable BODIPY dye, to observe tissue surface modification and the effectiveness of a cleavable linker. Pieces of bovine pericardium were treated with NHS-DBCO for one hour. Then, the modified tissue was reacted with one of the two dyes for a one-hour period. Finally, the tissues were washed and imaged using a fluorescence imager. The fluorescent intensity was quantified using Image J. A sample of imaged pericardium is shown below (figure 17).

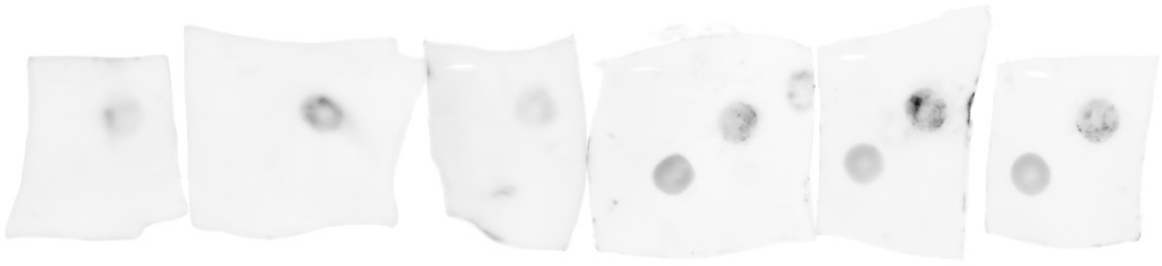

Figure 17: Example of modified bovine pericardium with chemical linker and dye.

We also observed the release kinetics of both dyes over a period of 90 hours. Our experimental results showed that the stable Alexa Fluor dye maintained a relatively constant intensity, while the cleavable BODIPY dye was cleaved over a period of 90 hours. The cleavable BODIPY dye cleaved most quickly during the first twenty hours (figure 18).

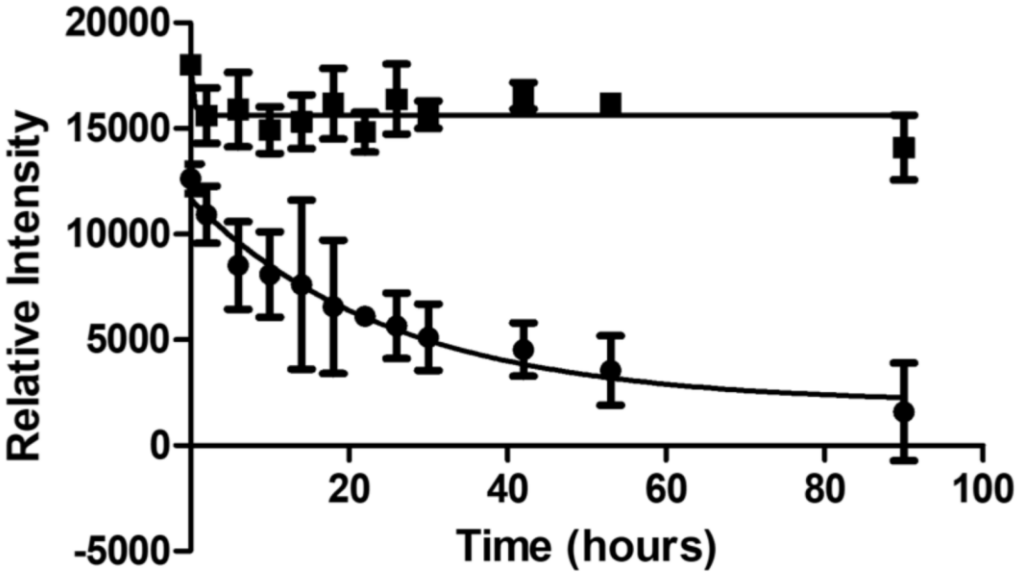

Figure 18: Alexa Fluor and BODIPY dye intensities measured over 90 hours

The results described above are part of our preliminary studies. We are now attempting to use two different linkers, which will covalently bond to the same dye. We believe this will provide a more robust and controlled model for future tissue surface modification experiments.

For these experiments, two linkers were synthesized: one cleavable linker and one stable linker. First, 6-bromohexanol was reacted with sodium-azide to make 6-azidohexanol[38,39,40].

Stable Linker Synthesis

The stable linker synthesis took the 6-azidohexanol and reacted it with triphosgene in pyridine to yield a 6-azidochloroformate. Finally, the chloroformate was reacted with tetrazine amine in pyridine to yield the stable linker (figure 19).

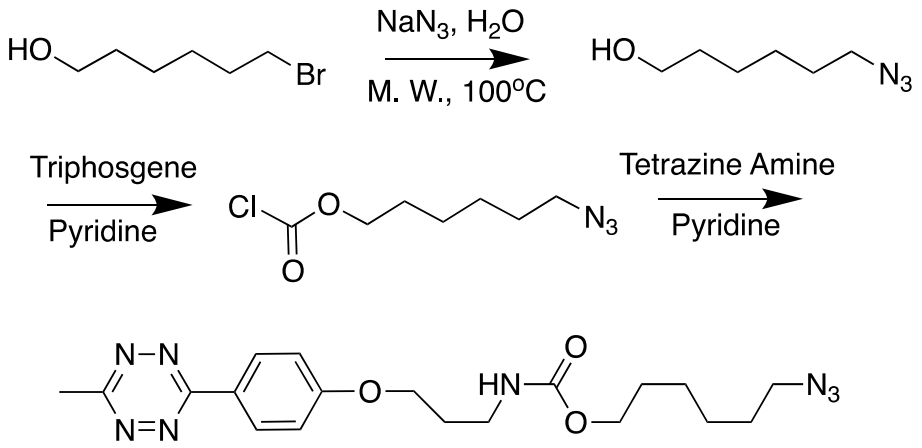

Figure 19: Stable linker synthesis from 6-bromohexanol

Cleavable Linker Synthesis

To synthesize the cleavable linker, 6-azidohexanol was reacted with TEMPO and $NAHCO_3$ in water to make 6-azidohexanal. 6-azidohexanal was reacted with n-butyllithium, methylphenylsulfone, and saturated ammonium chloride at -78°C. This yielded the linker alcohol shown below. The linker alcohol was then reacted with triphosgene in pyridine to make the linker chloroformate. Finally, the linker chloroformate was reacted with tetrazine amine in pyridine to yield the cleavable linker (figure 20).

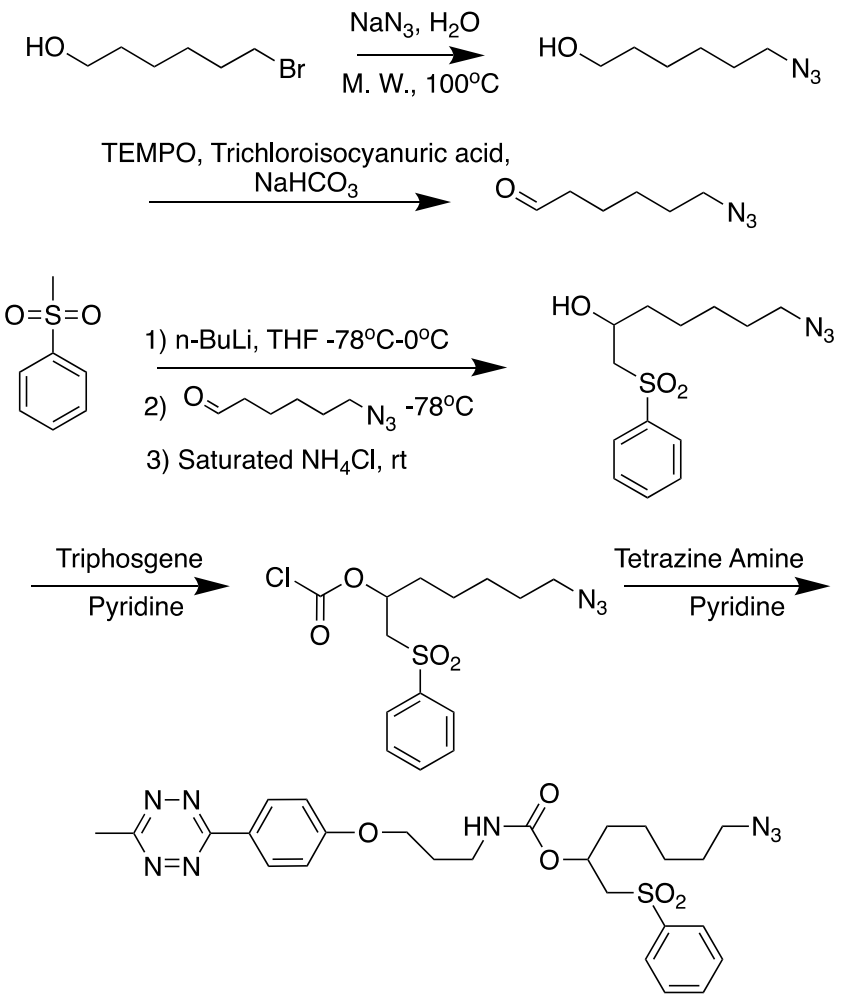

Figure 20: Cleavable linker synthesis from 6-bromohexanol

The stable linker will be used as a control to demonstrate a stable and continuous attachment to the tissue surface throughout the experiment. The cleavable linker will be slowly cleaved at the methylphenylsulfone attachment.

After synthesis of the two linkers, they will be reacted with a Cy-5 dye to yield a cleavable and stable dye (figure 21). The dye has an octene ring. This ring undergoes a Diels-Alder reaction with the nitrogen ring on the tetrazine amine to form the bond between the linker and the dye. The dye is very aromatic, which explains its ability to reflect light.

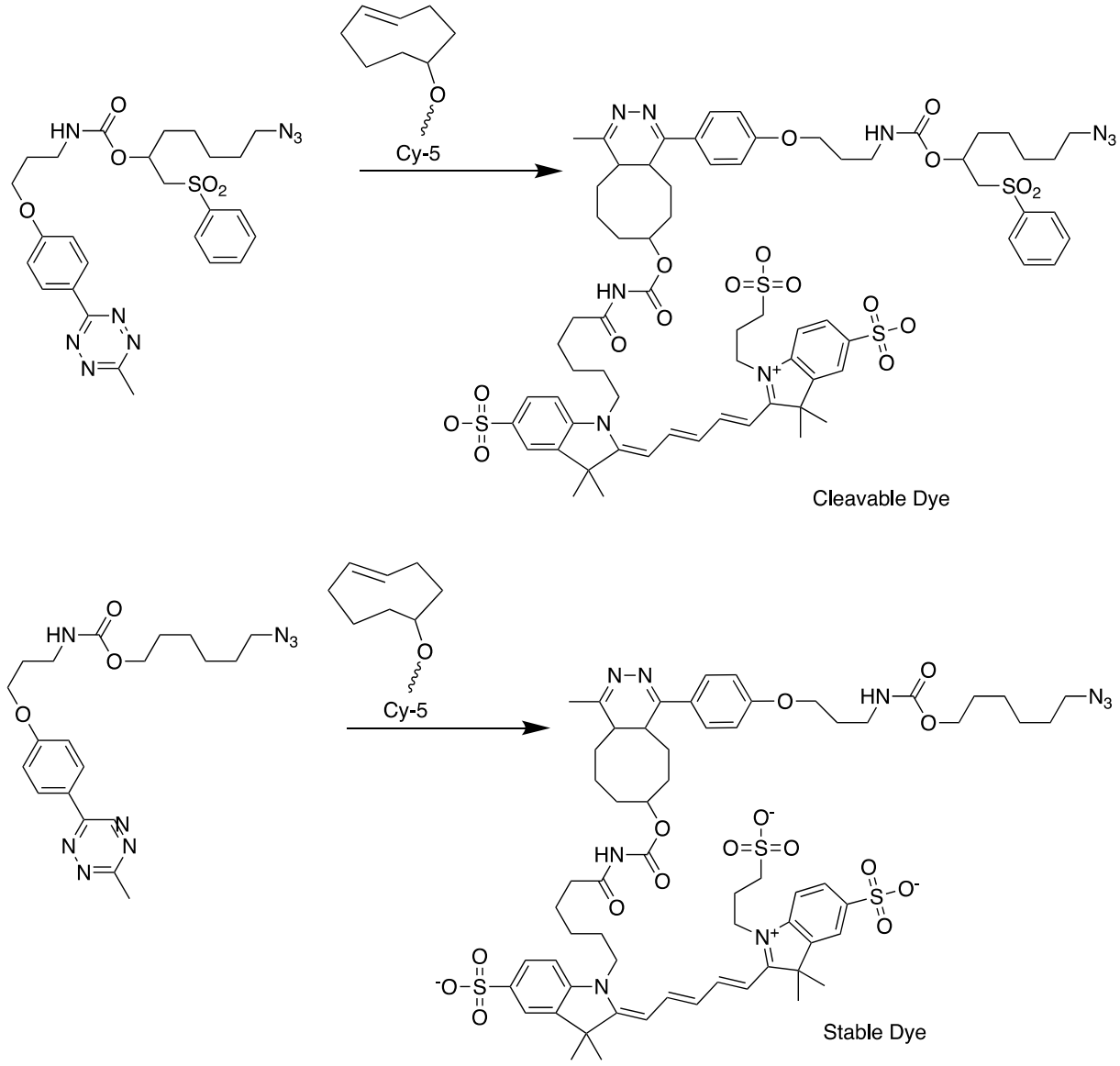

Figure 21: Stable and cleavable linkers reacted with Cy-5 dye

To create a model for tissue surface bioconjugation, we will first use DBCO-sulfo-NHS as our tissue surface linker. This molecule, shown below, reacts at its NHS group to bond with an amine on the tissue surface (figure 22).

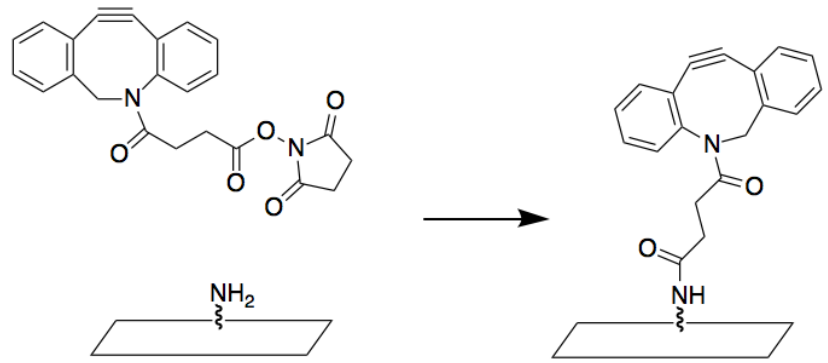

Figure 22: DBCO-NHS reacting with tissue surface amine

Next, the triple bond on the DBCO molecule's octyne ring will react with the azide group on the previously synthesized linkers to form the structures below (figure 23). Finally, the cleavable linker cleaves at the methylphenylsulfone bond to release the dye as shown below (figure 23).

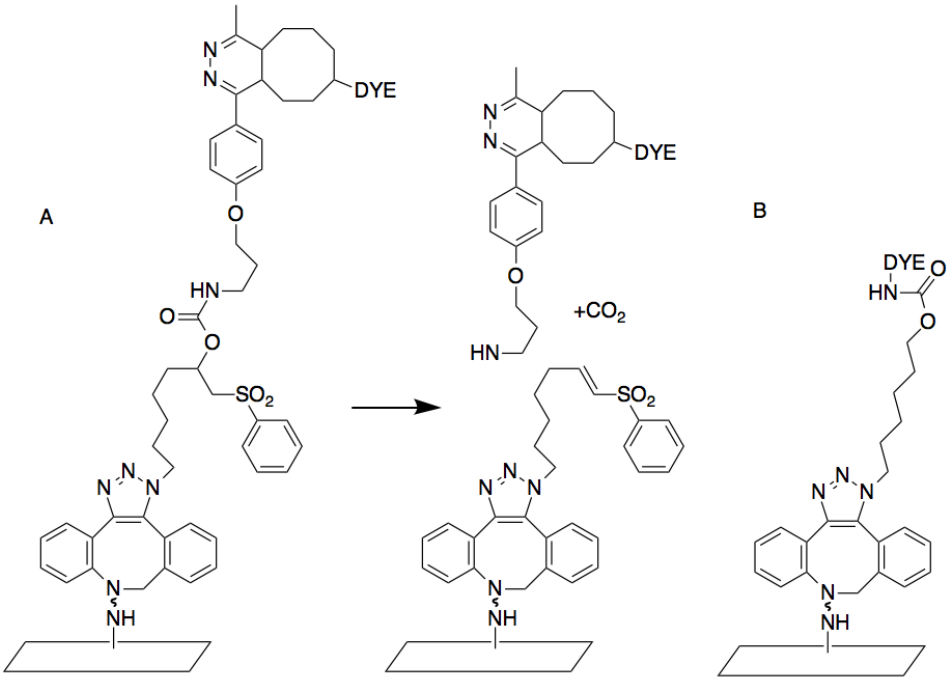

Figure 23: A) Cleavable linker dye attached to tissue surface and cleaved to form three products: CO_2, cleaved dye, and remainder of attached linker B) Stable linker dye attached to tissue surface

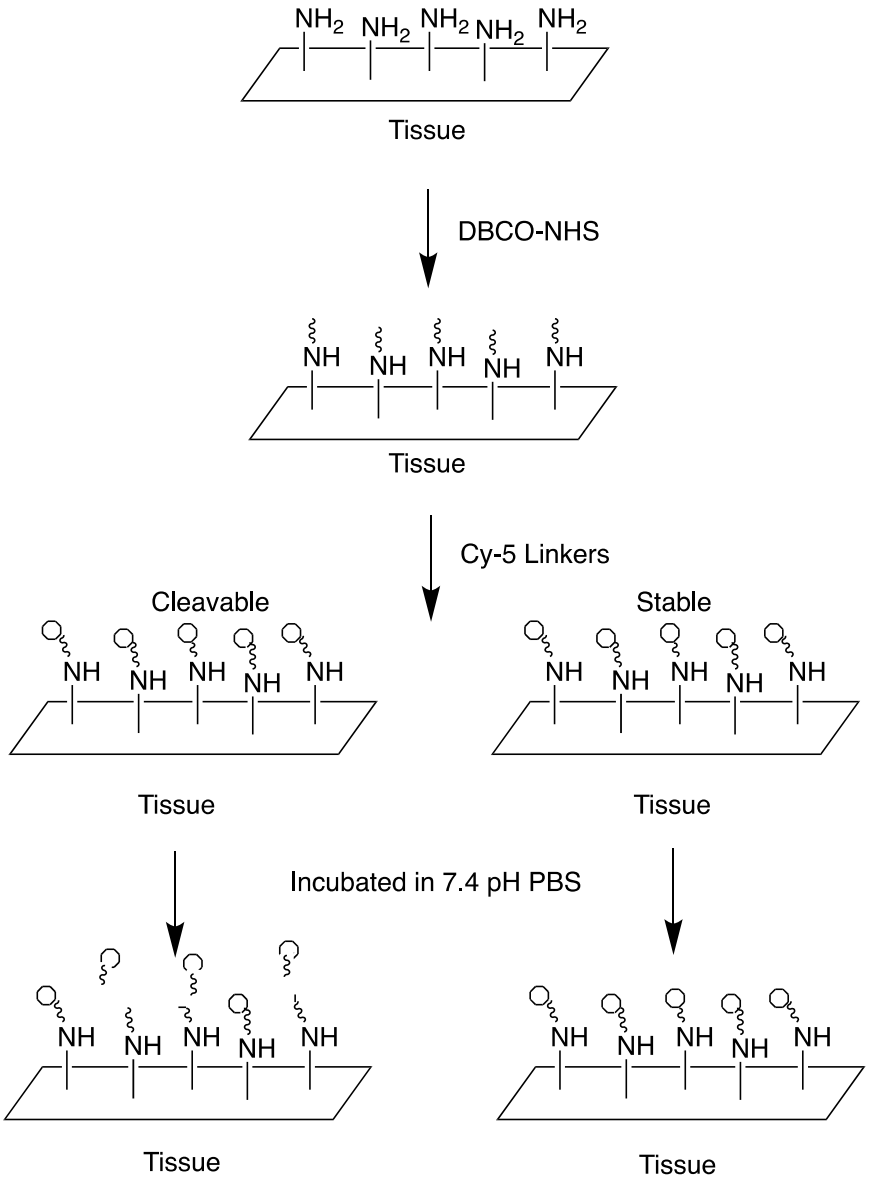

Figure 24: Overall schematic for tissue surface modification and release of cleavable linker; Black doodle=NHS-DBCO linker, Circle=cleavable and stable dye

CHAPTER FOUR

Other Experiments

A few other experiments not directly related to the overall story of this thesis are detailed here. These experiments were conducted over the past two years.

Previously at the Annette C. and Harold C. Simmons Transplant Institute, effective RNA isolations were thought to require around 100 islets. However, we conducted an experiment that sought to see how few islets were needed to isolate RNA. RNA isolation was performed on 15, 30, and 55 islets. The 18s primer was used since all cells should contain relatively equal concentrations. Since the ct values obtained after RT-PCR, were relatively the same between all groups, it suggests that RNA isolation can be performed on as few as 15 islets if done carefully (figure 25).

# of Islets	Ct Values	
15 islets	19.65	19.50
30 islets	19.15	19.72
55 islets	20.16	19.96

Figure 25: RNA isolation from 15, 30, and 55 islets.

We also stained islets to observe their viability. This is often performed after islet isolation to ensure that the islets are in suitable condition for transplantation. These islets show relatively good viability since very few cells are stained red (figure 26).

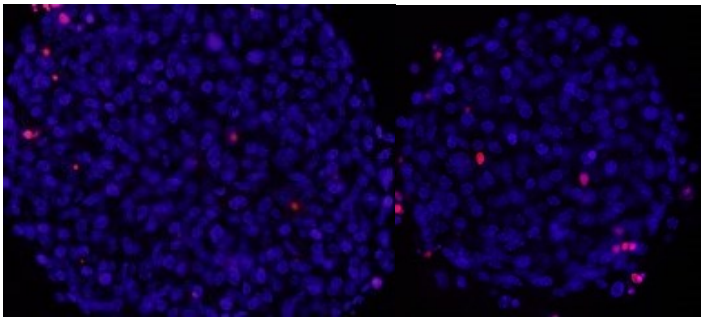

Figure 26: Islet viability staining, blue corresponds to cell nuclei and red corresponds to dead cells

During my time at the Annette C. and Harold C. Simmons Transplant Institute, I also had the opportunity to perform nephrectomies on our experimental mice. Islet transplant protocols for mice inject the transplanted islets into the kidney capsule where they vascularize. After islets are transplanted into the experimental mice and monitored for the desired amount of time, nephrectomies are performed to remove the islets. If the mouse's normoglycemic blood sugar levels are due to the presence of the transplanted islets, the removal of the kidneys should result in a return to hyperglycemia. This was noted in our mice from the experiments in chapter three.

Figure 27: Performing nephrectomies on experimental mice

CONCLUSION

Overall, this research aims to further improve transplant efficacy by covalently modifying tissue surfaces with immunosuppressant medications. Future directions of this work will include improving the robustness of our model by using more tissue types, such as bovine skin, porcine portal vein, and porcine thoracic aorta to simulate tissue modification on a variety of tissues important in common transplant procedures. Also, more linkers beyond DBCO-NHS will be tested to observe and compare their release kinetics. Hopefully this model will create a foundation for the bioconjugation of immunosuppressant medications onto tissue surfaces in animal models and eventually clinical models.

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