Pre-treatment of Renal Allografts to Modify Chemokine: Glycosaminoglycan Pathways Reduces Transplant Rejection

Development of a Novel Model to Test New Therapeutic Targets

by

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ABSTRACT

The need of organs for transplantation has become an increasing medical need due to a limited donor organ supply. Many organs fail within 10 years due to acute and chronic rejection. Acute or antibody mediated rejection leads to decreased long term graft survival and increases the need for a repeat transplant. In prior work, reducing endothelial heparan sulfation and blockade of chemokine-glycosaminoglycan (GAG) interaction with Myxomavirus-derived protein, M-T7, reduced aortic and renal graft vascular inflammation and rejection. Conditional endothelial Ndst1 deficiency and inhibition of chemokine-GAG interaction reduces early allograft damage and suggest new therapeutic options for graft rejection. Here acute renal rejection was examined in grafts with conditional endothelial N-deacetylase-N-sulfotransferase-1 knockout (Ndst1-/-) and in wildtype (WT) C57Bl6/J grafts treated with saline, M-T7, antisense oligonucleotides (ASO) for Ndst1 or a scrambled ASO control. Viruses have a highly adaptive ability to evade hosts defense and immune response. The immunomodulatory proteins derived from viruses provide potential therapeutic uses to alleviate this need for organs. The Myxoma virus derived protein M-T7 is a promising therapeutic for reducing kidney transplant rejection. Orthotopic transplantations in mice are extremely difficult and costly because they require a highly trained microsurgeon. This kidney to kidney subcapsular and subcutaneous transplant model is a practical and simpler method that requires fewer mice, one kidney can be used for transplants in 6 or more mice and there is much lower morbidity, pain and mortality. Heterotopic transplantation of allografts is a simple model for preliminary testing of treatments for early inflammation, ischemia, and graft rejection. Subcapsular kidney transplantation provides a first step approach to test virus-derived proteins as potential treatments to reduce transplant rejection and inflammation. This project reports on a broadly applicable platform on which to rapidly and conveniently test new treatments for transplant rejection. This finding will significantly lower the barrier to entry for labs which are interested in translating their laboratory findings to animal models of organ transplantation which is a complex surgical procedure, and thus accelerate the bench-to-bedside translation of novel, putative treatments for transplant rejection as an initial screening tool.

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CHAPTER 1

INTRODUCTION

Transplant Rejection

As of August 2017, there were 114,927 people awaiting an organ transplant. Of that number, 95% of them were waiting for kidney or liver transplants (Bentley, Phillips, & Hanson, 2017). The number of available donors however was staggeringly low. Only approximately 30,000 organs are typically available each year to fill this growing need for donor transplant organs (Abouna, 2008). There is thus a large gap between available organs for transplant and the need for organs for transplantation; this leaves an immense need for more organs. Kidney transplants are one of the most common organ transplants performed. Approximately 50% of the time that graft will fail in 5-10 years creating the need for a repeat transplant (Sood et al., 2016). These repeat transplants have the same statistics as the first or an even smaller chance of survival. In fact, any people receiving a solid organ transplant of any kind will often have kidney failure in 10 years (Sood et al., 2016). Thus, recipients of grafts are again in the same predicament as prior to their initial transplant and will eventually require an additional kidney transplant.

Both acute and chronic rejection reduce long-term graft survival, increasing the need for repeat transplantation and as noted are complicated by limited donor organ supply. Recurrent episodes of acute antibody-mediated immune rejection as well as persistent excess inflammation lead to transplant allograft vasculopathy which causes late graft loss and scaring associated with chronic rejection (Filippone & Farber, 2015; Guillen-Gomez et al., 2017; Kim et al., 2014; Roufosse et al., 2018; A. Shimizu, Yamada, Robson, Sachs, & Colvin, 2012; Tanabe, Takahashi, & Toma, 1996; VanBuskirk, Pidwell, Adams, & Orosz, 1997). Acute rejection is caused by a massive influx of alloactivated T cells that is seen early in the inflammatory response. Some immune responses are also induced by decreased blood flow (ischemia), surgical trauma and infections at the time of the transplantation (Pefanis, Ierino, Murphy, & Cowan, 2019). In some patients who have shock or brain death

there is a cytokine storm that causes excess immune and inflammatory cell activation causing damage to the donor organ even before transplantation and can be carried over with the donor graft to the recipient of the graft (Richmond & Harris, 2014). All these factors can contribute to early damage and excess inflammation in the donor organ at the time of implanting the transplanted graft.

When the organ is removed from the donor and cut off from the blood supply it causes ischemia reperfusion injury (IRI). IRI results in apoptosis and necrosis causing severe organ damage (Malek & Nematbakhsh, 2015; Pefanis et al., 2019). Kidneys being one of the most susceptible to this often exhibit many signs and have a decrease in function. Dying cells release a combination of damage associated molecular patterns (DAMPs), proinflammatory and chemotactic cytokines. Two major molecules involved are IL1α, a proinflammatory cytokine, and TNFα which is known to induce cell death by activating dendritic cells that activate T cells and macrophages (Pefanis et al., 2019). These invading inflammatory cells cause interstitial fibrosis and tubular atrophy both attributes of chronic allograft nephropathy (CAN) that are associated with late graft loss (Fletcher, Nankivell, & Alexander, 2009).

Chronic allograft nephropathy and vasculopathy are leading causes for graft loss in the organ after the first-year post transplantation (Fletcher et al., 2009). This can be seen by glomerular abnormalities for example a loss of structure which hinders the kidneys ability to filter fluids. The tubules also become shriveled up and the arteries thicken shutting off the blood supply to the kidney (Fletcher et al., 2009). Complement is also involved in organ rejection and important part of the innate immune system. During kidney rejection C4d is released as a cleaved product (Cicciarelli et al., 2017; Cohen et al., 2012; Filippone & Farber, 2015; T. Shimizu et al., 2012). This binds to antibodies which bind to the endothelial lining of the peritubular capillaries and glomeruli. This is used as a marker for antibody mediated rejection that can be detected in histology. All these signs are used in the clinic and backed by the Banff criteria widely used to identify renal failure (Roufosse et al., 2018).

In addition to histology from a biopsy, clinical signs of renal failure are often measured using creatinine and blood urea nitrogen (BUN) tests in urine or in blood.

Transplant Immunology

Chemokines play a large role in the immune system and fighting foreign organs. There are many different chemokines that can bind to many different receptors. Many of these receptors are associated with transplant rejection (Charo & Ransohoff, 2006; Dai et al., 2010; Filippone & Farber, 2015; Li et al., 2019; Tanabe et al., 1996; VanBuskirk et al., 1997). These chemokines are secreted at the site of injury. There are four families of chemokines C, CC, CXC, and CX3C. These chemokines direct the immune cell migration to the site of injury. Lining the organ arteries and capillaries is the glycocalyx, a polysaccharide (sugar) coat that allows for chemokines to bind to the glycocalyx causing a chemokine gradient to form at the site of injury (Gandhi & Mancera, 2008b). The glycocalyx is composed of glycosaminoglycans (GAG) and proteins. GAGs are a special type of polysaccharide with repeating disaccharide units and a modified negatively charged amino group (Chen et al., 2018; Gandhi & Mancera, 2008a; Hoogewerf et al., 1997). The glycocalyx is found on the apical side of endothelial cells facing the lumen of the vessel. Chemokine GAG complexes are involved in a variety of mechanisms including proliferation, angiogenesis, and immune and inflammatory cell adhesion as well as cell activation (Yaron et al., 2019; Zhang et al., 2018). A critical function of the glycocalyx in terms of graft rejection is its roll in chemotaxis. The primary waves of inflammation after transplantation comes from surgical trauma, ischemia reperfusion, and include cytokines like TNFα and IL1α. In mice CXCL1/2 and CCL2-4 attract the neutrophils and macrophages to the site of transplant (Schenk, Rosenblum, & Fairchild, 2008). All these incoming immune cells cause excessive damage early on eventually leading to a late graft loss.

Macrophages play an essential role in the innate immune response. These phagocytic cells clear the tissues by engulfing cellular debris and pathogens from the tissue. Macrophages are divided into two functions and either promote or suppress inflammation.

M1 macrophages, classically activated proinflammatory macrophages, release a storm of cytokines to promote inflammation in the presence of pathogens or interferon gamma (INF-γ). These macrophages release cytokines and chemokines such as TNFα, IL-1β, and IL-6 to name a few (Helm, Held-Feindt, Schäfer, & Sebens, 2014; Li et al., 2019; Mosser & Edwards, 2008). On the other hand, M2 macrophages suppress the immune response, promote angiogenesis, and promote tissue repair (Helm et al., 2014). M2 macrophages secrete IL-10, transforming growth factor (TGF-β), IL-4, and IL-13 which are anti-inflammatory and downregulate proinflammatory cytokines released by M1 macrophages.

Glycocalyx and NDST1

One method of blocking the chemokine from binding to the GAGs is through the use of a conditional N-deacetylase- N- sulfotransferase 1 knockout ($NDST1^{-/-}$). Heparan sulfate one of the major GAGs in the glycocalyx is responsible for binding to many of the chemokines that cause a massive inflammatory response. NDST1 is an enzyme critical for the sulfation of heparan sulfate (Collins & Troeberg, 2019). If the sulfation does not occur, then the chemokine cannot bind to the GAG thus preventing the chemokine gradient (Figure 1). Targeted modifications (e.g., sulfation) performed by specialized enzymes (Ndst1) mediate chemokine binding to the glycocalyx and facilitate immune cell migration. Conditional endothelial Ndst1 deficiency and inhibition of chemokine-GAG interaction reduces early allograft damage and suggest new therapeutic options for graft rejection (Chen et al., 2018). The knockout is designed to only knockdown specific cell types: endothelial and myeloid precursors (Rops et al., 2014; L. Wang, Fuster, Sriramarao, & Esko, 2005). A full knockout of the gene is lethal. Although the conditional Ndst1 knockout reduces acute rejection in mice, the specific organ knockout does not exist in humans thus targeted genetic modification or modulation of the pathway is required.

Myxoma Virus and M-T7

In the 1950s, there was a massive overpopulation of European Rabbits (Oryctolagus cuniculus) and this led scientists to release a virus to control the population. The chosen virus, Myxoma virus, induces a highly lethal disease myxomatosis in rabbits specifically European rabbits (Chan, Rahman, & Mcfadden, 2013). Less than 1% of the rabbits were able to survive infection, however, resistant rabbits began to emerge creating progressively attenuated strains of the virus. Myxoma has evolved the capability to replicate even in the presence of a highly active immune system which makes it a promising virus to use in therapeutics.

The use of viruses as therapeutics has become increasingly popular in the past few years. Viruses have evolved the capability to evade the hosts immune responses which makes them a promising choice for therapeutic targets. Viruses have been used as therapeutics for treating spinal muscular atrophy to targeting cancer cells (Chan et al., 2013; Pattali, Mou, & Li, 2019). Proteins derived from these highly adaptive viruses provide potential to be used as therapeutics to modulate the immune response that occurs during organ transplantation. In prior studies, reducing endothelial heparan sulfation and blockade of chemokine-glycosaminoglycan (GAG) interaction with the Myxomavirus-derived protein, M-T7, reduced aortic and renal graft vascular inflammation and rejection (Chen et al., 2018; Mossman et al., 1996; Yaron et al., 2019).

In European rabbits M-T7 acts as an interferon gamma receptor (IFNγR) homolog, however M-T7 only binds rabbit IFNγR, eg. binding is rabbit species specific (Mossman et al., 1996). M-T7 is a broad-spectrum chemokine binding protein that blocks C, CC, and CXC class chemokines that binds to glycosaminoglycans (GAG). GAGs are an immunomodulatory protein and a special type of polysaccharide with repeating disaccharide units with a modified amino and negatively charged group (Gandhi & Mancera, 2008a). The glycocalyx is comprised of GAGs and lies on the apical side of endothelial cells. The glycocalyx plays an important role in cell proliferation, inflammation, and cell adhesion. A critical function of the glycocalyx has been reported in graft rejection and specifically for the role of the glycocalyx in

chemotaxis. Virus-derived proteins that prevent the infiltration of immune cells thus may provide new potential therapeutics for combating antibody mediated organ rejection (Chen et al., 2018; Epperson, Lee, & Fremont, 2012; Yaron et al., 2020).

Functional allografts

In prior work, reducing endothelial heparan sulfation and blockade of chemokine to glycosaminoglycan (GAG) interaction with MT7, significantly reduced aortic and renal graft vascular inflammation and rejection. M-T7 was given to reduce aortic allograft inflammation and intimal hyperplasia (Bartee et al., 2014; Chen et al., 2018; Liu et al., 2000). This was done without any added immunosuppressants. The use of this unique virus-derived chemokine GAG inhibitor, M-T7, led to the investigation of new therapeutic targets, specifically Ndst1, as a sulfation enzyme for one of the predominant GAGs in the glycocalyx, heparan sulfate for treating antibody mediated transplant rejection and vascular disease (Chen et al., 2018). In a mouse kidney transplant model, M-T7 given alone significantly reduced rejection and improved survival. M-T7 effect was lost when given to mice with Ndst1 deficiency in a kidney transplant model. Donor kidney transplants deficient in Nsdst1 had reduced rejection providing evidence for new potential immune response pathways and new therapeutic targets. Thus, virus-derived immune modulating pathways have identified important regulatory pathways in immune responses and provide new therapeutics for inflammatory diseases.

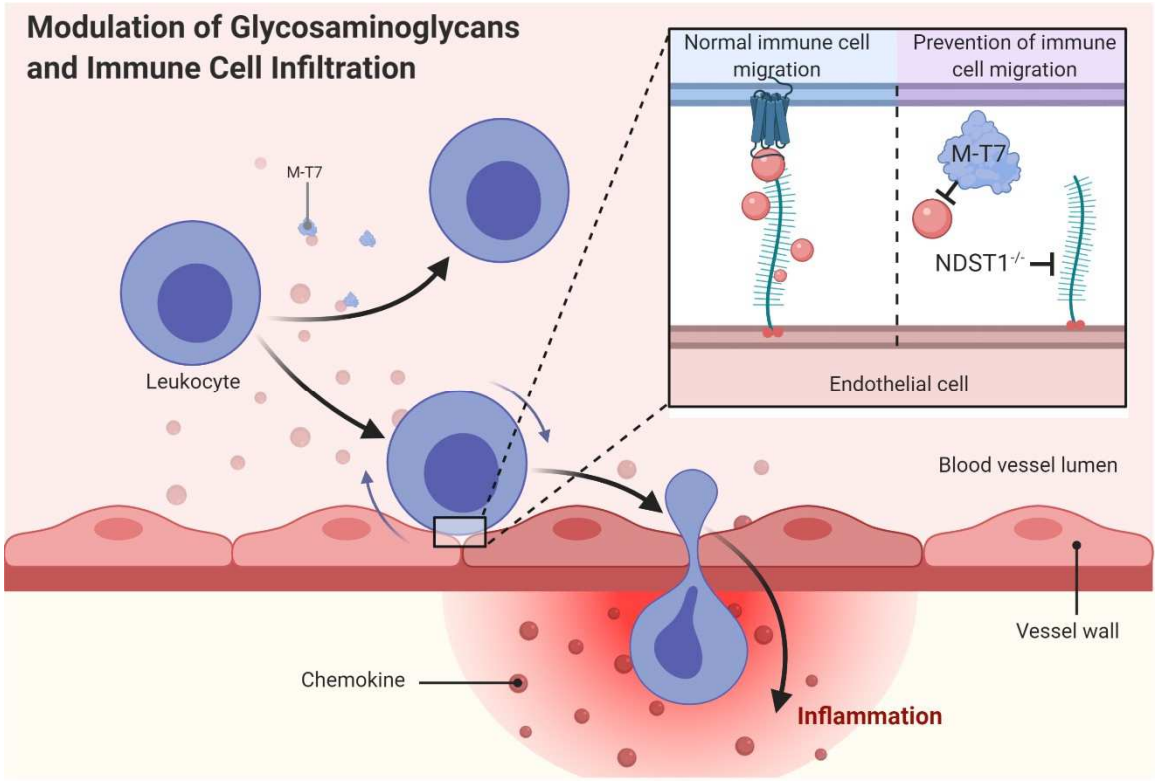

Figure 1. Inflammatory infiltration after modification of the glycocalyx. Chemokines form a gradient to the site of injury attracting immune cells. This causes inflammation and circulating immune cells to enter the site of infection or injury. M-T7, a chemokine binding protein, inhibits the chemokine from binding to the glycocalyx by interfering with the C terminus of the chemokine. NDST1 is a critical sulfation enzyme for GAGs. It adds the sulfation to the heparan sulfate GAGs, thus if there is a conditional knockout of NDST1 the chemokines would be unable to bind to the glycocalyx preventing the immune cell infiltration(Chen et al., 2018). Figure generated in Biorender.

Antisense Oligonucleotides

Antisense oligonucleotides (ASO) have been used in a variety of studies since the 1970s. Typically, ASOs are approximately 16-20 nucleotides in length and inhibit the expression of specific targeted genes (Bennett, 2019; Donner et al., 2015; Fusco et al., 2019; Southwell, Skotte, Bennett, & Hayden, 2012; Stepkowski, 1998). ASOs work by activating RNase H both intracellularly as well as extracellularly, and by inhibiting translation (Bennett, 2019). Their unique modified backbone structure allows them to easily be taken up by cells with increased nuclear resistance. The sugar ring is also modified to increase its binding capability to the target gene. ASOs have the capability to move through the body and collect in organs like the kidney (Donner et al., 2015). They have a unique capability of

targeting a single gene due to their short length. ASOs provide an easy route of administering treatments. As seen in Figure 2, the expression of the targeted genes is prevented through inhibition of the 5' cap formation, modulation of the RNA splicing, activation of RNase H in the nucleus or in the cytoplasm, modulation of polyadenylation, as well as the inhibition of translation (Fusco et al., 2019; Southwell et al., 2012). Many of these result in the cleavage of the mRNA and restrict the expression of the gene of interest.

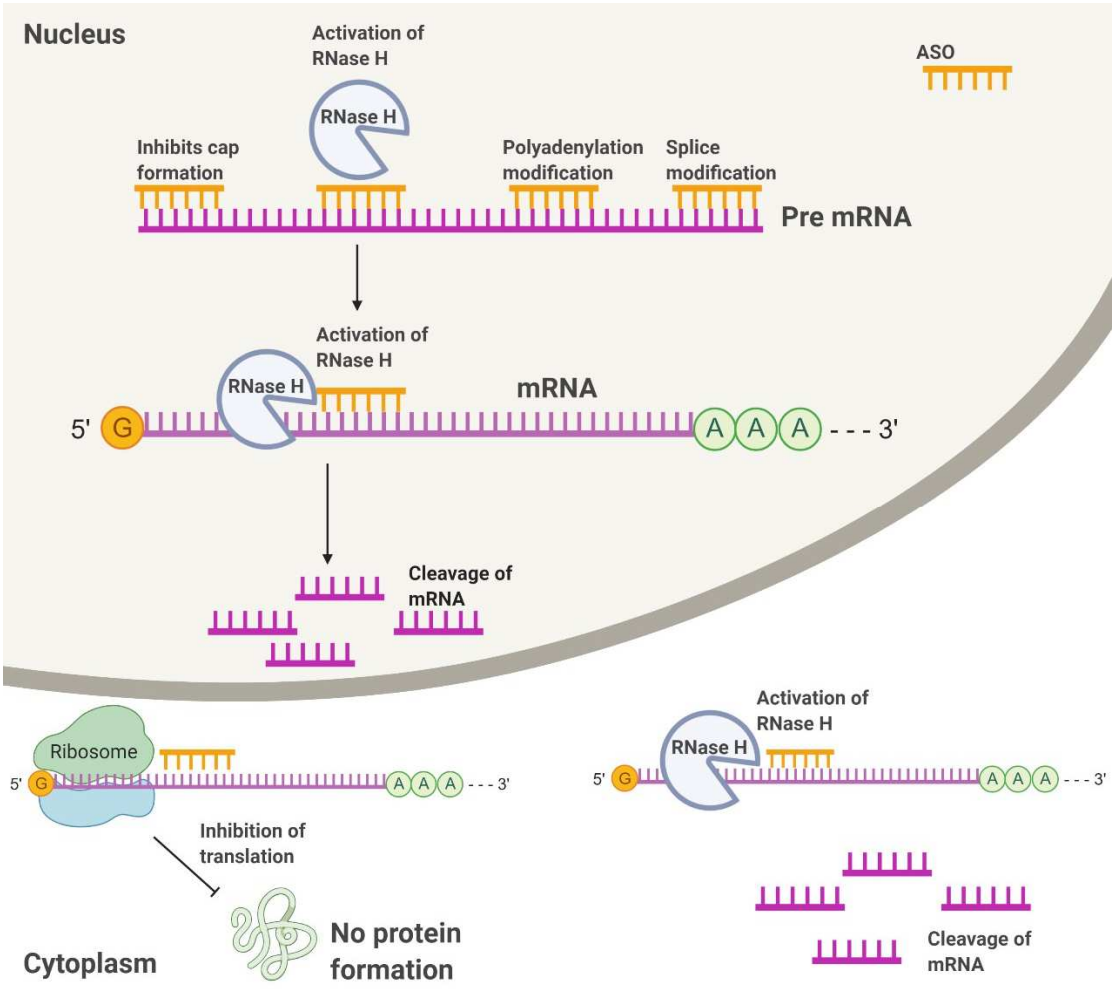

Figure 2. Functions of Antisense Oligonucleotides (ASOs). ASOs prevent gene expression by many different methods. A major function is the activation or RNase H which causes the cleavage of mRNA. This can happen in the nucleus or the cytoplasm. Other methods of preventing a specific protein from forming include inhibition of the 5' cap formation, modification of the polyadenylation, modification of the splicing, as well as inhibiting translation (Bennett, 2019; Southwell et al., 2012). Figure generated in Biorender.

Currently, there are a handful of drugs on the market that utilize this technology. IONIS, one of the leading developers, has three first in class drugs and many more in clinical trials that treat diseases from spinal muscular atrophy to hereditary transthyretin-mediated amyloidosis (Donner et al., 2015; Fusco et al., 2019; Southwell et al., 2012). ASO show a promising future in treating not only neurodegenerative diseases but also can be applied to treating disorders where there is a common protein that is vital to the pathogenesis of that disease. While transplant rejection is a combination of many proteins and immune cells flocking to the site, many of them require the sulfation of the polysaccharides to enter the site of injury. Targeting NDST1, the critical GAG sulfating enzyme, the immune cells would be prevented from binding to the sugary coat. This has been shown to work before a handful of models and is a suitable target for ASOs (Chen et al., 2018; Fusco et al., 2019). This will investigate the use of ASOs to reduce the expression of Ndst1 in subscapular and subcutaneous kidney allografts.

IONIS developed two ASOs for this project. The first ASO is a scrambled control. This is a random selection of nucleotides that will not bind to mRNA and has no function. The second ASO they developed is one that specifically targets NDST1. These are LICA ASOs that have an improved potency of 5-40 mg/week, longer dose frequency, lower cost of therapy, and can be administered through I.V., Sub Q, inhalation, oral, topical and many other routes (Donner et al., 2015).

To evaluate the toxicity of the ASO mice were given 3 doses of NDST ASO at day 0, 7, and 11. Albumin, alanine aminotransferase, aspartate aminotransferase, bilirubin, creatinine, and blood urea nitrogen tests were run on the serum and urine of the mice to determine toxicity. No toxicity was seen in the 10mg, 30mg, or 50mg. All values are within the normal ranges for mice and were confirmed by comparison to the PBS treated mice. Minimal toxicity was seen in the mice given doses of 100mg however this was only seen in one of the four mice tested. The levels of albumin decreased while alanine aminotransferase, aspartate aminotransferase, and bilirubin significantly increased (Figure 3).

Group	Rx NDST ASO	Mouse ID	1.ALB	3.ALT	5.AST	8.T.Bil	12.CRE	25.BUN
1	PBS	A1	2.7	16	33	0.13	0.08	25.5
		A2	2.437	18	44	0.15	0.09	22.4
		A3	2.63	22	37	0.12	0.09	24.7
		A4	2.729	20	44	0.13	0.1	25.5
2	10mg	B1	2.823	16	38	0.13	0.11	23.5
		B2	2.701	13	37	0.09	0.11	23.2
		B3	2.786	24	45	0.16	0.13	27
		B4	2.705	20	38	0.14	0.16	26.9
3	30mg	C1	2.552	26	154	0.25	0.14	24.5
		C2	2.622	12	39	0.1	0.14	21.5
		C3	2.542	16	36	0.11	0.12	23.1
		C4	2.522	22	36	0.1	0.16	24.1
4	50mg	D1	2.735	15	34	0.15	0.14	23
		D2	2.867	19	41	0.15	0.15	23.8
		D3	2.527	11	34	0.1	0.14	21.1
		D4	2.659	14	38	0.1	0.14	25.6
5	100mg	E1	2.18	293	500	0.32	0.06	12.6
		E2	2.583	13	36	0.12	0.13	19.5
		E3	2.451	16	53	0.13	0.12	25.9
		E4	2.389	19	37	0.1	0.14	25.9

Figure 3. Low level of toxicity for NDST1 ASOs in mice. Values for albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin (T.Bil), creatinine (Cre), blood urea nitrogen (BUN) in mice. Each group contained 4 mice that were given various doses of NDST1 ASO or PBS as a control. The values in the blood serum and urine were measured at day 14 after 3 doses in that time period. Mouse E1 displayed significant increases in ALT, AST, and T.Bil while it had a decrease in albumin. Mouse C1 also displayed small increases in AST and T. Bil. All other mice values were within a normal range and had similar values to the mice treated with PBS. (Data supplied by IONIS)

To test the functionality of the ASO that targets NDST1, PCR was performed to determine the expression of the target enzyme in the heart, liver and kidney. Figure 5 shows the most effective organ to treat is the liver which displayed the largest decrease in NDST1. The kidney which is the organ of interest in this study showed approximately a 50% reduction of NDST1. After 10mg doses the reduction tapers off and remains the same even as the dose increases. To ensure the ASO did not increase the amount of immune cells in the organ of interest levels of MCP1 were tested. Expression of MCP1 remained the same in the kidney even when the dose increased (Figure 6). The heart and liver displayed a significant increase at the higher doses. These tests were used to determine the optimal

dose of ASO that can be given to the mice with the least amount of NDST1 expression and toxicity.

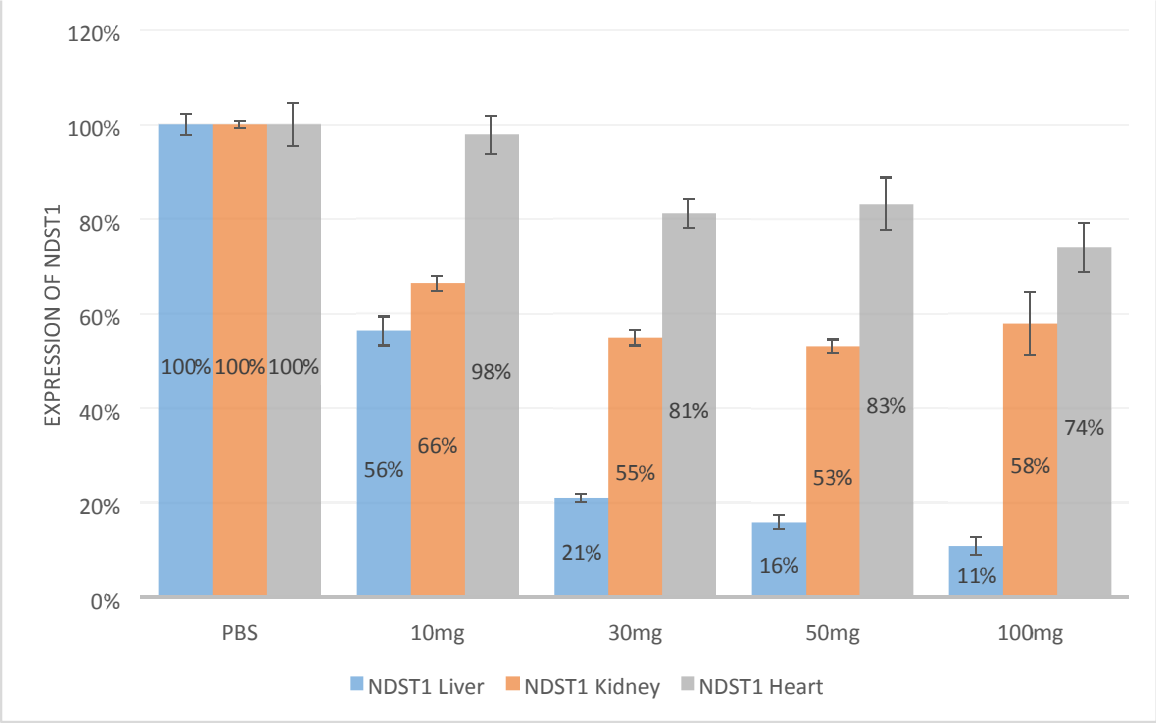

Figure 4. Decreased expression of NDST1 after injections of NDST1 ASO. Levels of NDST1 were measured by PCR in the liver, kidney, or heart after treatment with 3 doses of NDST1 ASO. As dosage of ASO increased the percent expression of NDST1 compared to PBS treated mice decreased. The lease effective organ was the heart and displayed the smallest decrease in expression only getting to 25% decrease with the highest dose. Kidney performed slightly better in its capability to reduce the expression of NDST1 resulting in a 50% decrease after 30mg dosage. Liver displayed the largest change in expression reaching a staggering 80% decrease at the 30mg dose range. (Data supplied by IONIS)

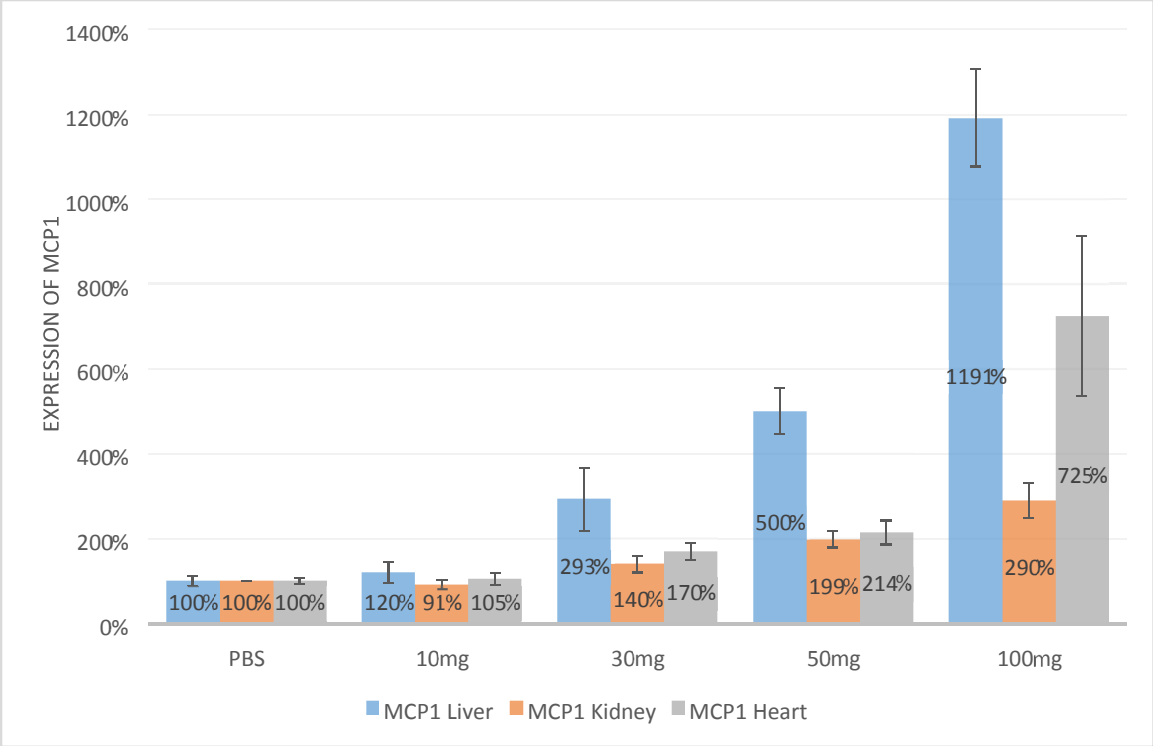

Figure 5. Levels of MCP1 remain low at lower concentrations of NDST1 ASO. Levels of MCP1 were measured by PCR in the liver, kidney, or heart after treatment with 3 doses of NDST1 ASO. The expression in the liver and heart increased significantly in doses higher than 10mg. The kidney displayed significantly lower values compared to the liver and heart. After 30mg doses levels of MCP1 expression while higher than PBS remained lower and close to the values of the control group. (Data supplied by IONIS)

Despite some increases in toxicity at the highest dosage of 100mg of NDST1 ASO, these ASOs remained at an extremely low level of toxicity. The purpose of these tests was to determine the best dose to administer to the mice that will give the largest effect without compromising the heath of the mouse. Figure 3 showed that any dose under 100mg will have little if not no toxic side effects on the mice. As the kidney is the targeted organ in this study, any dose larger than 10mg will give approximately a 50% decrease in expression of NDST1. This narrows the dose range down to 10-50mg. Lastly, levels of MCP1 needs to remain low in order to give the kidney its best chance of survival and not induce further inflammation from the drug. Figure 5 displayed in kidneys relatively normal ranges in 10-30mg doses. These experiments suggest the best possible dosage for the most NDST1

reduction with the least side effects would be between 10-30mg. In this study 25mg dose was selected.

These experiments display the versatile use of ASO to treat various diseases and hints towards the possibility to use ASOs to reduce transplant rejection. In Figure 5, the levels of NDST1 expression never decrease to zero and remain at only a 50% decease. It has been postulated that the inflammatory response is necessary for the transplant survival. Having a partial deletion of the NDST1 could allow for the helpful proinflammatory immune cells to come to the transplant while the mass of immune cells are minimized preventing the destruction of the new organ.

Pretreatment of grafts

Traditionally, the approach to reduce acute or recurrent acute transplant rejection episodes is to flood the organ recipient with immunosuppressants and steroids. Many transplanted patients are reliant on immunosuppressants for the rest of the functioning organ's life which typically lasts about 10 years before a repeat transplant is necessitated. This also causes them to be highly susceptible to infections and have an increased risk of cancer. Immunosuppressants are also known to cause bone loss and diabetes (Yaron et al., 2019). Much of the damage explained above has already occurred. Most therapies for transplants are post-operative, very few tackle treatments of the graft donor prior to transplantation. Treating the donor organ shows a promising method of reducing the initial damage which often leads to chronic rejection and thus graft loss (Chen et al., 2018; Yaron et al., 2019). Current methods to modify the organ prior to transplantation are through human leukocyte antigen (HLA) matching and perfusion however only about 30% of them can find a match (Yaron et al., 2019). To provide more organs for transplantation, the transplant criteria have been expanded to allow the use of organs from older donors, donors with poor HLA matching, and from patients after cardiac arrest and brain death, but transplantation of these organs has proved to be less successful owing to ischemic damage and marked systemic

inflammation. Other researchers are exploring other methods of modifying the donor organ prior to transplantation.

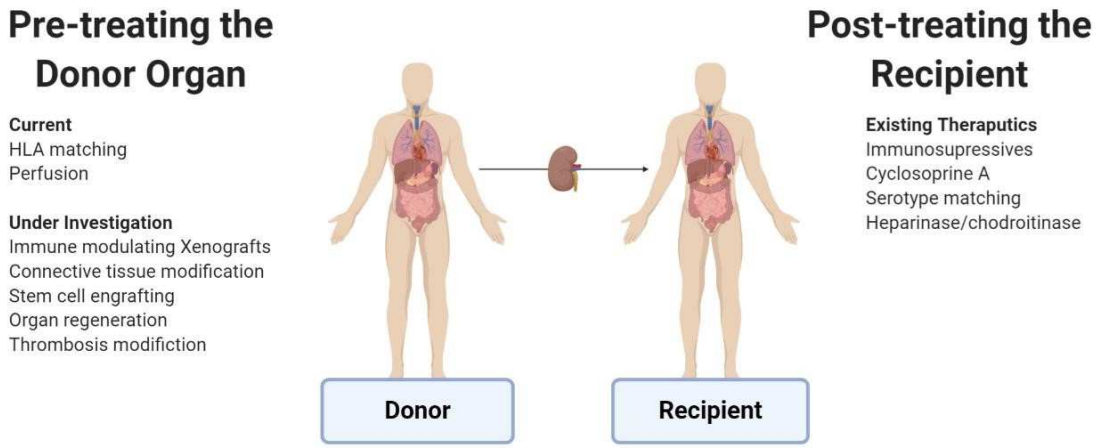

Figure 6. Modifying the donor organ compared to the traditional treatment of the recipient organ. Organ donation is a critical need that is ever increasing each day. Typically, the traditional way to treat a transplant I by flooding the recipient with immune suppressants that they will have to remain on for the rest of the organ's life. Pretreatment of grafts provides a promising method to give the organ its best survival. HLA matching a perfusion are already options however only work a small fraction of the time. Some methods under investigation include immune modulating proteins, xenografts, organ regeneration, and stem cell engrafting (Yaron et al., 2019). Figure generated in Biorender.

Recently, cardiac c-kit+ stem cells were found to be minimally regenerative in adult tissues, contrary to previous reports (Chugh et al., 2012). This revelation poses a challenge to clinical progress and at least one major trial paper (NCT00474461) has been retracted due to manipulation of data relevant data (Bolli et al., 2011; Chugh et al., 2012). In another example, patients treated with autologous stem cells for macular degeneration experienced near-complete vision loss (Y. Wang, Tang, & Gu, 2020). CRISPR methods have facilitated the production of genetically engineered pigs, including the genome-inactivation of porcine endogenous retroviruses (PERVs)(Figure 6). Other researchers are investigating growing organs in other animals and transplanting them into humans however this method is an extremely time-consuming process. Therapeutics under investigation include immune modulation, genetic engineering, xenografts, connective tissue, stem cell, and organ regeneration. All these methods provide promising new ways to help alleviate the issue of a lack of acceptable donor organs.

CHAPTER 3

SUBCUTANEOUS TRANSPLANTATION

Background

Testing new therapeutics on kidney transplants can be a difficult procedure to perform on mice due to their small size. An alternative method for initial therapeutic target screening can be performed using subcutaneous transplants. This is a simpler method than performing a full orthotopic transplant. Orthotopic transplants while requiring an immense amount of skill and often a well-trained microsurgeon, also costs significantly more due to the need for extensive training, the prolonged surgical time, and the need for larger numbers of mice specifically one donor for two recipients, the higher surgical loss and the increased need in surgical supplies. A reoccurring problem is the need to test therapeutics in a rapid manner. Subcutaneous transplantation of allografts could provide a simple model for preliminary testing of treatments for early inflammation and ischemia. During a subcutaneous transplantation, a small piece of tissue is inserted between the shoulders of the mice in the subcutaneous subscapular pocket. This is a simple surgery that can be performed by the most beginner researchers.

Much research has already been done using this method. Most commonly done is the transplantation of hepatocytes underneath the skin. One group was able to transplant sheets of fabricated functional liver in the subcutaneous space to test treatment for hemophilia (Tatsumi & Okano, 2017). Subcutaneous transplantation was also tested with trachea, pancreatic islets, temporal arteries, and stem cells (Chan et al., 2013; El-Halawani et al., 2020; Hertz, Jessurun, King, Savik, & Murray, 1993; Obokata, Yamato, Tsuneda, & Okano, 2011). The skin has a vast array of immune cells that are normally reside there making it a good spot to transplant tissue and receive an immune response (Richmond & Harris, 2014). This placement however lacks blood supply and the circulating immune cells naturally present in the blood and kidney.

Materials and Methods

Materials

Mice

All surgical procedures where performed after Institutional animal care and used committee (IACUC) approval and conformed to appropriate national, international and university guidelines for animal care. Mice used included C57BL6/J, BALB/c, and NDST$^{-/-}$ at 8-12 weeks of age. Their approximate weights were 20-25g. Equal numbers of male and female mice were used in these studies. Numbers for the study where determined using power calculations. Table 1 shows numbers of mice used for each treatment.

	Transplant Type	Numer of Days Follow up	Treatment	Number of Mice
Subcutaneous Soaked	BALB/c - BALB/c	15 Days	Saline	5
	NDST KO - BALB/c		Saline	6
	C57BL/6 - BALB/c		Saline	6
	C57BL/6 - BALB/c		MT7	3
	C57BL/6 - BALB/c		Scramble ASO	6
	C57BL/6 - BALB/c		NDST ASO	6
	C57BL/6 - BALB/c		CD40 ASO	6
	BALB/c - BALB/c	3 Days	Saline	6
	NDST KO - BALB/c		Saline	6
	C57BL/6 - BALB/c		Saline	2
	C57BL/6 - BALB/c		MT7	4
	C57BL/6 - BALB/c		Scramble ASO	4
	C57BL/6 - BALB/c		NDST ASO	8

Table 1. Numbers of subcutaneous transplants

Surgery

Insulin Syringe (1 cc U, 28G 1/2), Anesthetic (Ketamine (100 mg/mL, 120 mg/kg)/xylazine (20 mg/mL, 6 mg/kg) mixture), Electric heating pads- 36 to 38˚C, Electric hair clippers, Sterile cotton swabs and pads, Chlorhexidine Gluconate 2% solution, Sterile sodium chloride, 0.9% (w/v) isotonic saline, 70% ethanol, Puralube vet ointment, Sterile drape with 15mm centrally located fenestration, Sterile iris forceps, serrated and 1 x 2 teeth, straight and curved, Sterile dissecting scissors , Surgical blades, size 10, Scalpel handle, #3, Sterile Dumont forceps, Olsen-Heger needle holder with suture scissors, 4-0 sterile absorbable suture with a 13mm 1/2 c tapered needle, Reflex clip applier, Buprenorphine 0.03 mg/ml, 0.05-1.0 mg/kg

Histology

Formalin solution, neutral buffered, 10%, Cassettes, Embedding station (LEICA EG 1160), Microtome (LEICA RM 2165), Glass histology slides and coverslips (HARETA AHS90WH), Paraffin, Oven, Mixture of Xylenes (also called Xylene), Ethanol (70%, 95%, 100%) diluted in DI water, Gill No. 3 hematoxylin (6 g/L), Eosin Y (5% in aqueous solution), 1% Acid Alcohol (1ml Hydrochloric acid and 100ml 70% ethanol), 0.2% Ammonia water (2ml ammonium hydroxide and 1000ml distilled water), Microscope and imaging system, Slide tray, Tris-Buffered Saline, 0.1% Tween (TBST), Protein block (5% w/v Bovine Serum Albumin diluted in TBST), DAB kit (VECTOR SK-4105), Immunohistochemical stains, Primary antibody: Anti-mouse C4d Cat: HP8033, Ra pAb to F4/80 ab100790, Rb pAb to CD3m ab 5690, Secondary antibody: Goat antiRat IgG2a Hrp conjugated Cat: A110-109P, Microscope and imaging system (Olympus BX51 microscope with 4x-100x objectives, a Prior ProScan II stage and Olympus DP74 CMOS camera and cellSens software analysis system)

Methods

Preparation for surgery

Balb/C mice were anesthetized by an IP injection of 100µl of Ketamine/xylazine mixture per 25g mouse and placed on a heating pad to be kept warmed. The levels of anesthesia were checked by firmly pinching the toes of the mice looking for flinching. If movement occurred, boosts were given of 20µl of Ketamine/xylazine. Clippers were used to remove the hair over the surgical site and cleaned both 70% ethanol and chlorhexidine gluconate twice by swabbing in an outward motion. Puralube vet ointment was added to the eyes to prevent drying.

Subcutaneous surgery

Both kidneys were removed from donor mice placed in saline and then cut in half. The fat is removed and placed in a 15ml tube with oxygenated DMEM with treatment or

control solution for 1 hour prior to transplantation. M-T7 concentration was 1µg/ml M-T7 (5µl to 5ml of oxygenated DMEM). ASO concentration was kept at 25 mg/g of scramble or NDST1 ASO. Kidneys were cut in half for the subcutaneous surgeries and injected using an insulin syringe with the control or treatment solution to ensure profusion throughout the tissue. Time started when the tube of kidneys was removed from the ice. The tube was laid on its side and gently agitated occasionally.

A 1x1 inch section was shaved and prepared on the back of the mouse between the shoulders. Using forceps and scissors a small incision approximately 0.25 inches in length was made in the skin of the mouse without cutting the abdominal wall. A pocket was made using blunt forceps underneath the skin to make a pocket for the organ to sit in. Half the donor kidney that had been soaked an hour prior to transplantation was carefully inserted into the subscapular pocket using forceps. To close the skin 2-3 sutures were made and mice were checked daily for signs of discomfort such as hunching, piloerection and weight loss.

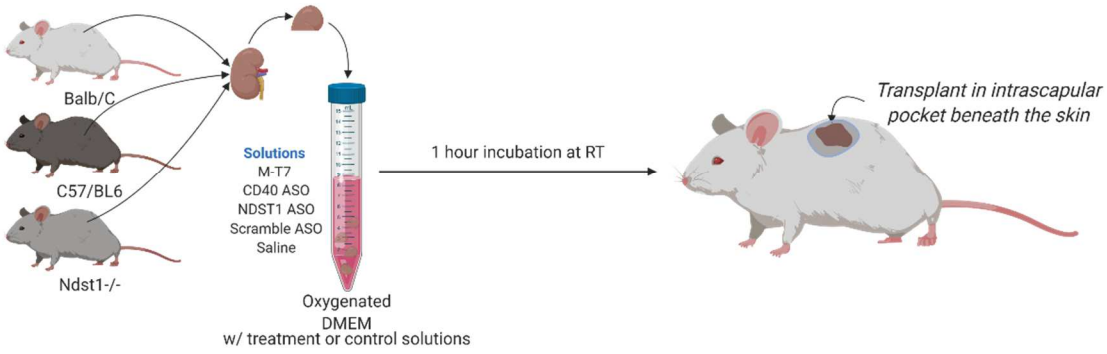

Figure 7. Subcutaneous surgery overview. Donor kidneys were removed from either Balb/C, C57BL6, or NDST$^{-/-}$ mice. Kidneys were cut in half and place in oxygenated DMEM with one of the treatments or control solutions. Kidneys were injected with the solution to ensure complete perfusion in the kidney. After one hour the kidney halves were inserted into a subscapular subcutaneous pocket and left for 15 or 3 days post operation.

Collection of tissues

After 3- or 15-days post-transplant, mice were euthanized using CO2 gas followed by a cervical dislocation. The abdomen of the mouse was sprayed with 70% ethanol before cutting open the abdominal cavity. A vertical incision was made parallel to the spine along the belly of the mouse. The abdominal wall and skin were pinned to the side. Blood was collected up to 400µl by puncturing the heart with a syringe. The blood was spun down, and plasma was collected and frozen at -80 ˚C until needed. First the transplanted tissue was removed using blunt forceps and scissors. The ureter and renal vein/artery was cut and the kidney was placed in formalin fixed overnight. The spleen was collected next and placed in a 15 ml tube with formalin. Spleens collected first due it rapidly changing following death. Then the rest of the organs were collected and placed in the same tube.

Histology

After the tissues were fixed in formalin overnight the organs were embedded in paraffin wax and placed into cassette blocks prepped for sectioning. 5 micron sections were made and placed on charged slides. Slides were stained for Hematoxylin and Eosin for the timing listed in Table 2. Immunohistochemistry was performed on the slides. Markers tested included CD3 T cells, F4/80 Macrophages, and Ki67 proliferation marker.

Step	Time
1. 60 C oven	30 minutes
2. Xylene	5 min
3. Xylene	5 min
4. Absolute alcohol	2 min
5. Absolute alcohol	2 min
6. 95% alcohol	2 min
7. 70% alcohol	2 min
8. Water wash	2 min
9. Hematoxylin	5 min
10. Water wash series	
a. Dip in first water(up and down)	
b. Second water	1min
c. Third water	5min
11. Acid alcohol	12 dips
12. Water wash	2 min
13. Amm H2O (.05%)	30 sec
14. Water wash	2 min
15. Eosin	5 min
16. 95% alcohol	2 min
17. 95% alcohol	2 min
18. Absolute alcohol	2 min
19. Absolute alcohol	2 min
20. Xylene	2 min
21. Xylene	2 min
22. Dry and mount	

Table 2.

Using microscope and counting cells

Interstitial inflammation, scarring and tubulitis was observed on stained sections using a brightfield microscope. The equipment used was an Olympus BX51 microscope with 4-100×

objectives, a Prior ProScan II stage and Olympus DP74 CMOS camera and cellSens software analysis system. Comparisons to the Banff criteria was made to score level of rejection [6].

Statistical analysis

Statistical Analysis Data were analyzed in GraphPad Prism version 8.43 (GraphPad, USA) or in StatView version 5.0.1 (SAS Institute Inc.) using one-way ANOVA (Analysis of vaiance) with a Tukey's post-hoc, Fischer's LSD (Least significant difference) comparison, or a Student's unpaired T test. Results were considered significant if $p < 0.05$.

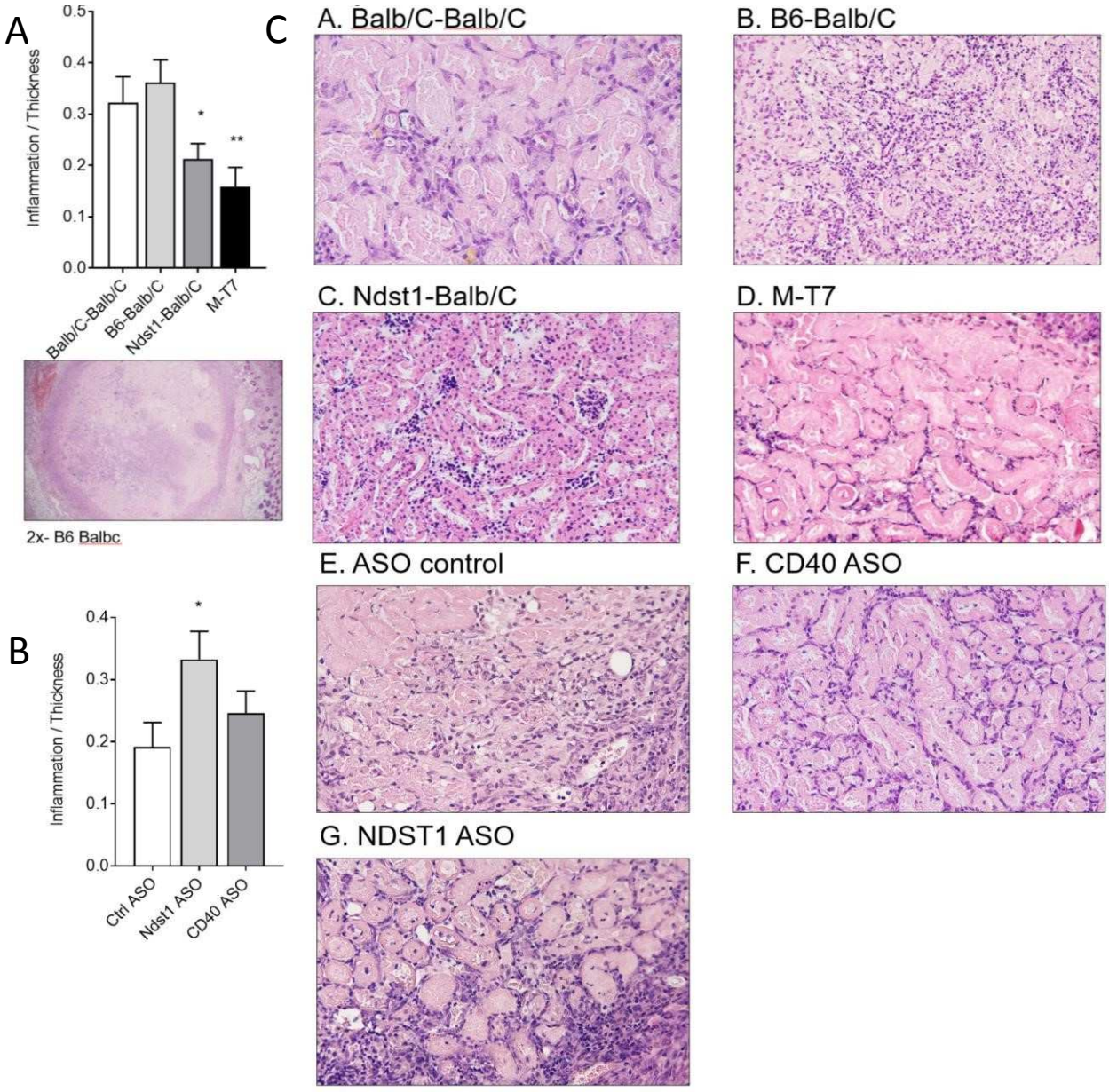

Figure 8. Infiltrating Immune cells. Histopathological analysis on hematoxylin and eosin staining on subcutaneous kidneys after 15 days post operation. Mice were pretreated by soaking the donor organ with either a control solution (Saline), M-T7, tissue from an NDST1$^{-/-}$mouse, NDST1 ASO, scramble ASO as a control. Inflammatory infiltration was compared to the total thickness of the transplant. A) Bar graph demonstrates that M-T7 and NDST1$^{-/-}$ mice both displayed a significant decrease in inflammation. B) NDST1 ASO displayed an increase in inflammatory infiltrate. C) Histological analysis showed increased inflammation in C57BL6Balb/C, scramble ASO and Balb/C-Balb/C transplanted sections.

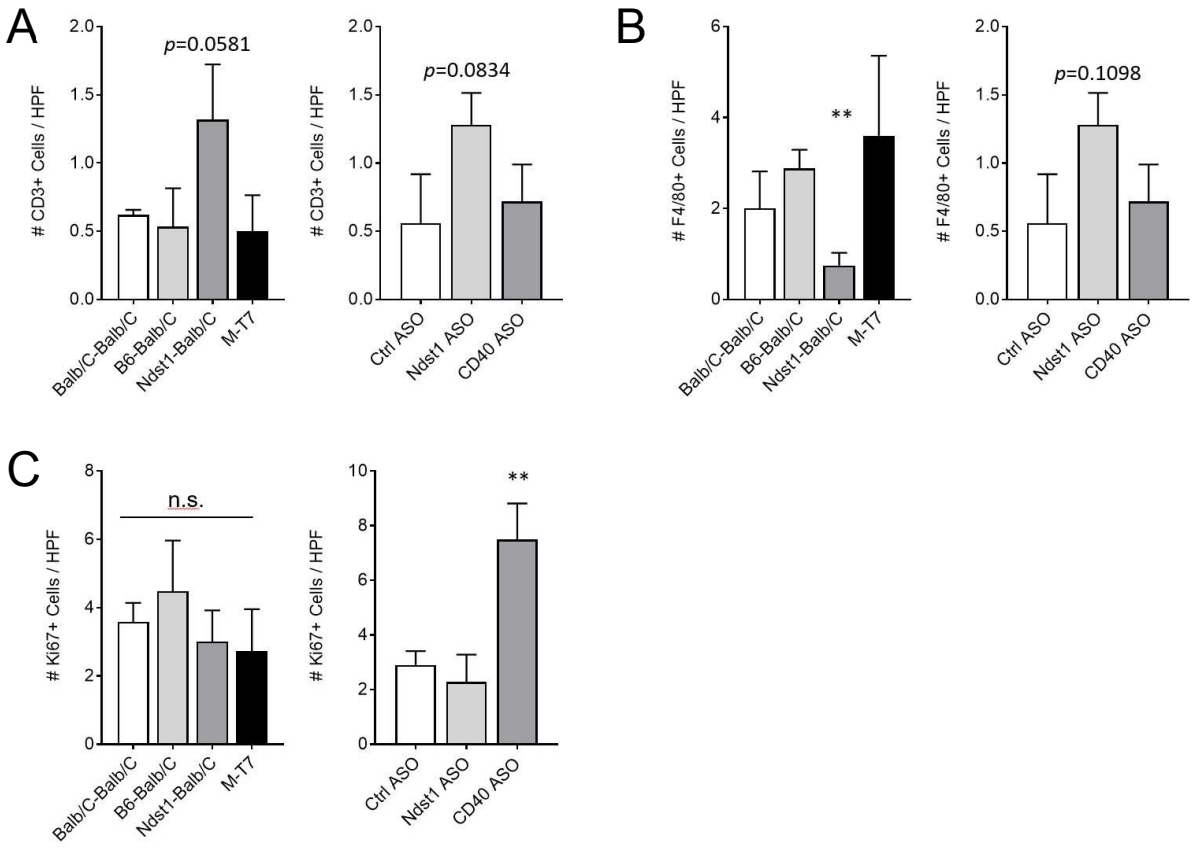

Figure 9. Immunohistochemical analysis of immune cells present after subcutaneous surgery. Immunohistochemistry staining for T cells, macrophages, and proliferation. We examined acute renal rejection in grafts with conditional endothelial N-deacetylase-Nsulfotransferase-1 knockout (Ndst1$^{-/-}$; N=6; C57Bl6/J background) with comparison to wildtype (WT) C57Bl6/J grafts treated with saline (N=6), M-T7 (N=3) treatment, and treatment with antisense oligonucleotides (ASO) including a scramble targeting Ndst1 (N=6), CD40 (N=6), or a scrambled ASO control (N=5). Ndst1$^{-/-}$ had reduced numbers of F4/80 macrophages (p<0.05). Ndst1 ASO trended towards reduced F4/80 macrophage infiltration (p=0.1098). Ndst1 ASO reduced positive staining for a proliferation marker, Ki-67 (p=0.0473), while CD40 ASO increased the number of Ki-67 positive cells (p=0.0132). Both Ndst1$^{-/-}$ and Ndst1 ASO trended towards significance in reducing positive CD3+ T cell infiltration (p=0.0581 and p=0.0834, respectively).

NDST1 and M-T7 significantly reduced infiltrating immune cells. Histopathological inflammation was compared in grafts treated with M-T7, NDST1 ASO, or tissue from nice containing a conditional knockout of NDST1. C57BL6 mice were used as donors to simulate a mismatched transplant going into a Balb/C mouse. Balb/C tissues were also used as a control donor for a transplant that would have been a match for the recipient. Firstly, inflammation and kidney structures were analyzed. Ndst1$^{-/-}$ and M-T7 both significantly

(p<0.05) reduced inflammation when compared to transplanted C57BL/6 wildtype grafts (Figure 8, A). NDST1 ASO displayed a significant increase in amount of immune cell infiltration compared to the scramble ASO (Figure 8, B). Subcutaneous kidneys displayed obliterated tubules and very minimal intact glomeruli (Figure 8, C).

Macrophage and T cell infiltration is associated with acute renal rejection and ischemia. To determine which types of immune cells were present immunohistochemistry was performed for macrophages, T cells, as well as a proliferation marker. F4/80 was used as a marker for general macrophages, CD3 which is present on all T cells was used as a marker to identify T cells, and Ki67 was used as a marker for proliferation as it is a protein that cells increase before they divide into new cells. Ndst1$^{-/-}$ had reduced numbers of F4/80 macrophages (p<0.05). Ndst1 ASO trended towards reduced F4/80 macrophage infiltration (p=0.1098) (Figure 9, B). Both Ndst1$^{-/-}$ and Ndst1 ASO trended towards significance in increasing positive CD3+ T cell infiltration (p=0.0581 and p=0.0834, respectively) (Figure 9, A). Ndst1 ASO reduced positive staining for a proliferation marker, Ki-67 (p=0.0473) (Figure 9, C). These findings indicate that NDST1 expression and chemokines are influential on the number of immune cells present during acute kidney injury.

Discussion

Acute cellular rejection and ischemia are leading causes of graft loss and chronic rejection. The initial invading wave of inflammation does significant damage to the organ (Chen et al., 2018). If this could be prevented or minimized the graft would likely have a better change for long term survival. The result of this study showed significant decreases in inflammation both the NDST conditional knockout and the M-T7 treated transplants. These organs also displayed slightly more preserved glomerular and tubular structure. The application of utilizing subcutaneous transplantations as an initial screening tool for testing therapeutics on kidney transplants was tested in this study. The results displayed variable levels of inflammation and inflammatory cells.

While subcutaneous transplantation provides a quick method the immune microenvironment is vastly different than that of the kidney (Hori, Joyce, & Streilein, 2000; Ng, Osawa, Hori, Young, & Streilein, 2019). This method also displays many signs of irreversible damage to the organ.

Subcutaneous transplantation of allografts is a simple model for preliminary testing of treatments for early inflammation, ischemia, and graft rejection. Conditional endothelial Ndst1 deficiency and inhibition of chemokine-GAG interaction reduces early allograft damage and suggest new therapeutic options for graft rejection.

After analyzing the tissues from the subcutaneous transplant, it was concluded that this method of testing new treatments for transplant rejection may not the be best approach out there. It lacked a proper blood supply as well as having a completely different immune environment that that organ would normally not be in. Most of the transplants were analyzed for amounts of inflammation in an ischemic tissue. Next, we turned towards an alternative method: subcapsular transplantation. This is where one transplants a small piece of tissue and places it underneath the capsule of the kidney. This provided a unique approach that allowed for a controlled environment for the transplanted tissue to reside in. Underneath the capsule provides an immune privileged environment as well as access to the circulation blood system.

CHAPTER 4

SUBCAPSULAR TRANSPLANTATION

Background

Heterotopic transplantation provides a simpler method for testing transplant rejection. While subcutaneous transplantation showed promising results as an initial screening tool to analyze early immune responses and ischemia, the method still lacks access to the tissue specific microenvironment naturally present in the kidney. Another well researched method utilized a subcapsular transplantation. A small clump of cells is inserted underneath the capsule that surround the kidney. The capsule provides a protected area for the transplant and keeps it contained in a highly vascularized site. Here the allograft has access to the circulating immune cells and the immune environment naturally present in the kidney. This surgical procedure while more difficult than a subcutaneous transplant is still significantly easier to perform than a full orthotopic transplant. One donor mouse can be used for many recipient mice since the transplant size is small. The easily accessible site is well contained and held in place by pressure from the capsule.

The idea of using subcapsular model to transplant foreign tissue has been utilized many times in the past. For example researchers have implanted not only kidney cells but also pancreas, liver, and cornea cells (Miyabe et al., 2019; Ng et al., 2019; Shultz et al., 2019). Because of the unique and well contained environment under the capsule, it provides an ideal area for tissues to be inserted. While whole kidney organ pieces have not been tested before, researchers have been able transplant kidney organoids that were capable of fully functioning and even formed nephron, glomerular, and tubular structures (Berg et al., 2018). This suggested that vascularization and some immune response is required to maintain a healthy organ. Subcapsular transplantation was chosen as a more viable option to testing therapeutic targets than the previously described subcutaneous method.

Materials and Methods

Materials

Mice

All surgical procedures where performed after Institutional animal care and used committee (IACUC) approval and conformed to appropriate national, international and university guidelines for animal care. Mice used included C57BL6/J, BALB/c, and NDST$^{-/-}$ at 8-12 weeks of age. Their approximate weights were 20-25g. Equal numbers of male and female mice were used in these studies. Numbers for the study where determined using power. Table 3 shows numbers of mice used for each treatment.

Subcapsular Soaked	C57BL/6 - BALB/c	15 Days	Saline	10
	NDST KO - BALB/c		Saline	4
	C57BL/6 - BALB/c		MT7	5
	C57BL/6 - BALB/c		Scramble ASO	4
	C57BL/6 - BALB/c		NDST ASO	7
	C57BL/6 - BALB/c	3 Days	Saline	4
	NDST KO - BALB/c		Saline	4
	C57BL/6 - BALB/c		MT7	4
	C57BL/6 - BALB/c		Scramble ASO	0
	C57BL/6 - BALB/c		NDST ASO	4
Subcapsular Pretreated (7 Days Prior to Transplantation)	C57BL/6 - BALB/c	15 Days	Saline	6
	C57BL/6 - BALB/c		MT7	6
	C57BL/6 - BALB/c		Scramble ASO	6
	C57BL/6 - BALB/c		NDST ASO	6
	C57BL/6 - BALB/c	3 Days	Saline	6
	C57BL/6 - BALB/c		MT7	6
	C57BL/6 - BALB/c		Scramble ASO	6
	C57BL/6 - BALB/c		NDST ASO	6

Table 3. Numbers of subcapsular transplants.

Surgery

Insulin Syringe (1 cc U, 28G 1/2), Anesthetic (Ketamine (100 mg/mL, 120 mg/kg)/xylazine (20 mg/mL, 6 mg/kg) mixture), Electric heating pads- 36 to 38˚C, Electric hair clippers, Sterile cotton swabs and pads, Chlorhexidine Gluconate 2% solution, Sterile sodium chloride, 0.9% (w/v) isotonic saline, 70% ethanol, Puralube vet ointment, Sterile drape with 15mm centrally located fenestration, Sterile iris forceps, serrated and 1 x 2 teeth, straight and curved, Sterile dissecting scissors , Surgical blades, size 10, Scalpel handle, #3, Sterile spring-loaded Vannas scissors, Sterile Dumont forceps, Oral gavage needle, 22 gauge, Stereoscope, Olsen-Heger needle holder with suture scissors, 4-0 sterile absorbable suture

with a 13mm 1/2 c tapered needle, Reflex clip applier, Mosquito hemostatic forceps, Wound reflex clips, Buprenorphine 0.03 mg/ml, 0.05-1.0 mg/kg

Histology

Formalin solution, neutral buffered, 10%, Cassettes, Embedding station (LEICA EG 1160), Microtome (LEICA RM 2165), Glass histology slides and coverslips (HARETA AHS90WH), Paraffin, Oven, Mixture of Xylenes (also called Xylene), Ethanol (70%, 95%, 100%) diluted in DI water, Gill No. 3 hematoxylin (6 g/L), Eosin Y (5% in aqueous solution), 1% Acid Alcohol (1ml Hydrochloric acid and 100ml 70% ethanol), 0.2% Ammonia water (2ml ammonium hydroxide and 1000ml distilled water), Microscope and imaging system, Slide tray, Tris-Buffered Saline, 0.1% Tween (TBST), Protein block (5% w/v Bovine Serum Albumin diluted in TBST), DAB kit (VECTOR SK-4105), Immunohistochemical stains, Primary antibody: Anti-mouse C4d Cat: HP8033, Ra pAb to F4/80 ab100790, Rb pAb to CD3m ab 5690, Secondary antibody: Goat antiRat IgG2a Hrp conjugated Cat: A110-109P, Microscope and imaging system (Olympus BX51 microscope with 4x-100x objectives, a Prior ProScan II stage and Olympus DP74 CMOS camera and cellSens software analysis system)

Methods

Pretreatment of mice

C57BL6 mice were given daily 200µl intraperitoneal (IP) injections of saline, 25mg/g ASO, or 1ng/g M-T7 dissolved in saline for 7 days. On day of surgery mice were euthanized via CO2 asphyxiation at a rate of 1.8-2 L/min followed with a cervical dislocation. The kidneys were then placed in petri dish with the DMEM and cut using a scalpel into 1-1.5 mm pieces for the subcapsular surgeries.

Preparation for surgery

Balb/C mice were anesthetized by an IP injection of 100µl of Ketamine/xylazine mixture per 25g mouse and placed on a heating pad to be kept warmed. The levels of

anesthesia were checked by firmly pinching the toes of the mice looking for flinching. If movement occurred, boosts were given of 20µl of Ketamine/xylazine. Clippers were used to remove the hair over the surgical site and cleaned both 70% ethanol and chlorhexidine gluconate twice by swabbing in an outward motion. Puralube vet ointment was added to the eyes to prevent drying.

Subcapsular surgery

The recipient mouse was placed in left lateral recumbent position with a sterile drape covering the mouse fenestration (hole) over the surgical site. Forceps and scissors were used to make a vertical incision approximately 0.7 – 1.0 cm in length midway between the bottom of the rib cage and the iliac crest. A similar incision was made in the abdominal wall over the area of the kidney found by palpating the craniolateral aspect of the abdomen. Pressure was applied to the dorsal and ventral sides of the abdominal cavity and curved forceps were used to exteriorize the kidney. Applying direct pressure to the kidney causes bruising or capsular rupture which results in additional damage and inflammation. Sterile saline was periodically added to the kidney to prevent drying. Under the stethoscope, spring-loaded Vannas scissors were used to make a small incision 2mm in length on the capsule of the kidney. Dumont forceps were used to hold the edge of the capsule and gently inserted the bulb of the oral gavage needle between the capsule and the renal parenchyma to make a subcapsular pocket for the transplanted tissue. The donor tissue was inserted into the pocket using the oral gavage needle and Dumont forceps. Pressure from the capsule will ensure the transplant is kept in place after the surgery. The capsule incision did not need to be closed if the capsule is not torn because the tissue was held in place by the capsule pressure. To return the kidney to the abdomen, gentle pressure was applied to the kidney while the edges of the incision were held open with forceps. Mice were ear tagged and the location of the graft was noted after each mouse to ease the process of finding the transplant later when sectioning. The abdominal wall was closed with 2-3 sutures using 4-0 sterile absorbable suture with a 13mm 1/2 c tapered needle. Then to close the skin, sterile wound clips were added. Approximately, 2 clips were used for each 20g mouse. To minimize pain,

mice were given an injection of Buprenorphine about 0.1 mg per/kg mouse after the mouse had righting capability. Additional injections of subcutaneous sterile saline (200-500µl) were provided postsurgically to aid in recovery. Mice were kept on a heating pad until fully awake and then placed in a clean cage. Cages were checked daily for signs of pain and discomfort (hunching, piloerection, weight loss, ect.). If signs of excess pain are seen, another dose of buprenorphine was administered, or the mouse was euthanized. Mice were monitored by the veterinary staff as well as the experimental group.

Collection of tissues

After 3 or 15 days post-transplant, mice were euthanized using CO2 gas followed by a cervical dislocation. The abdomen of the mouse was sprayed with 70% ethanol before cutting open the abdominal cavity. A vertical incision was made parallel to the spine along the belly of the mouse. The abdominal wall and skin were pinned to the side. Blood was collected up to 400µl by puncturing the heart with a heparin coated syringe. The blood was spun down, and plasma was collected and frozen at -80 ˚C until needed. First the transplanted tissue was removed using blunt forceps and scissors. The ureter and renal vein/artery was cut and the kidney was placed in formalin fixed overnight. The spleen was collected next and placed in a 15 ml tube with formalin. Spleens collected first due it rapidly changing following death. Then the rest of the organs were collected and placed in the same tube.

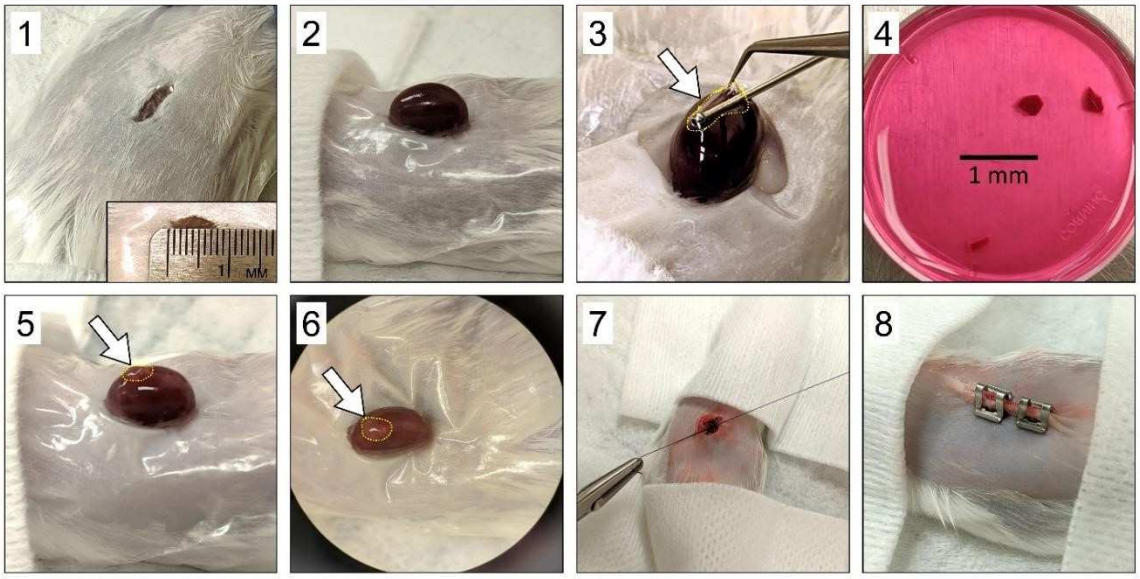

Figure 10. General overview of surgical procedure. 1) Creation of the vertical incision in skin and abdominal wall. 2) Exteriorized kidney. 3) Creation of pocket underneath the capsule for transplanted tissue. 4) Example size of donor tissue to be inserted underneath capsule. 5) Exteriorized kidney with donor tissue inserted beneath the capsule. 6) Kidney with donor tissue viewed under the stereoscope. 7) Closure of abdominal wall with sutures. 8) Closure of skin with wound clips.

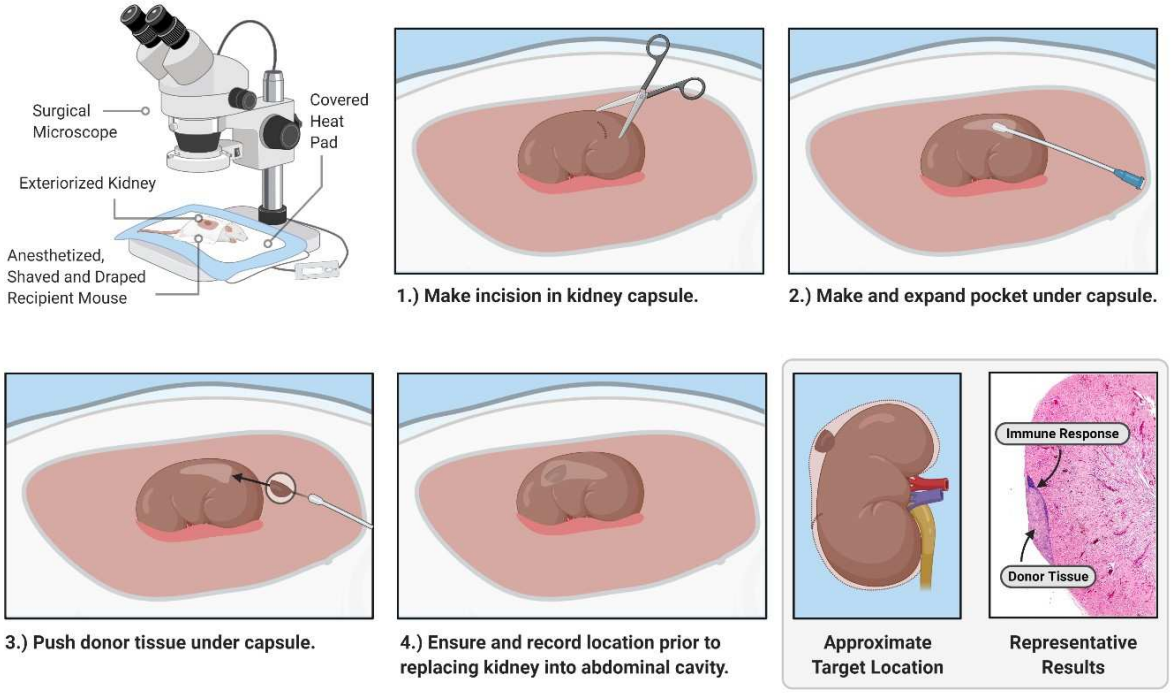

Figure 11. Illustration of the procedure to insert donor tissue underneath the capsule of the recipient mouse and is performed underneath the stereoscope. After the kidney

is exteriorized the mouse is placed underneath the scope for better lighting and closer view of the work being done. Images in the grayed area demonstrate approximate location of transplanted tissue with representative hematoxylin and eosin (H&E) stained section of a subcapsular kidney transplant. Immune cells are stained dark purple and a boundary is clearly seen surrounding the transplanted tissue.

Histology

After the tissues were fixed in formalin overnight the organs were embedded in paraffin wax and placed into cassette blocks prepped for sectioning. 5 micron sections were made and placed on charged slides. Slides were stained for Hematoxylin and Eosin for the timing listed in Table 2. Immunohistochemistry was performed on the slides. Markers tested included CD3 T cells, F4/80 Macrophages, and C4d. Stained sections are measured by counting in 10 random fields of staining the number of positive cells in a high-powered field (40X or 20X) or by measuring the area of positive staining and inflammation compared to the total area of transplant. Interstitial inflammation, scarring, and tubulitis was observed on stained sections using a bright-field microscope. We use an Olympus BX51 microscope with 4–100X objectives, a Prior ProScan II stage, Olympus DP74 CMOS camera, and cellSens software analysis system. Comparisons to the Banff criteria was used to score the level of rejection [6].

Using microscope and counting cells

Interstitial inflammation, scarring and tubulitis was observed on stained sections using a brightfield microscope. The equipment used was an Olympus BX51 microscope with 4-100× objectives, a Prior ProScan II stage and Olympus DP74 CMOS camera and cellSens software analysis system. Comparisons to the Banff criteria was made to score level of rejection (Roufosse et al., 2018)**.**

BUN and creatinine tests

Kits were used from Pointe scientific. Plasma from mice were collected and frozen at -80C till needed. BUN test wavelength was kept at 340nm while the creatinine test was measured at 510nm. Using a plate reader and a 96 well plate, plasma was added to the reagents and measured at specific time points. In the BUN tests, 2 µl of sample was added to 200µl of

reagent. This amount was optimized by testing various concentrations of the standard. Measurements were taken in 30 second intervals for a minute and a half. Creatinine tests used 20 µl of sample and 200 µl of reagent. Reads were taken at 60 second intervals for 3 minutes. Calculations were made using the formulas listed in the kits.

Statistical analysis

Statistical Analysis Data were analyzed in GraphPad Prism version 8.43 (GraphPad, USA) or in StatView version 5.0.1 (SAS Institute Inc.) using one-way ANOVA with a Tukey's posthoc, Fischer's LSD comparison, or a Students unpaired T test. Results were considered significant if $p < 0.05$.

Results

Initial screening displayed promising results. Smaller numbers of mice were first analyzed for inflammatory infiltrate. These measurements were done with only 1-3 mice per group however reached significant values of reduced inflammation. Both $NDST^{-/-}$ and NDST1 ASO display significant decrease in inflammation and improved histological structure (Figure 12, A, B). The bar graph displays initial measurements of H&E sections from the 15 day soaked allografts showed a significant reduction of inflammation infiltration in the $NDST1^{-/-}$ and NDST1 ASO when compared with saline or the scramble ASO control (Figure 12, C).

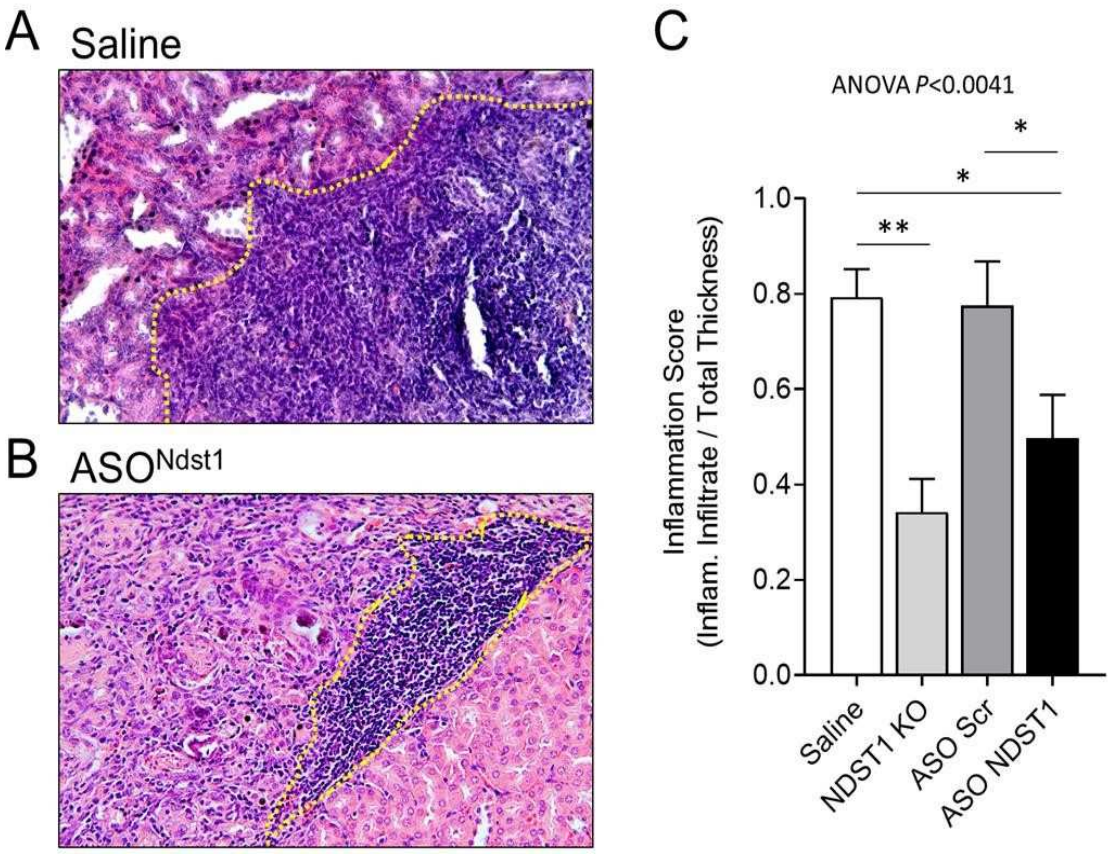

Figure 12. Reduction of inflammatory cells in soaked transplants (Preliminary data). Preliminary data was taken on 2-3 mice per group. Levels of inflammatory cells infiltration was measured and compared to the size of the whole transplant. Both NDST knockout and NDST ASO displayed significant decreases in general inflammation. A,B) Histological images display difference in purple immune cells compared to the transplant.

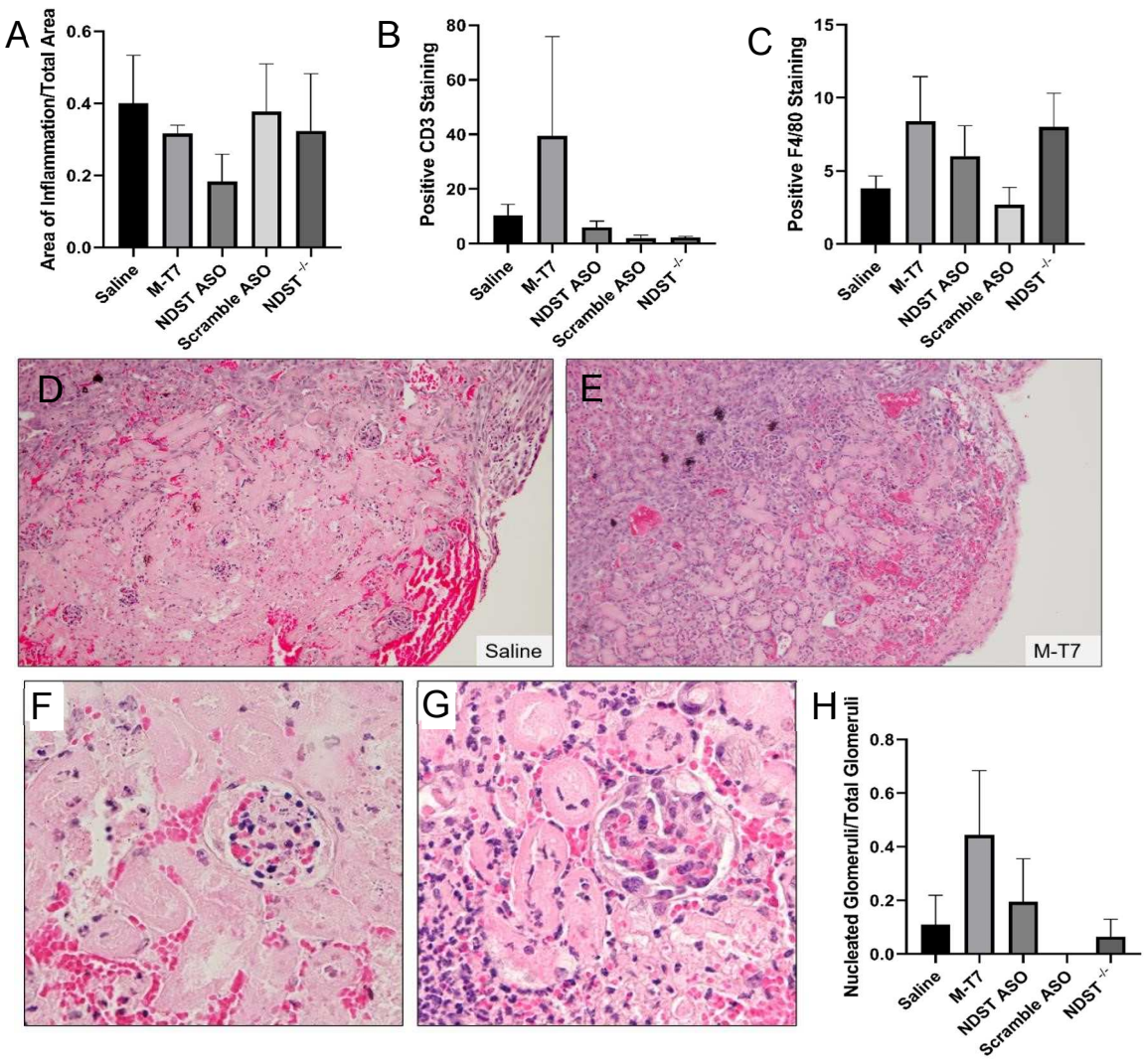

Figure 13. Histological analysis of soaked transplants shows variable changes in inflammation. NDST1 ASO displayed small decrease I overall inflammation however showed an increase in macrophages compared to saline treated tissue. M-T7 increased the number of macrophages. M-T7 most interestingly increased number of nucleated glomeruli compared to the total number of glomeruli in the tissue. F,G) H&E sections displaying a saline treated graft F with a obliterated glomeruli to the M-T7 treated transplant G with a retained structure. No statistical significance was found for any of the measurements however some displayed small changes.

M-T7 and NDST ASO trend toward a decrease in histopathological markers associates with acute rejection in 15 day soaked transplants. To obtain the number of mice determined by the power calculations more surgeries were conducted. 4-10 mice per group were given a subcapsular transplant with one of the control or treatment solutions. The graph displays the proportion of inflammation compared to the total are of the transplant. M-

T7, NDST ASO and NDST$^{-/-}$ showed slight decreases of inflammatory cells (Figure 13, A). H&E stained tissues treated with saline (Figure 13, D) or M-T7 (E). Image D has little to no retained tubular and glomerular structure while image E displays less severe tubulitis and glomerulitis. M-T7 treated transplants retained more glomerular structure than saline treated transplants (Figure 13, H). Histology images illustrate saline (F) with minimal structure of the glomerulus when compared to M-T7 (G). M-T7 while there are more inflammatory cells shows a more preserved structure. Immunohistological analysis of the grafts displayed variable results. M-T7 treated mice had an increase in CD3 as well as F4/80 saining while NDST ASO and NDST $^{-/-}$ only had an increase in F4/80 macrophages.

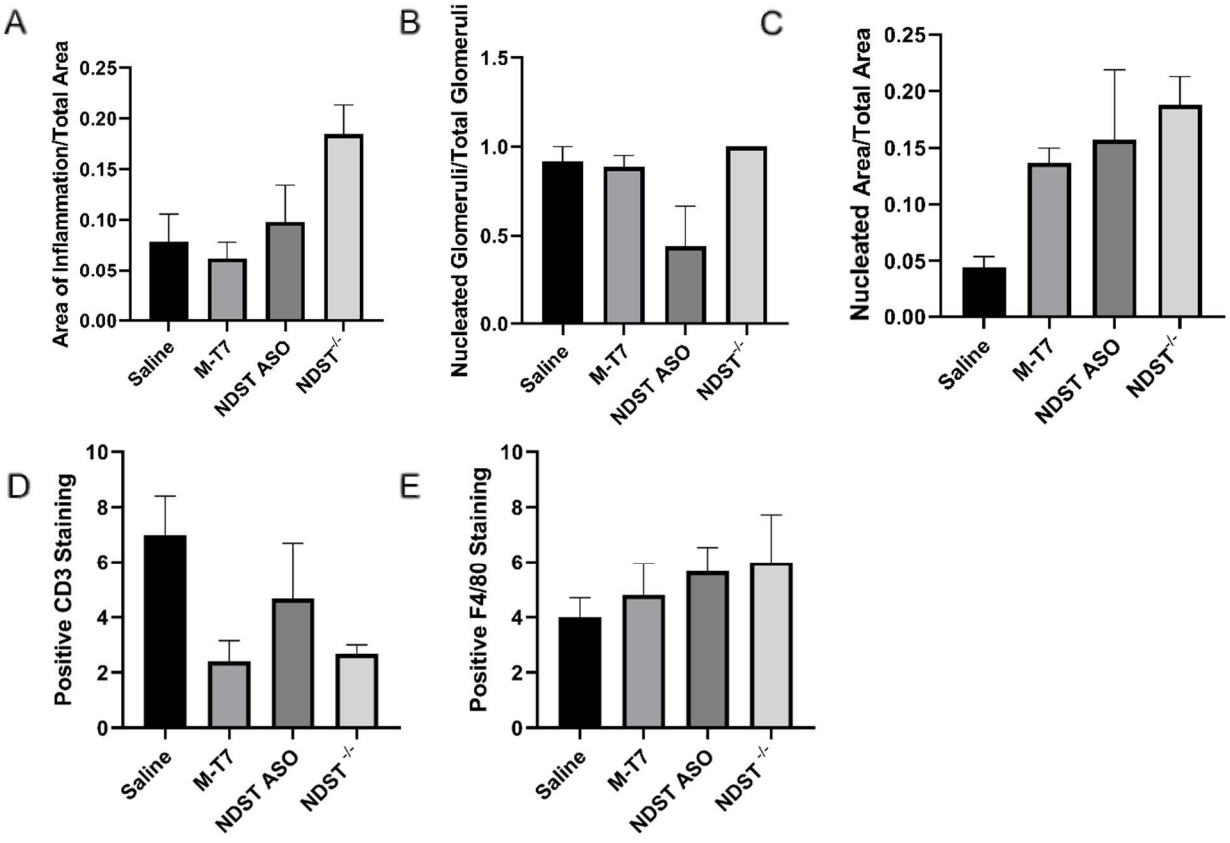

Figure 14. 3 day follow up of soaking transplants display slight changes in inflammatory cells. Transplanted tissue was collected 3 days after surgery to evaluate early changes in inflammation. NDST knockout displayed an increase in overall inflammation as well as more nucleated area in the transplant. M-T7 and NDST ASO displayed increases in nucleated area however shoed a decrease in CD3 staining. No statistical significance was found for any of the measurements however some displayed small changes.

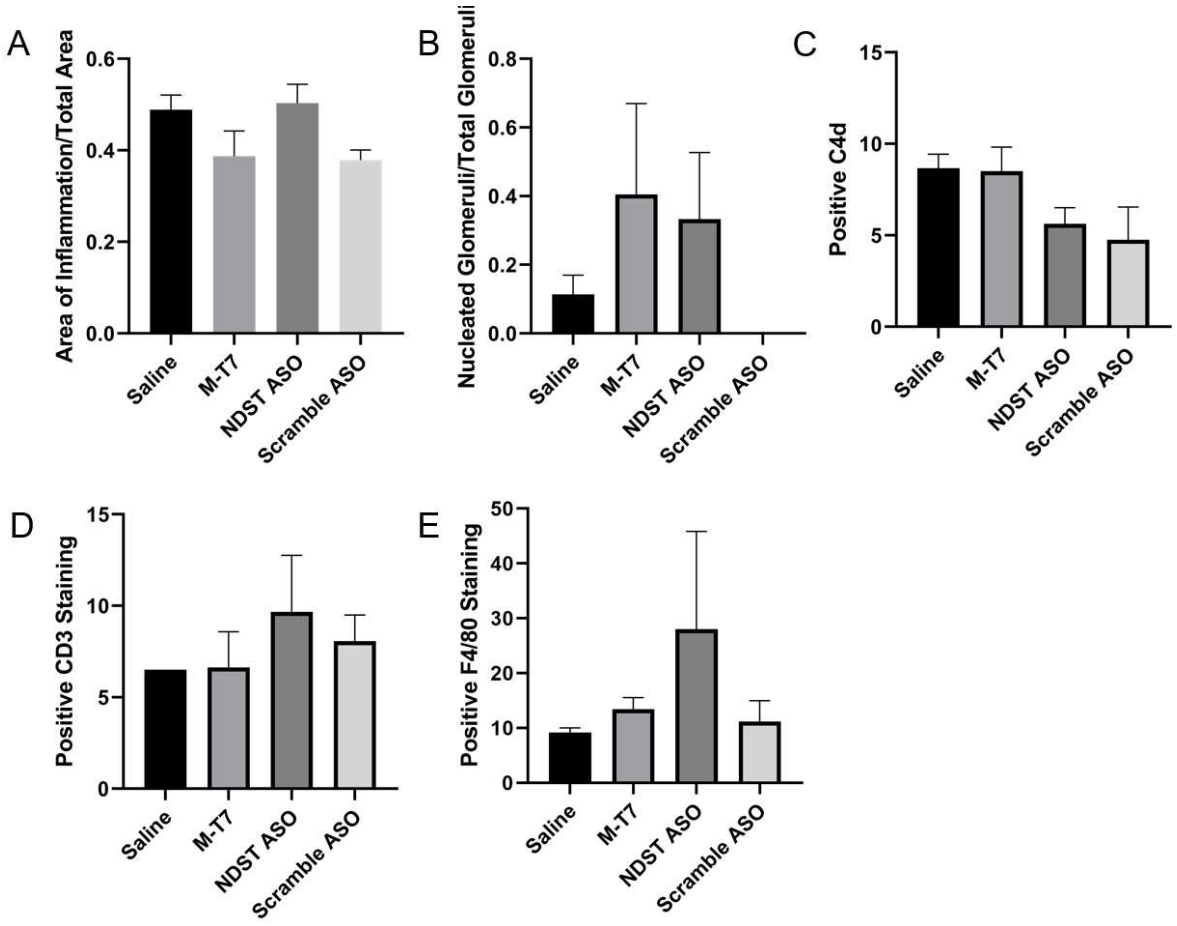

Figure 15. Pretreatment of transplants 7 days prior to surgery. The effect of injecting the donor with treatment prior to transplantation was measured using markers of inflammation. No statistical significance was found for any of the measurements however some displayed small changes. Conditional deletion of NDST1 displayed an increase in inflammatory cells while having a increase in nucleated cells and preserved tubular structure. M-T7 increased the number of nucleated glomeruli. None of the treatments increased CD3 T cell staining at a high-powered 20X field however NDST ASO increased staining for F4/80.

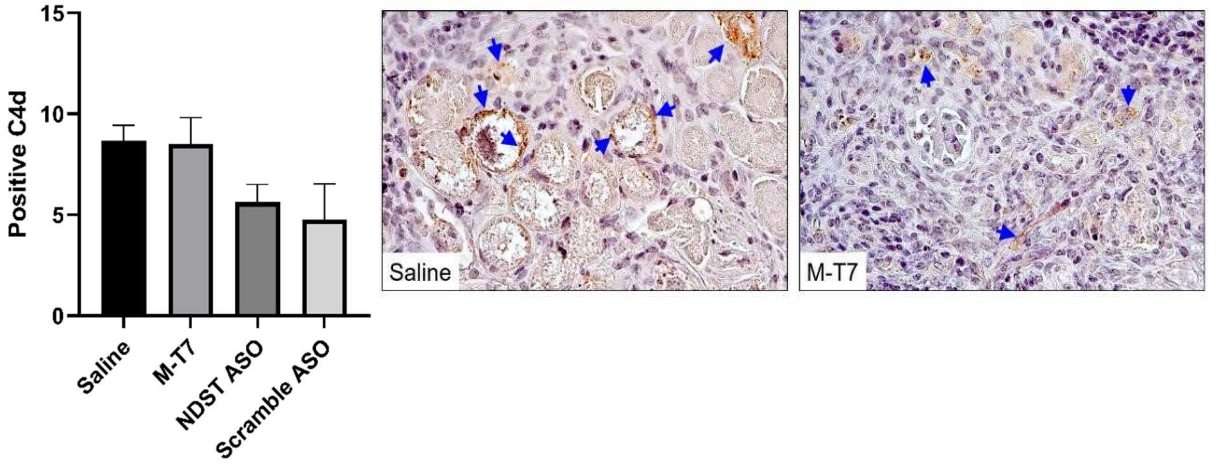

Figure 16. Markers of rejection has variable changes. IHC sections (40X objective) demonstrating C4d deposition in saline- and M-T7-treated subcapsular kidneys with a 15-day follow-up. C4d depositions indicate antibody-dependent rejection and are included in the Banff scoring criteria. Scoring of C4d is determined by the ratio of peritubular capillaries and vasa recta that has positive staining. Lesion scores range from 0 to 4 with no staining to more than 50% deposition. Blue arrows indicate C4d-positive staining. Saline section on the left shows greater amounts of positive staining around the peritubular capillaries. Approximately 50% of capillaries stain positive giving a Banff score of 2. In comparison, M-T7-treated section on the right shows minimal deposition of C4d and a maintained structure of a glomerulus in the center of the field. This section would receive a score of 1 according to the Banff lesion score. IHC staining using a C4d marker provides a molecular indication of antibody-mediated rejection and indicates poor graft survival. No statistical significance was found for any of the measurements however some displayed small changes

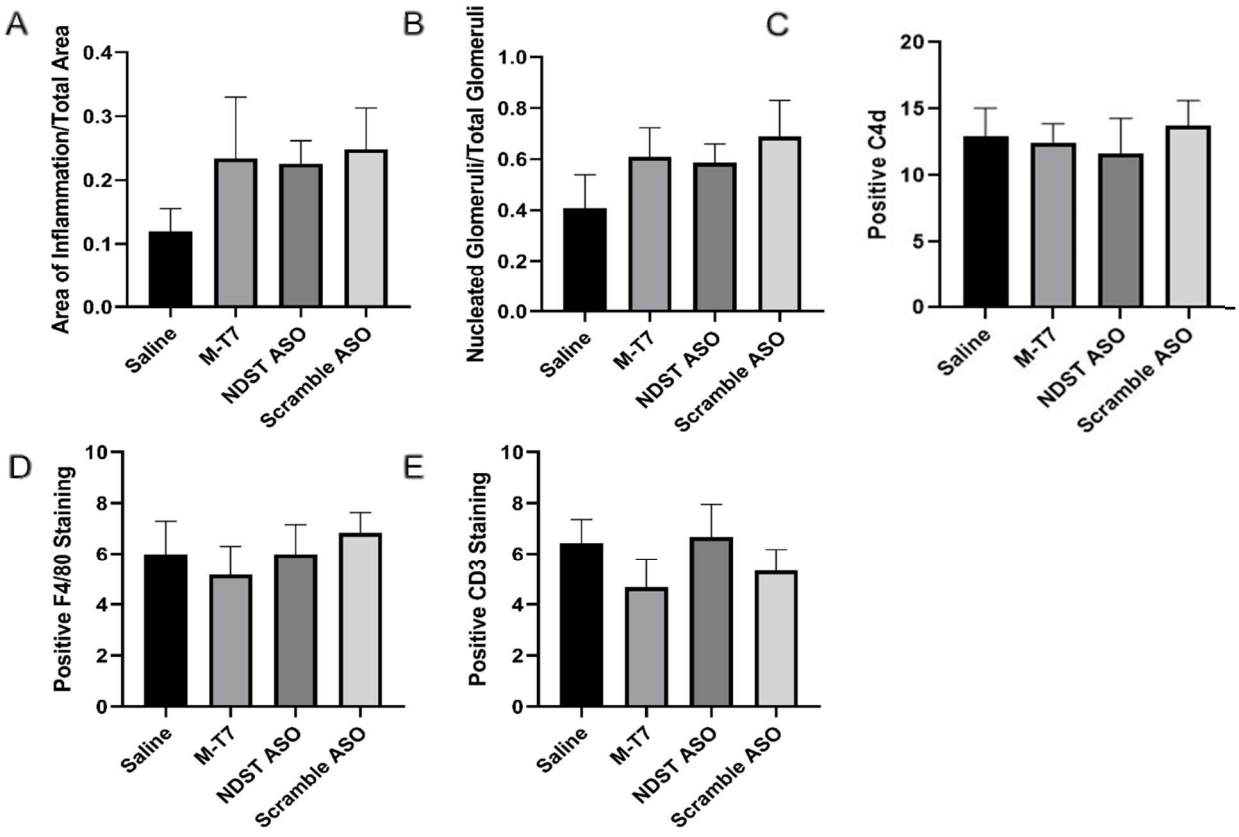

Figure 17. Short term follow-up provides no significant changes in inflammation or markers for rejection. Tissues were collected 3 days after transplantation of the pretreated tissues. The donor organs were pretreated for 7 days. No statistical significance was found for any of the measurements however some displayed small changes. There was a slight increase in M-T7, NDST ASO, and Scramble control ASO levels of inflammation as well as nucleated glomeruli.

Discussion

Subcapsular transplantation is a simple method for testing new therapeutics against initial waves of inflammation however would not be idea for longer term studies. There were many changes in T cells and macrophages however it is not known which specific immune cells were present. As seen in Figure 13 and 15 there was an increase in inflammation however also an increase in nucleated area. Not all inflammation is considered “bad”. The majority of the immune cells present could be M2 macrophages that are anti-inflammatory and initiate tissue repair (Helm et al., 2014; Li et al., 2019; Mosser & Edwards, 2008).

The varying numbers of mice is due to the transplant being lost or passed during sectioning. Initially there was no method to determine where the transplant was in the tissue. To find it one needed to section 5 microns at a time and stain for H&E every 100 microns. We section 5 microns at a time to find a transplant that is approximately 600 microns thick. When cutting through the kidney that is over 10,000 microns thick finding a piece that is 600 microns is extremely difficult. This method proved to be extremely time consuming and often resulted in the loss of the transplant. It is extremely helpful to note the location of each graft implant into the receiving kidney. Difficulty finding the site of transplant thus did not arise later in the histology processing. Mice were also ear tagged before the surgery and noting each mouse's transplant location at the time of engrafting to make finding the site easier when sectioning.

As seen in this study there was mostly variable changes that did not have a pattern. This amount of tissue transplanted under the capsule has not been well investigated. It is a possibility the transplant size is too large. There is also significant damage done by cutting the piece of kidney out of the whole kidney. This method will likely work better using kidney organoids instead of a large piece of tissue. These organoids can function on their own while the piece of kidney cannot. The tissues were also not read by blinded pathologist and could have a completely different result than the ones discussed above.

Modifying the donor organ prior to transplant, either in conditional endothelial Ndst1 deficiency or with Ndst1 ASO reduce early graft inflammation. M-T7 inhibition of chemokine-GAG interaction reduces early allograft damage, suggesting new therapeutic options for treating donor organs prior to transplantation as an approach to reduce rejection.

CHAPTER 5

BROADER EFFECTS ON THE RECIPIENT KIDNEY

Background

It has been postulated that the subcapsular transplant surgery causes damage to the kidney from the surgery alone. A sham surgery is when the researcher performs the exact surgery without inserting any tissue or cells. In some studies, utilizing this method the researchers compare the sham transplant to the treated transplants (Miyabe et al., 2019). The treated transplants are normalized to the shams to create a fair conclusion. Little work however has investigated the exact negative effects on the recipient kidney of the subcapsular transplantation. Often around the grafted tissue there is a mass of immune and red blood cells that surround the area. The study described in this paper, transplants an even larger amount of tissue than what has been previously inserted under the capsule. We compared the damage done to the kidney performing the subcapsular transplantation and inserting in that amount of tissue.

Materials and Methods

BUN and creatinine tests

Kits were used from Pointe scientific. Plasma from mice were collected and frozen at -80C till needed. BUN test wavelength was kept at 340nm while the creatinine test was measured at 510nm. Using a plate reader and a 96 well plate, plasma was added to the reagents and measured at specific time points. In the BUN tests, 2 µl of sample was added to 200µl of reagent. This amount was optimized by testing various concentrations of the standard. Measurements were taken in 30 second intervals for a minute and a half. Creatinine tests used 20 µl of sample and 200 µl of reagent. Reads were taken at 60 second intervals for 3 minutes. Calculations were made using the formulas listed in the kits.

Histology

After the tissues were fixed in formalin overnight the organs were embedded in paraffin wax and placed into cassette blocks prepped for sectioning. 5 micron sections were made and

placed on charged slides. Slides were stained for Hematoxylin and Eosin for the timing listed in Table 2. Immunohistochemistry was performed on the slides. C4d marker was used as stated in previous chapter.

Using microscope and counting cells

Interstitial inflammation, scarring and tubulitis was observed on stained sections using a brightfield microscope. The equipment used was an Olympus BX51 microscope with 4-100× objectives, a Prior ProScan II stage and Olympus DP74 CMOS camera and cellSens software analysis system. Comparisons to the Banff criteria was made to score level of rejection [6].

Statistical analysis

Statistical Analysis Data were analyzed in GraphPad Prism version 8.43 (GraphPad, USA) or in StatView version 5.0.1 (SAS Institute Inc.) using one-way ANOVA with a Tukey's post-hoc, Fischer's LSD comparison, or a Students unpaired T test. Results were considered significant if $p < 0.05$.

Results

Subcapsular transplantation recipient kidneys display signs of decreased function. To measure the functioning ability of the kidneys the plasma was collected from the recipient mice and ran through the creatinine and blood urea nitrogen tests. Creatinine levels significantly increases suggesting a decrease in filtering ability of the kidneys (Figure 18). There also was a significant increase in blood urea nitrogen. Both these tests confirm that there is significant damage to the recipient kidney that happens because of the transplant and surgery. Next a marker for rejection C4d was used on the recipient kidneys. C4d depositions indicate antibody dependent rejection and are included in the Banff scoring criteria. Figure 19 displays a significant increase in staining for C4d but also more scar area in the kidney. This suggests that the whole kidney could be rejecting.

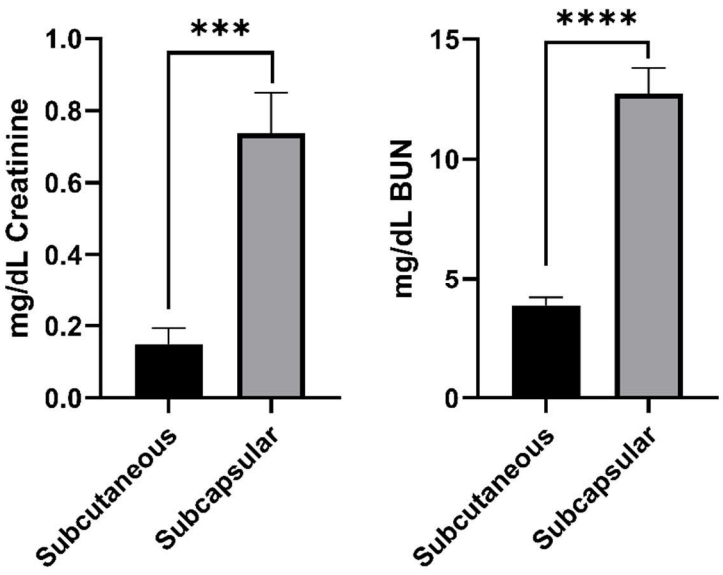

Figure 18. Subcapsular transplant mice display decreased kidney function. Subcutaneous transplants both showed a significant decrease in creatinine and blood urea nitrogen (BUN) levels when compared to those mice that received a subcapsular transplant. All transplant treatments were tested and displayed the same levels of decreased kidney function. Values were considered significant with P≤0.001 for creatinine and P≤0.0001 for BUN.

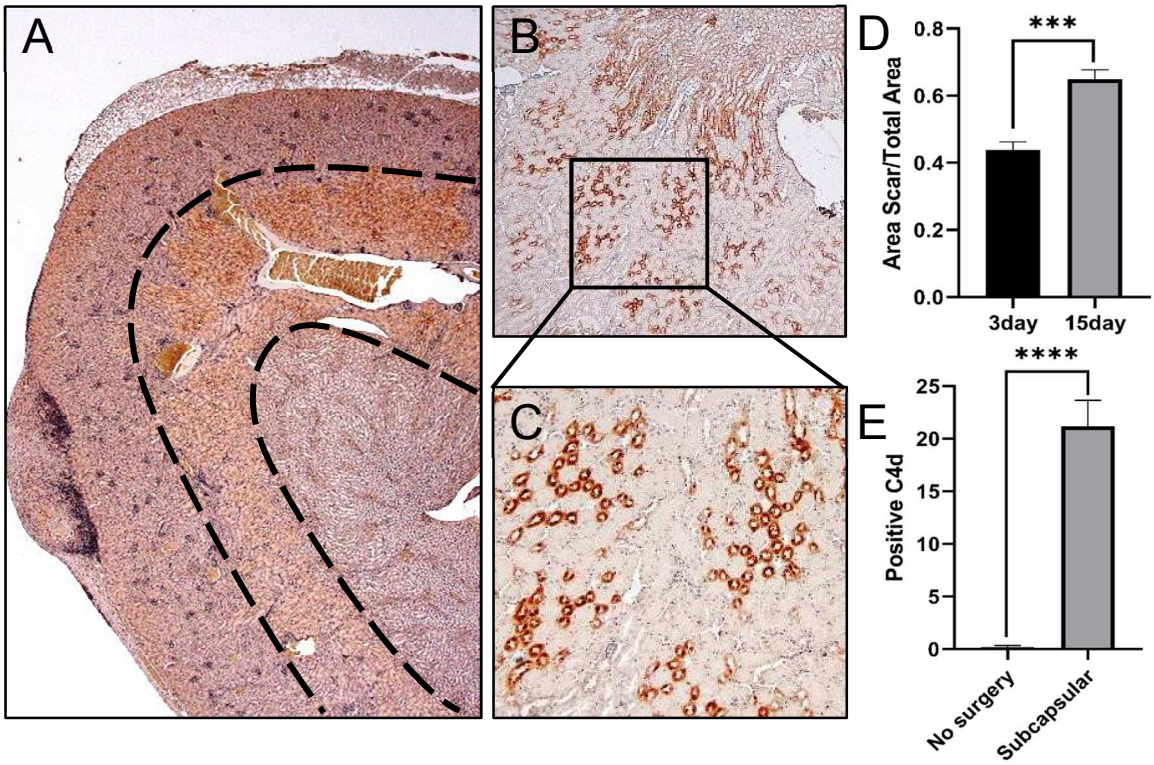

Figure 19. C4d deposition in recipient kidney of subcapsular transplants. IHC sections demonstrating C4d deposition in subcapsular transplant. A) 2X objective overview of recipient kidney. Dashed line indicates area with positive C4d staining and scar area. B, C) Brown staining in tubules suggest C4d positive cells. These count under the Banff criteria and would receive a score of 4. D) Graph displays comparison of 3 day and 15 day follow up after transplantation. The area of the scar was measured and compared to the total are. A significant increase in scar was seen in the 15-day tissues. E) Five 20X fields were measured and positive tubules were counted. Subcapsular transplant recipients displayed a significant increase in rejection marker than kidneys from a healthy mouse.

Discussion

Mice given a subcapsular transplant displayed both signs of decreased kidney function but also increased staining of C4d, a clinical marker for rejection. Significant damage is done during the surgery. This could be due to the size of the transplant or from the surgery itself. In future studies, sham mice should be used, and treatments should be normalized against the sham. Many studies have been done utilizing sham surgeries however few look at the effects on the recipient kidney itself. This study hints at a previously unknown relationship between subcapsular

transplant surgeries and a decreased kidney function in the recipient organ. As seen in figure 18 there was significant damage done to the filtering ability of the recipient kidney. The increase in creatinine and blood urea nitrogen confirmed that. Next when the marker for kidney rejection, C4d, was tested there was surprisingly a large amount of deposition in the whole kidney itself rather than just in the transplant where it was expected. This suggests the possibility the whole kidney is rejecting. There were little differences between treatments however more numbers would be needed to confirm this finding. It would be interesting to see the long-term effects of this surgery. In the future, long term effects should be investigated along with the effects of the size of the transplanted tissue.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Applicability of subcutaneous and subcapsular models. Full orthotopic transplants as previously discussed are extremely difficult to perform. Subcutaneous and subcapsular transplantation could provide quick methods to assay new therapeutic targets for changes in acute rejection. This study expands on a novel model that will greatly impact and reduce the difficulty of testing new drugs for reducing rejection in organ transplantation. While subcutaneous transplantation provided a simpler model subcapsular was found to be a potentially better option. There are circulating immune cells that naturally reside in the kidney as well as fully contained environment in the capsule. Subcapsular kidney transplantation provides a first step approach to testing virus-derived proteins as new potential immune modulating therapeutics to reduce transplant rejection and inflammation. Traditionally, the approach to reduce transplant rejection is to flood the organ recipient with immunosuppressants. Many transplanted patients are reliant on immunosuppressants for the rest of their graft's functional life. However, much of the damage to the organ has already occurred. Most therapies for transplants are post-operative, very few tackle treatments of the graft donor prior to transplantation. Treating the donor organ shows a promising method of reducing the initial damage that leads to chronic rejection and thus graft loss. M-T7 and inhibition of NDST1 provides a suitable target for reducing graft rejection. Reducing endothelial heparan sulfation and blockade of chemokine-glycosaminoglycan (GAG) interaction with Myxomavirus-derived protein, M-T7, reduced aortic and renal graft inflammation and rejection. Conditional endothelial Ndst1 deficiency and inhibition of chemokine-GAG interaction reduces early allograft damage and suggest new therapeutic options for graft rejection.

Future directions. There were some complications when it comes to this study. Firstly, the data was not analyzed by blinded pathologists and would benefit to send the slides off to have them analyzed by experts in the field before any permanent conclusions can be made

about this method. This would give more significant results that would be free of bias. The pretreatment of grafts that were soaked in the treatments prior to transplantations displayed the more significant changes in inflammatory response. This could be due to the immediate and large amount of drug present at the time of transplantation. Treating for longer or with different concentrations should be explored. he injections should be tested at varying concentration and for multiple time periods. The possibility that the treatments need to be applied both before and after transplantation should also be investigated in future. In prior studies when mice were given treatments of M-T7 along with cyclosporine, an immune suppressant, they showed a prolonged survival (BEDARD et al., 2003). It would be interesting to explore if treating both prior to transplantation as well as a few days or weeks after would give the graft an even better survival rate with minimal damage. Specific immune stains would also shed light on the exact immune cells present during rejection in these transplants. There is a large possibility a portion of the inflammatory influx is good immune cells that promote tissue repair like M2 macrophages.

Significance. This project establishes the first subcapsular renal transplant model which can be applied to a wide variety of organs. It reports on a widely applicable platform on which to rapidly test new treatments for transplant rejection. The project has explored over 162 mice, six different timepoints, two simple surgical procedures, and investigated four different therapeutic targets. This finding significantly lowers the barrier to entry for labs which are interested in translating their laboratory findings to animal models of organ transplantation which is a complex surgical procedure, thus accelerating the bench-to-bedside translation of novel, putative treatments for transplant rejection.

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